

Bioactive Fibers and Polymers

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Bioactive Fibers and Polymers

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Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previously published papers are not accepted.

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Preface

This American Chemical Society (ACS) symposium series book addresses the design and activity of bioactive fibers and polymers, which are high molecular weight natural and synthetic macromolecules and their complexes. The focus is on research in wound healing and antimicrobial materials where the design and mechanism of the biologically active molecule plays a key role in the textile fiber function. The symposium on which this book was based was held to bring attention to work at the interface of biologically active molecules and natural and synthetic textile fibers. Because it is by deriving more insight into the actual activity of the molecule on the textile surface that new products will emerge in wound healing and antimicrobial textiles. The symposium was convened to understand biological function of natural and synthetic fibers in light of the mechanistic issues of fiber design. Mechanism of fiber activity is directly related to the complex biological environment surrounding the fiber. Thus, this interdisciplinary subject area brought together physical science disciplines from synthetic, analytical, textile, and polymer chemistry with life science disciplines of medicine, biochemistry, biophysics, and microbiology.

Scientists have been working on the issues that underpin making more efficient wound dressings and antiseptic textiles for more than a century. The molecular bases of disease processes are better understood now, and our basic understanding of the structure and function of biologically active molecules does enable the creation of bioactive fibers that can selectively interact with their biological environment. Some scientists have coined the term 'smart fabrics' to depict the targeted function these types of textiles have and their ability to perform a specific function in wound healing, arterial implants, or antimicrobial activity.

The current worldwide crisis in microbial infection and health care of wounds suggests that more research is needed to understand practical and effective ways of creating safe bioactive textiles. The future development of biomedical and protective textiles with selective properties that benefit the consumer will be based on applying scientific and clinical advances in wound healing, antimicrobial, and enzyme-based fabrics as are reviewed in this book.

The chapters of this book seek to deal with understanding the issues in the challenging task of both creating biologically active fibers with selective function as well as the impact of biologically active molecules on fabric function and durability. It is hoped that the reader will derive a basic understanding of some of the current mechanistic, design, and analytical approaches that are being taken in applying biomedical research to textile fibers.

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Chapter 1

Biologically Active Fibers in Health Care

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The concept of bioactive fibers is rooted in biomaterials for medicine and surgery and has relevance to apparel for public health. Considerable progress has been made with nonimplantable fibers for wound care and with the incorporation of antimicrobial functional groups durable to laundering and refurbishing. Extracorporeal fibers are being improved upon by grafting chemical and biochemical ligands onto kidney dialysis tubing. A rational design for biologically active fibers has been under development for over a decade in the area of implantable fibers. The formulation of integrin recognition sequences into biomedical implants has been a model for the incorporation of compounds with pharmacological activity into textile fibers such as polyurethane, poly(ethylene glycol) and crosslinked acrylate polymers. Some of the issues in modifying fibers chemically or biochemically to produce bioactive textile surfaces with a targeted function and/or bioselectivity are discussed. Fibers that afford a bio-barrier and thus minimize transmission of infectious disease are considered. Textile fibers such as wool, cotton, alginates, polyesters, and synthetic composites may be optimized as high performance bioactive fibers when more information is available about the fundamental mechanisms of wound pathology, hygiene, resistant antibiotics and cell surface signaling. The relation of structure to function and some of the biophysical issues in designing textiles with selective properties in biological fluids are discussed.

The design of new fibers for use in health care textiles has increased dramatically over the last twenty-five years due to advances in polymer, wound, hygiene and textile sciences. Innovations in fiber technology and chemistry have led to improvements in nonimplantable, implantable, extracorporeal and hygiene textile products. Traditionally, empirical design and processing techniques have been

utilized to incorporate synthetic and natural fibers into health care textiles. Processes utilizing textile engineering and chemical technologies to improve fibers that function on human skin and wound contact have evolved over the centuries since the dawn of civilization (1) when people first began to construct fabrics (Fig. 1). However, approaches for modifying fibers based on the biochemical mechanism of disease and molecular design to create high performance health care textiles have appeared only within the last decade (2). The discovery of new pharmaceuticals with de novo approaches that address the biochemical mechanism of disease has revolutionized medicine through the use of biotechnology (3), combinatorial chemistry (4), rational design (5), and drug discovery. Similar approaches are needed in fiber science to improve the quality and performance of health care fibers.

Unmet patient needs related to chronic wounds (6,7) hospital-acquired infections (8) and organ transplantation (9) are examples of challenging areas of health care where improved fibers possessing a biologically active component are needed. Moreover, the trend of global warming, rapid population growth and worldwide travel are concomitant with the concern that there are at least 30 new infectious diseases that have been characterized over the past two decades. It has been estimated by the World Health Organization (WHO) that infectious diseases are responsible for 17 million deaths per year worldwide and that half of the 5.8 billion world's population are at risk for many endemic diseases (10). There is also evidence accumulating that many other animal species serve as reservoirs for pathogenic transmission to humans. Thus, textile bio-barriers to inhibit the transmission of infectious disease are considered as an increasingly important technology in foreign, domestic and multi-cultural environments.

To address these trends, new textile fiber designs are currently being explored with a view to improving an understanding of the activity of molecules at the fiber surface in biological fluids. Continued expansion of the medical textile market (increasing overall by 10 per cent in annual gross sales) suggests that new products will be appearing as new fibers are designed with value-added properties to meet consumer needs (11). The chapters in this book focus on the use of rational biologically-based design methods in wound, microbial, genetic engineering and hygiene research, and represent an emerging dialogue on bridging textile chemistry to the molecular mechanism of disease and hygiene pathology across several scientific disciplines (12). This overview discusses some of the recent advances in fiber design of health care textiles and surveys the future possibilities for rationally designed and genetically engineered textile fibers as biomaterials.

Nonimplantable Fibers

Nonimplantable textile fibers are those which are applied outside of the body. They include: dressings and bandages used in wound and orthopedic care, bedpads, sheets, diapers, and protective clothing such as patient and medical personnel gowns, gloves, face masks and related items.

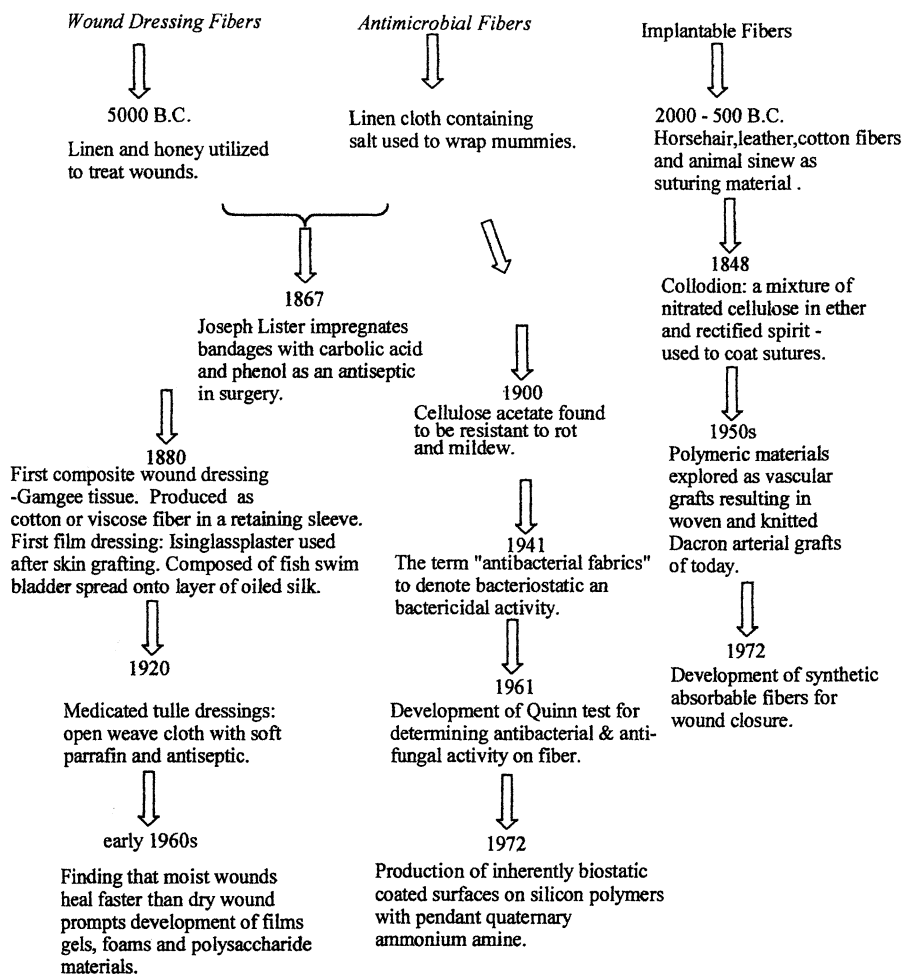


Figure 1: Historical milestones in the development of wound dressings, antimicrobial fibers and implantable fibers.

Nonimplantable Wound Dressings

Recent reviews (13, 14, 15) have treated the categorical description and discussion of conventional and some types of specialty fibers. The chapters in this book focus on a variety of new approaches to designing selective properties into natural and synthetic fibers of wound dressings. A wound dressing must effectively isolate the wound from the harmful environment so that effective healing can take place. Fiber designs continue to be optimized to promote hemostasis, limit edema, reduce pain and improve gas exchange between blood and tissue.

Issues related to the biodegradable and absorbable properties of collagen, and polysaccharides such as alginate and chitosan are being addressed (15). Alginate fibers, which continue to have a market increase of 40% per year, are being improved upon for their absorbency and application to heavily exuding wounds (16). Alginate co-polymers (Figure 2) contain α -L-guluronic acid (G) and β -D-mannuronic acid (M) arranged in three types of blocks (GG, MM, and MG). Alginate forms a gel when the divalent metal ion calcium binds with GG and water is sequestered between the polymer chains. Ion exchange of calcium with sodium upon contact with a wound draws water into the alginate fiber and results in swelling and contouring to the wound surface. Alginate fibers with high M content can be easily removed from the wound surface without disruption of delicate tissue.

Both chitin and chitosan have shown promise in wound management; although their structures are similar to cellulose, they have been manipulated to produce different beneficial effects in wound management. The degree of acetylation in chitin and chitosan (Fig. 2) increases the level of hydrogen bonding and promotes tensile strength in the crystalline fiber. A variety of modifications to the glucosamine have been shown to promote interesting effects in wound healing. For example, N-carboxybutyl chitosan has been reported to promote more ordered tissue regeneration (17).

Occlusive dressings that promote a moist wound environment and promote healthy gas and water vapor exchange are composed of alginate, collagen, chitosan, hydrocolloids, and hydrogel polymers. Although these dressings have significantly improved wound care management since the moist healing concept of the early 60s (18), they must still be carefully controlled because the same environment that improves healing may enhance pathogenic growth. Wound healers have begun to ask mechanistic questions about the biochemistry of inflammation and associated positive and negative effects of cytokines and proteases that are a part of tissue damage associated with chronic wounds. Undoubtedly, wound dressings of the future will be improved based on better mechanistic approaches. Thus, we are far from reaching the ideal wound dressing which would have specific functions designed for the pathology while allowing the clinician a transparent view of the healing progress of the wound. In chapters two through ten some of the research that will take us in that direction is addressed.

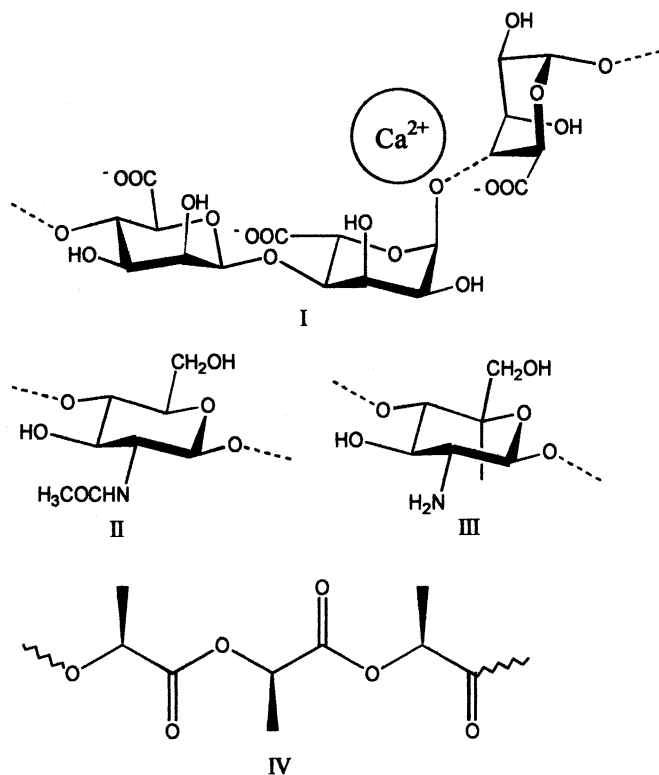


Figure 2: Structures of aglinate (I) represented as (1-4) β -D-mannuronyl-(1-4) α -L-galuronyl-(1-4) α -L-galuronate, chitosan (N-acetylglucosamine) (II), chitin (glucosamine) (III). D,L polylactide (IV)

Nonimplantable Hygienic Textiles

Issues in the design of nonimplantable textiles that absorb biological fluids from the body have changed based primarily on comfort, convenience, and lifestyle. The design of disposable diapers has undergone continual changes to provide advantages in skin care, dryness, and leakage protection. The transition from cloth diapers to disposable diapers occurred gradually when the latter was introduced in the late 1940s, but more dramatically in the last twenty-five years. Until recently, more emphasis was put on the design and marketing of disposable diapers rather than on their actual effectiveness in preventing infection and associated problems such as diaper rash. The essential components of a disposable diaper (Fig. 3) include (a), a moderately porous cover sheet usually made of a hydrophobic fiber such as polyethylene or polypropylene to rapidly transfer urine into (b), a very thin and

porous hydrophobic acquisition layer that uniformly spreads the urine into (c), an absorbent core, which is usually made of cellulose wood pulp and powdered superabsorbent such as poly(acrylic acid), attached by an adhesive to (d), a hydrophobic backing to prevent leakage of fluid. Performance improvements have been made over the years to minimize channeling of fluid and clumping due to uneven distribution of the powdered superabsorbent. Design and marketing improvements that are currently available include clothlike backsheets, tricot landing strips, elastic waists, breathable side panels, stretchable fastening tapes and leg cuffs (19). There is still room for improvement in reducing leakage and incidence of diaper rash (usually due to microbes such as *Candida albicans*). There is also an ongoing debate about the enormous amount of waste generated by use of disposable diapers with regard to methods of disposal (landfill, incinerating, recycling and composting) and the feasibility of using more biodegradable components (20).

Incontinent bedpads and adult diapers are much more recent in their development and use (only about 20-25 years old), but have many of the same designs and features as baby diapers. However, as this class of nonimplantables has become more popular, certain design features and performance requirements have evolved, particularly for heavy incontinence. These attributes include rapid absorption of urine and very high absorbent capacity (exsorbency), isolation of wetness from the skin contact layer, odor reduction, user comfort especially in the wet state and ease of use and removal (21). The crosslinked polyol process developed by one of the authors was demonstrated to be particularly amenable for bedpads since selected nonwovens

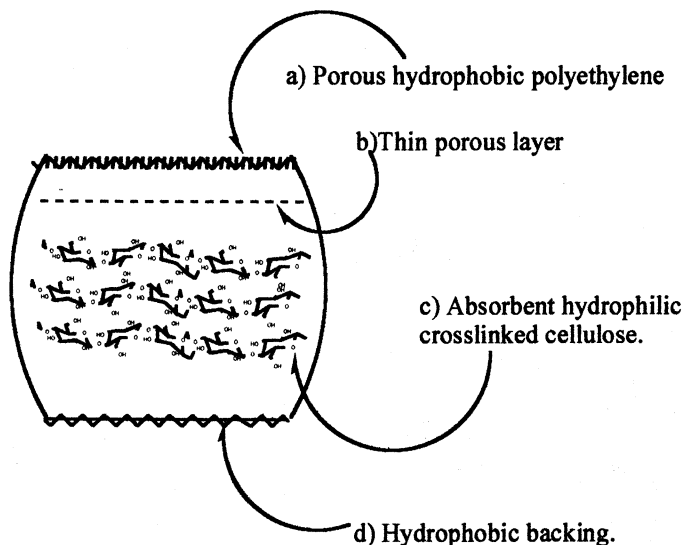


Figure 3: Diagram of the essential fiber components of a disposable diaper. An absorbent hygiene product used to absorb and contain the urine and faeces of a baby.

treated by this process had very high exsorbency and the additional benefit of antimicrobial activity (22).

Protective clothing against biohazards has been critically reviewed with regard to performance and available products (23) and suitable test methods (24). The impetus for the development of hospital gowns, face masks, gloves and related items was the passage of an OSHA standard in 1991 that mandated use of protective clothing for over 7 million persons in the health care and medical professions and other industries. The emphasis was on protection of transmission of bloodborne pathogens (primarily HIV and various forms of hepatitis) (25). Although there are many hydrophobic materials that dramatically reduce airborne particles in operating rooms and computer assembly facilities and prevent liquid strikethrough (simulation of bloodborne pathogen transmission), the difficulty has been in also providing garments and materials that are breathable and afford thermal comfort.

Extracorporeal Fibers

Extracorporeal fibers are those used in mechanical organs such as hemodialysers, artificial livers and mechanical lungs. Historically regenerated cellulose fibers in the form of cellophane have been utilized to retain waste products from blood (26). Cuprophane, a cellulosic membrane has been the material of choice due to the selective removal of urea and creatinine while retaining nutritive molecules such as vitamin B-12 in the bloodstream (27). Attempts have been reported to improve on the removal of unwanted cytokines formed from inflammatory pathologies associated with dialysis and antibodies from transplant rejection. Klein et. al. (28) reported on a method for capturing anti-(Gal α 1-3Gal) antibodies formed from hyperacute rejection of pig xenografts by immobilized Gal α 1-3Gal oligomers derived from carrageenan. The use of bioactive epitopes containing galactose in an α -1-3 linkage (Figure 4) were obtained from γ -carrageenan oligosaccharides which were immobilized on hydrazide-modified microporous nylon membranes. These subsequently were shown to decrease human anti-(Gal α -1-3 Gal) antibody level in normal human plasma. Layers of needlepunched fabrics varying in density have been reported to efficiently remove waste materials as an alternative in dialyser filters (29).

The potential for artificial livers as extracorporeal support of acute liver failure has been assessed (30). The development of a hollow fiber bioreactor, where hepatocytes were entrapped in a collagen gel was found to be effective after five days based on hepatocyte viability, oxygen uptake, and sustained albumin activities. The application of polymer chemistry to the development of bioartificial organs has been recently reviewed and an evaluation of the polymer structure/biomaterial function relationship made concerning an artificial pancreas (31).

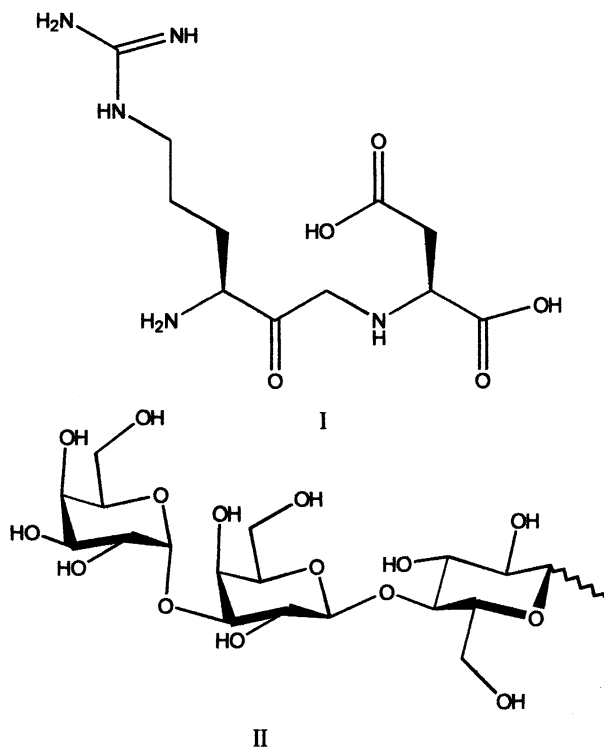


Figure 4: Structures of integrin recognition sequence Arginyl-Glycyl-Aspartate, (RGD) (I); Galactosyl $\alpha(1-3)$ β Gal(1-4) β Glc (III)

Implantables

Implantable fibers include materials placed in the body that are used for wound closure or replacement surgery. Some of the considerations that are involved in determining the biocompatibility of the textile material by the body are; 1) biodegradability 2) non-toxicity 3) fiber size, porosity and tissue encapsulation. For example a collagen composite of poly(2-hydroxyethyl methacrylate) – polymer (HEMA) has been shown experimentally to improve the hard tissue replacement biocompatibility of the polymer (HEMA) by slowly undergoing biodegradation, and allowing uniform mineralization (32). Other synthetic polymers which have good biocompatibility and meet the above criteria are the family of polyesters including poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and copolymers of lactic and glycolic acids (PLGAs) (33). These synthetic polymers have been approved by the

FDA for *in vivo* use and have been used in sutures, support fabrics, and controlled release technology. Fibers and fiber meshes have been formed from thermoplastics of these polyesters, which can be formed into the desired shape by molding, extrusion and solvent processing thus giving high surface area implants. Lactic acid polymers of poly(ethylene glycol) prepared from UV copolymerization demonstrate good potential in tissue engineering.

Poly(ethylene glycol) is a hydrophilic synthetic polymer that has been demonstrated to have biocompatible potential in fibers (34) due to its low protein adsorption, platelet, cell and bacterial adhesion. Low protein adsorption and cell adhesion are a result of the low interfacial free energy with water molecules, steric stabilization, and absence of ionic binding sites. Lactic acid polymers of poly(ethylene glycol) prepared from UV copolymerization demonstrate good potential in tissue engineering. Entangled protein gels or polymeric meshes have been designed using fiber meshes of poly(glycolic acid) and poly(L-lactic acid). Chondrocytes that secrete collagen and glycosaminoglycan matrix have been included in the fiber matrix (35). The synthetic polymer is replaced with slow hydrolysis of the ester bonds of the fiber by a cartilaginous matrix that resembles the shape of the original implant.

Integrin-Recognition Sequences on Biomaterials

In recent years implantable textiles have been increasingly designed with a biologically active component. Implantable textiles have been designed with specific functions compatible with the surrounding tissue. The functions have been targeted to blood flow and cardiovascular pressure as well as the forces and interactions of surrounding tissue. These include sutures for dermal and tissue repair, fabrics that have traditionally been employed in heart repair as reinforcement meshes, vascular grafts, velours for blood contacting surfaces, fiber reinforcements for hard and soft polymer bone and ligament prosthetics and intraocular lenses. The usefulness of actual biologically active molecules as a part of the fiber's function in a tissue environment has been explored. Research in this area is a model for bridging bioactive molecules on fibers with performance textiles. Modern drug design approaches are based on enzyme and cell receptor recognition principles, and the analogous development of pharmacologically active molecules on textile fibers is targeted to biological recognition originating on the textile fiber. The concept of a bioactive fiber with pharmacological activity has been developed with implantable textiles in the cell adhesion domain.

Cell adhesion is important to cell spreading and migration and occurs within the extracellular matrix (ECM) which is composed of proteins and glycoproteins such as fibronectin, laminins, collagens, and vitronectin. Cell surface receptors termed integrins bind ECM proteins to the matrix and mediate mechanical and chemical signals from it (36). In the early 1980s it was shown that the integrin binding and activation of these large proteins of the ECM resides in a short three amino acid sequence, arginine-glycine-aspartic acid (RGD in single-letter code: structure shown in Fig. 4) (37). Cell adhesion activity was found to be effected with a small tripeptide motif. More recently integrin receptors have been found to trigger other cell cycle signals involved with cell proliferation and differentiation. The cell

signaling cascade is also connected to growth factor receptors, ion channels, and organization of the intracellular actin cytoskeleton.

Medicinal chemists have attempted to use the RGD and related integrin recognition sequences to develop a variety of pharmacological approaches to treating cardiovascular disease. Development of the RGD pharmacophore has been the basis of a therapeutic drug design approach with receptor agonist or antagonist activity (38). Diseases that have been identified that are targetable with RGD antagonist activity include cardiovascular (39), cancer (40, 41), osteoporosis (42), and inflammation (43). Diseases that potentially may be treated with agonist activity of RGD include those related to organ and tissue engineering (44), chronic wounds (45,46), and cardiovascular (47). Implants directed to the latter pathologies represent a new direction in improving biocompatibility based on fiber modifications that promote a constructive biological response.

The design of biomaterials having integrin recognition activity has been performed on what are considered, otherwise, inert surfaces to promote cell attachment. Understanding the biophysical issues in a rational design approach is in part dependent on knowing how to control cell adhesion signaling on inert surfaces. It has recently been observed that the presentation of cell adhesion signals influences the rate of cell migration and cell adhesion strength. Thus, engineering the quantity and activity of cell adhesion ligands on the fiber surface will have a direct effect on cell migration. Optimum cell migration on a grafted implant fiber or in a non-healing wound may better be understood by learning how to control cell adhesiveness with strategic spatial distribution of the receptor recognition sequence. Understanding the role of receptor ligands such as galactose multiantennary clusters and the asialoglycoprotein receptor (48) on hepatocytes has provided a valuable model in understanding spatial distribution of ligand clusters on cell spreading. Multiantennary galactose ligands are of interest in targeting hepatocytes for gene therapy technology, but may hold some promise for bolstering the efficacy of artificial hepatocyte fiber reactors.

Some of the biophysical considerations in designing a fiber that would exert biological activity are: 1) protein adsorption on the fiber surface; 2) lipid protein interactions between cells and proteins or biologically active compounds on the fiber surface; 3) the effect of electrostatic charge in conferring biologically active properties to the fiber and 4) the relative hydrophilic and hydrophobic properties needed to provide optimal adsorption. When biological fluid containing proteins is exposed to a fiber surface spontaneous accumulation of protein molecules at the solid-liquid interface is usually observed. The adsorption of proteins at the interface is a result of the interaction between the protein molecules, the sorbent surface, ions and water molecules. Dehydration of a hydrophobic surface can be the major contributing factor in the energetics of protein adsorption (49). Since the cell adhesion proteins fibronectin and vitronectin occur in blood plasma and other biological fluids the adsorption of these proteins onto surfaces in the form of monolayers will trigger cell adhesion on the material surface.

Previously, two salient issues have been cited as important when dialing in integrin recognition activity on the textile surface: 1) covalent and non-covalent attachment through a molecular tether and 2) sterically allowed orientations of the integrin recognition sequence on the surface of the fiber. Massia and Hubbell reported the

Biologically Active Proteins on Fibers

Work on the use of small integrin recognition sequence peptides either immobilized to biomaterials or attached as a controlled release agent has demonstrated the cell-type selectivity that can be accomplished on biomaterials using a small peptide as shown in Table 1. The approach of using receptor recognition on biomaterials may not be limited to small peptides, and may include native proteins that contain the receptor recognition sequences. Clinical studies with endothelial cell seeded vascular grafts have shown that grafts where the fiber is coated with the adhesion protein fibronectin perform better in high blood flow regions where stresses on the attached endothelial cells are greatest (52).

Table 1: Integrin sequences and their cell surface receptors, biomaterials and potential use.

| Integrin receptor recognition sequence | Cell Receptor | Biomaterial | Potential Use |
|--|--|--|---|
| RGD | $\alpha_v\beta_3$ on bone, fibrinogen, fibronectin, laminin, thrombospondin, vitronectin, vonWillebrand factor | Glass, polyurethane, polyethylene glycol, elastomeric peptide polymers, silk | Cardiovascular textiles where selective cell-adhesion is desired. |
| REDV | $\alpha_4\beta_1$ on endothelial cells | Polyethylene glycol | Tissue engineering, antithrombotic graft |
| YIGSR | $\alpha_1\beta_6\&7$ and $\alpha_v\beta_3$ on a variety of cell types including tumor cells | Polyethylene glycol, glass | Tissue engineering, antimetastatic, neural adhesion, modulation of inflammation in healing. |

Note : Single letter designation for the amino acid sequences.

Fibronectin and other ECM proteins such as vitronectin and laminin may also support cell adhesion through electrostatic interaction of positively charged sequence regions of the protein with the anionic cell-surface, anticoagulant heparin sulfate (53). Heparin-binding non-peptide amines when immobilized to surfaces at high density

induced cell adhesion in a way that was dependent on both charge and structure. Heparin has been placed on biomaterials (54), and recently a bioactive material made through sulfonation and carboxylation (55) of polyethylene has been proposed as a heparin mimetic (Fig. 5).

The recent demonstration that some growth factor receptors, such as for epidermal growth factor (EGF) (56) and transforming growth factor B (57), can trigger biological responses when bound to a solid phase. This opens up a range of new potential uses for proteinaceous growth factors that may be tethered to the fibers of biomaterials. In linking EGF to the solid substrate Kuhl and Griffith-Cima utilized tethering chemistry by employing the single amino-terminal group (Fig. 6) of epidermal growth factor removed from the receptor recognition portion of the oligopeptide. This approach assured a high ratio of receptor binding EGF sequences ($\sim 3 \text{ ng/cm}^2$) on the glass surface of derivatized PEO.

The potential use of spun silk-like fibers consisting of engineered protein polymers has been studied from the perspective of incorporating a fibronectin cell attachment protein sequence through recombinant DNA technology (58). The protein is termed SLPF (silk-like polymer with fibronectin cell attachment functionality). Crystal structures of SLPF protein films revealed a microstructure formed of woven sheaves where the sheaves were well-defined whisker crystallites (59). Integrin recognition sequences of fibronectin and laminin were displayed at the crystal surface in polymers of this type that have been produced to be used as a coating reagent to promote cell attachment and growth on synthetic substrates in the absence of serum-supplemented media. Wool keratin is a protein with helix-containing salt bridges of arginine-aspartate in the proteinaceous fibers (chapter 16) and may have similar potential biomaterial applications. The genetic engineering strategy for synthesis of poly-D(-)-3-hydroxybutyrate, a thermoplastic polymer, in cotton fibers has been reported (60). Cotton fibers may also present some potential for biopolymer display in biological fluid. As more is learned about the genes that control the synthesis of proteins in the cotton fiber new vistas in the selective use of cotton based biomaterials may be opened.

The potential to use bioactive fibers with selective enzymatic properties through grafting or covalent attachment of the enzyme on the surface of fibrous materials has been attempted. Immobilization of alpha-amylase (61) and glucose oxidase (62) have been reported on a variety of fabrics. Edwards et al. (63) have recently compared the antimicrobial activity of glycine-linked lysozyme on cotton cellulose fabrics (Fig. 7). The enzyme-cellulose conjugates were shown to be more effective in the lysis of gram-positive bacteria than freely soluble lysozyme.

Molecular modeling of the cellulose conjugate linkage assessed the possible residue linkages removed from the active site that would putatively enable activity. The potential utility of immobilizing numerous other enzymes on fabrics as a source of biologically active textile surfaces would offer highly selective functional properties while providing an environmentally acceptable textile finish. A similar approach is described in chapter two with regard to detoxifying enzymes covalently bound to cotton cellulose.

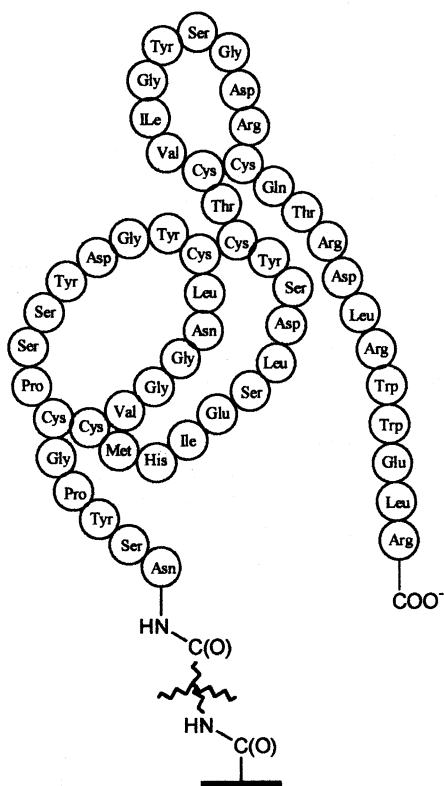


Figure 6: Structure of epidermal growth factor linked at the amino-terminus to a solid surface.

Sutures

Vascular grafts of woven and knitted Dacron that are now being modified with integrin recognition sequences, were discovered from research that originated with observations of Voorhees in 1952 (64). He hypothesized from observations of a silk suture coated with endothelial cells hanging free in the heart that cells would coat the fabric graft and thus resist the clotting problem. Thus a variety of fabrics and textile fibers were experimented with prior to the development of knitted Dacron arterial grafts currently used (65). In fact the progress of suture development throughout history parallels textile development in the many materials used to tie (ligate) or sew (suture) tissue. These materials range from bark and animal tendons to synthetic

polymers. More recently gene delivery via sutures of a DNA plasmid (66) to smooth and cardiac muscle was demonstrated to give efficient expression of protein by cells in the wound-healing environment.

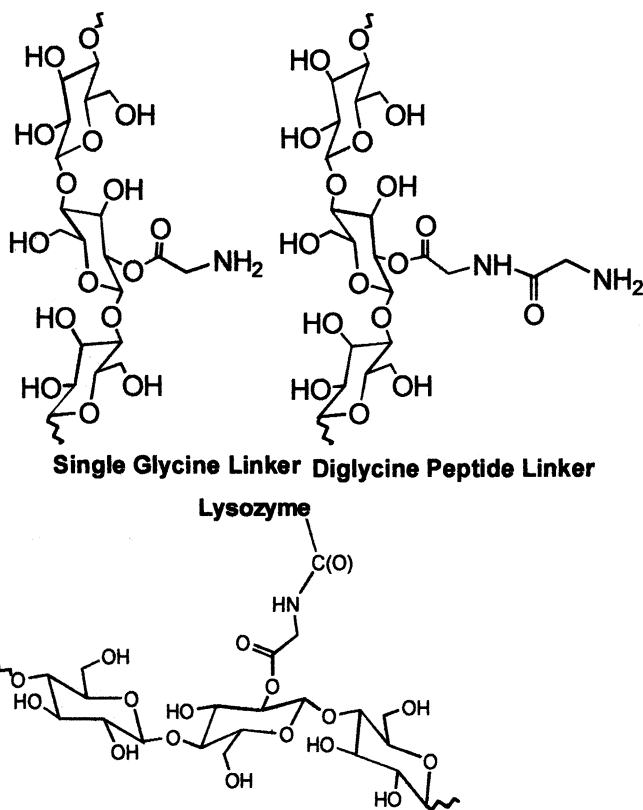


Figure 7: Structure of glycine-linked cellulose conjugates of lysozyme.

Mechanisms of Imparting Antimicrobial Activity to Fibrous Substrates

The mechanisms by which modified fibrous substrates resist microbial growth and contamination depend on the type of microbe (see detailed discussion in Chapter on Antimicrobial Agents: Retrospective and Prospective). It was recognized almost a century ago (Figure 1) that the acetylation of cellulose rendered it hydrophobic and thus blocked attack and degradation by fungal hyphae. However, the concept of

antibacterial and antimycotic (fungi pathogenic to humans) activity was not even formulated until 1941 (see also Figure 1). An important paper by Gagliardi (67) critically discusses strategies for producing antibacterial fibers by various techniques (homo- and copolymerization, resin treatment, covalent bond formation and the regeneration principle) by a controlled release mechanism. With very few exceptions, most commercial and experimental antibacterial and many antifungal materials operate through the controlled release mechanism. The challenge has been to have the release occur at a slow and predictable rate so that the materials would have long term durability to laundering and weathering. The regeneration principle defined by Gagliardi is that an inactive chemical species attached to the fiber could be continually regenerated by physical or chemical agents to become biostatic or biocidal. This concept was recently demonstrated by the attachment of a nitrogen-containing species to cellulosic fibers that were rendered antibacterial by formation of an N-Cl bond activated by bleach which is described in chapters fourteen and fifteen.

The first instance of a different mechanism for antimicrobial action for materials was an inherently biostatic coating based on a silicon polymer with a pendant quaternary ammonium group (see Figure 1) discussed in another chapter on antimicrobial agents. More recently, Vigo and Leonas described the antimicrobial action of fabrics containing crosslinked polyols as a physicochemical mechanism rather than a chemical effect caused by the inability of microbes to adhere to the coated surface of the fibers (68). Some of the more innovative approaches for imparting antimicrobial activity to fibrous substrates include compounds/polymers that trick the microbes into attaching to fake copies of carbohydrate receptors on cell surfaces, anti-adhesion compounds that block construction of microbial cell walls and chemicals that disorient biochemical signals to microbes (69). To accomplish these advances, more fundamental information is needed on how viruses attach to surfaces, particularly prions because of their difficulty in decontamination by most chemical agents.

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Chapter 2

Retention, Unfolding, and Deformation of Soluble Proteins on Solids

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The behavior of proteins on solids has been studied over several decades. Recent developments have expanded the variety of methods that can be used to examine protein-surface interactions. This paper summarizes the adsorptive behavior of proteins on various surfaces including cotton and synthetic polymers. In general, it is postulated that surfaces act like catalysts for protein unfolding. We give specific examples of the unfolding of cytochrome c and show a lowering of the unfolding temperature from solution to an anionic surface. Additionally, we show how chromatography can be used to screen adsorbents, such as cotton for possible commercial applications. The ability of each surface chemistry to act as a catalyst for unfolding is discussed.

This chapter focuses on the adsorption of proteins and discusses the application of protein adsorption to cotton. Cotton is a special natural product that is nearly pure cellulose. Table I displays the composition of cotton. Cotton was not used because of its high purity, but rather for the potential application as a gauze to assist in wound healing. Whether the purity of cotton helps its success in wound healing is not clear. However, using a pure substrate for adsorptive studies helps in the understanding of its adsorptive properties. Benefits to studying the adsorption of proteins to cotton include not only wound healing, but also fabric cleaning and staining.

PROPERTIES OF COTTON AND CELLULOSE

The molecular structure of cellulose is well known. Cellulose is typically non-ionic but has polar characteristics. Its hydrophilic properties help cotton resist excessive adsorption of water soluble proteins. This resistance is due to the matrix

having a greater attraction for water than protein. We modified cotton to enhance its binding properties for a specific protein, elastase. This allowed us to study the protein binding properties of various cotton derivatives (1).

Table I. Chemical Composition of Cotton

| <i>Composition of Cotton Fiber</i> | <i>Proportion of dry weight (%)</i> |
|--|---|
| Cellulose | 94.0 |
| Protein | 1.3 |
| Pectin | 1.2 |
| Wax | 0.6 |
| Ash | 1.2 |
| Other Substances | 1.7 |

The information for this table was from "Encyclopedia of Chemical Technology, 4th Edition, Jacqueline I. Kroschwitz executive ed., Copyright 1993 by John Wiley & Sons, Inc. Reprinted by permission of John Wiley & Sons, Inc.

THE IMPORTANCE OF UNDERSTANDING PROTEIN ADSORPTION

Protein adsorption is crucial to numerous industrial processes. Examples are biomaterial fouling and barnacle attachment. Barnacles adsorb after receiving chemical cues from the surface (2). Bacterial adhesion has also been attributed to protein adsorption and is known to take place in two stages. These have been described as adhesion of the extracellular membrane proteins in the primary and secondary minimum (2). The primary minimum involves molecular recognition. The secondary minimum is governed mainly by non-specific van der Waals forces.

Similarly, protein adsorption to biomaterials involves two stages. The first is a reversible binding in which the native protein retains its shape. The second involves protein unfolding or spreading on the surface (3). This stage is irreversible, although severe conditions can cause these proteins to desorb (4).

Protein adsorption from protein mixtures can be complex. For example, when plasma proteins from whole blood bind to biomaterials, albumin often binds initially and is later displaced by fibrinogen. Fibrinogen can then be displaced by other blood proteins (5). This is the Vroman Effect. Although the Vroman Effect was discovered for blood proteins, it may take place in other situations in which multiple components can bind.

PROTEINS

Proteins are polymeric amino acids and are the major component of dried living material. Some proteins are glycosylated (glycoproteins), still others are closely associated with lipids (membrane proteins, and lipoproteins). Each amino acid has a different chemical characteristic so that when they are assembled in a protein, they give the protein a unique series of chemical properties. Each protein varies by the

sequence and/or number of amino acids. The amino acid sequence is partially responsible for the tertiary (3D) structure. The other contribution to protein structure occurs during the folding process (6). The final, native 3D structure is typically near or at the lowest energy state of the polypeptide under native conditions.

Proteins can be classified in a number of different ways. For example there are enzymatic and non-enzymatic proteins. They can be classified by other means, such as those which are conjugated and non-conjugated, soluble and membrane-bound, or by their origin (viral, bacterial, plant, animal, blood, milk, etc.). There are believed to be about 40,000 different proteins in humans, with a large number of variations for each protein type. For example, it is believed that there are over 1 million different forms of the blood protein fibrinogen in each human (7).

This large variety of proteins and protein forms suggest that when different types of proteins are present, the adsorption of one or more of them to any given surface is more likely than if they all had similar properties. Each surface may have an affinity to at least one of the thousands or millions of proteins available.

WHEN DO PROTEINS ADSORB?

Proteins are more likely to adsorb when surfaces have the least attraction for water. Water can prevent proteins from reaching a surface, minimizing protein adsorption (8). When protein adsorption does occur, the subsequent biological response depends on how the binding takes place. That is, the details of the adsorption process are important. An example is the adsorption of blood proteins and blood coagulation. Surfaces with either high or low interfacial energy with water cause proteins to adsorb, but only surfaces with low interfacial energy with water accelerate blood coagulation (9). Some implants appear to resist coagulation more than others, probably due to the manner in which proteins bind to them. Another important consideration is how strongly water binds to the surface. A strongly bound water layer can prevent proteins from penetrating and binding. Yet, even strongly hydrophilic supports will promote protein binding and a biological response (6). This needs to be investigated further in order to improve the life expectancy of implant materials.

Protein adsorption is a well-established field that melds the disciplines of surface science and biochemistry. In this field, significant knowledge can be gathered from some simple measurements. For instance, proteins can be loaded onto a surface from solution and the total amount of absorbed protein can be measured. This is the approach used for developing adsorption isotherms. Example isotherms are shown in Figure 1. Here, proteins were adsorbed either to a porous diethylaminoethyl (DEAE) derivatized agarose, or alumina. From these examples, one can observe that large amounts of protein can be bound to the DEAE support (Figure 1a) except when high concentrations or amounts of NaCl are present (IgG, Figure 1b). However, albumin and fibrinogen remained bound after introducing 0.15 M NaCl, indicating they would not desorb under these near-physiological conditions. In contrast, IgG would not bind at that concentration, due to the ability of NaCl to occupy its ionic binding sites.

ADSORPTION ISOTHERMS

Adsorption isotherms are used not only to examine the amount of binding on a surface, or in a sorbent, but also to estimate the number of layers bound. When the protein structure or dimensions are known, and the surface is non-porous, plateaus in the isotherm (such as those observed in Figure 1c) could help elucidate the molecular orientation (assuring close-packed monolayer, bilayers, and etc. are formed). Figure 1 compares the amount of protein bound on alumina and a porous DEAE support. Surface area is usually a known variable used to create an isotherm. For this study, the mass of the beads was known, but not the surface area. Therefore, mass was used in place of surface area. When the size of the adsorbed molecule is much smaller than the dimensions of the pores, the surfaces of the pores are available for adsorption. However, proteins can be larger than some pores, which restrict the available total area. The area, including that from the pores,

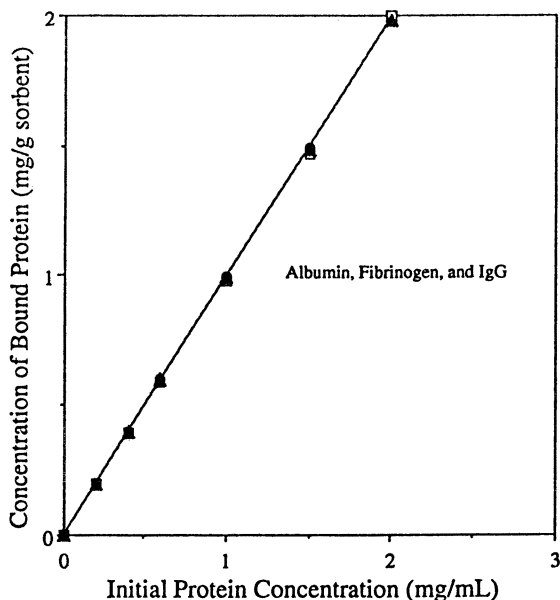


Figure 1a. Adsorption Isotherm for Albumin, Immunoglobulin G, and Fibrinogen in Dilute (5mM) Tris Buffer (pH 7.4) on DEAE BioGel A.

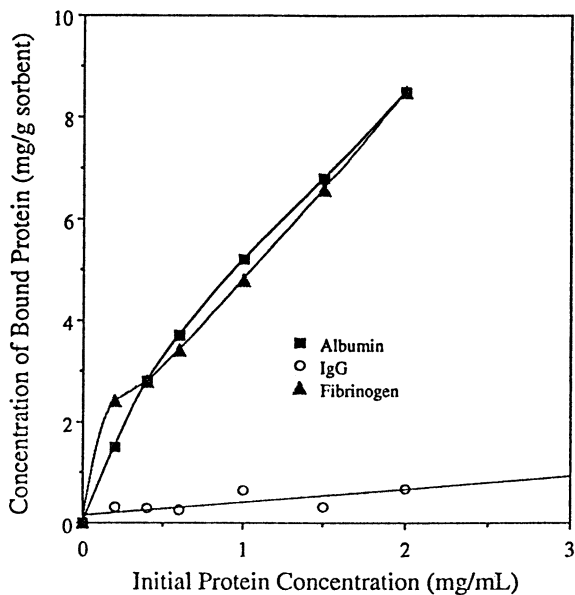


Figure 1b. Adsorption Isotherm for Albumin, Immunoglobulin G, and Fibrinogen in 0.15 M NaCl and (5 mM) Tris Buffer (pH 7.4) on DEAE BioGel A.

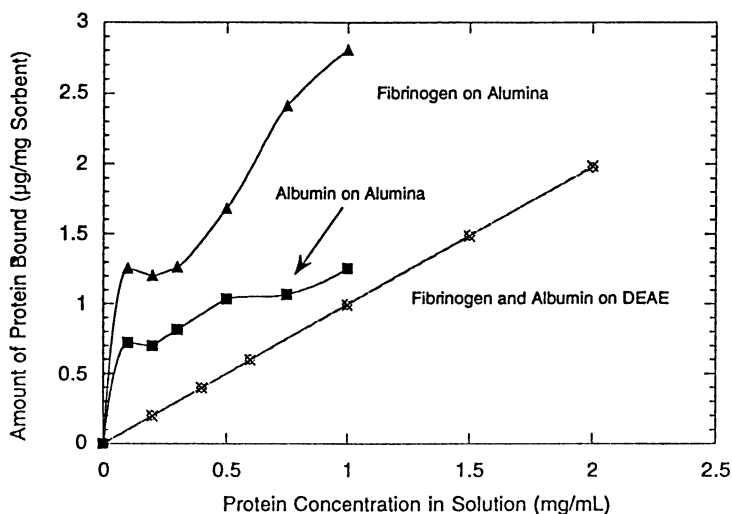


Figure 1c. Comparison of Adsorption Isotherms from Alumina and DEAE for Fibrinogen and Albumin in Dilute (5 mM) Tris Buffer (pH 7.4).

must be included when the area is indicated in an isotherm. Pores complicate the determination of available surface area and the accurate interpretation of the isotherm. For alumina (Figure 1c), the pores were much smaller than the diameter of either of the blood plasma proteins shown. However, the pore size of agarose varies in commercially available gel permeation products (Figure 1-c) but in this case it was large enough (Bio-Gel A-0.5m, Bio Rad Laboratories, Hercules, CA, exclusion limit = 500,000 daltons) for most proteins to penetrate the beads. The significance of this difference is that the DEAE support could adsorb a greater protein mass due to its greater surface area per gram of material. However, alumina bound more protein than the porous DEAE, possibly due to the formation of multiple layers of protein on the alumina surface.

OTHER METHODS FOR STUDYING PROTEIN ADSORPTION

Adsorption isotherms are often used to define the amount of material adsorbed under equilibrium or steady state conditions. Other methods of studying surface properties include examining the surface tension. Surface tension influences the extent and rate of protein adsorption (10). The surface tension between water and a solid can be measured by placing a drop of water on the surface and measuring the contact angle. The contact angle is related to the affinity of the water for the surface. The water adhesion tension is negative for hydrophobic surfaces, resulting in a greater

contact angle. Polar surfaces such as silica have a much higher water adhesion tension, resulting in water spreading.

It would be difficult to investigate the interfacial tension between a protein in solution and a surface in this manner. Proteins are generally in their native structure when they are in an aqueous buffer. When a protein binds to the surface in a multi-component solution (protein, buffer, and water), the interfacial tension changes, as does the contact angle. In such a dynamic system, the contact angle would have to be followed with time and the data may be difficult to interpret. However, the surface tension between water and various solids has been measured, and correlated with various surface phenomena for which protein adsorption is believed to be responsible. One example is blood coagulation (9). Here, blood was believed to coagulate more rapidly when the water adhesion tension of a surface was increased. Protein binding does not necessarily correlate with biological response. There is another factor, which is probably related to the orientation and organizational structure of the bound protein.

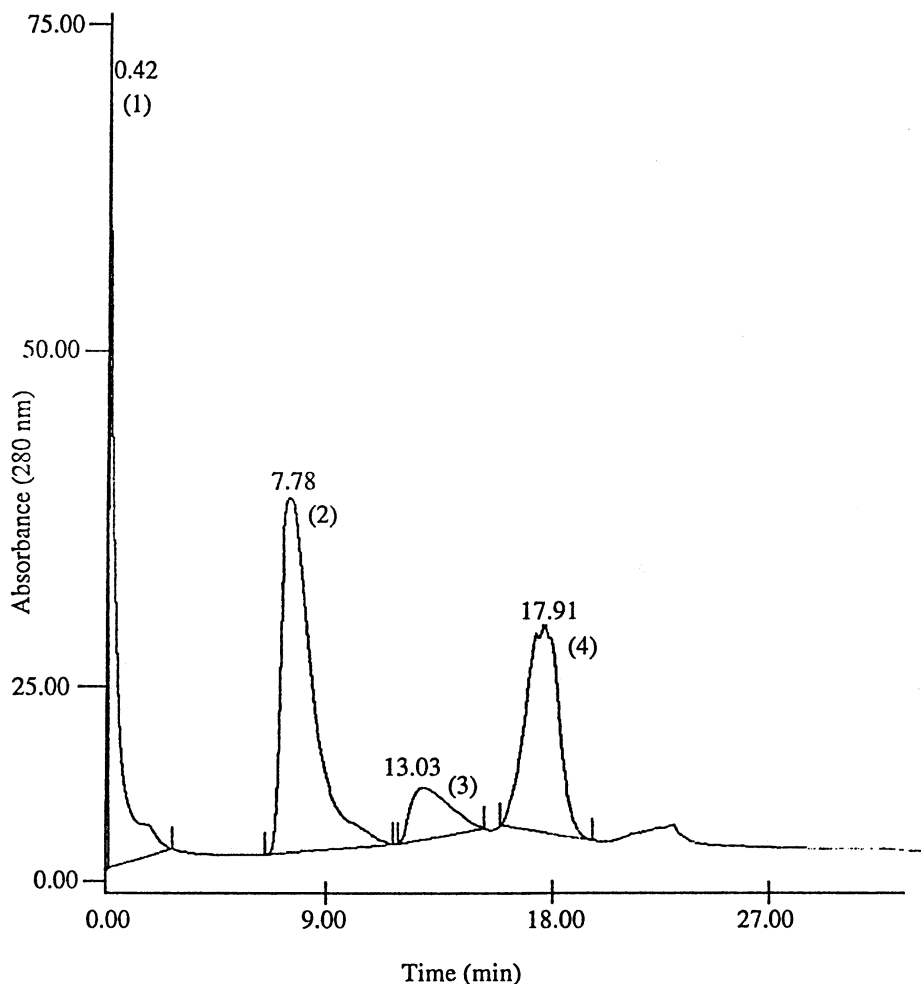
Liquid Chromatography

Surface-protein interactions have also been studied by a variety of more recent technologies such as ellipsometry, NMR, fluorescence, Auger spectroscopy, etc. A method we have used to study protein adsorption is liquid chromatography (LC). LC is typically used to perform separations to help purify components of a mixture. The separation mechanism is by adsorption-desorption processes between the analytes and packing material surface. This affinity, or recognition process, is commonly used to purify proteins. LC allows us to examine three different properties of protein adsorption. One of these properties is the capacity of the material to adsorb protein. The same parameter is measured by adsorption isotherms (11). LC also allows the exploration of the relative retention of proteins on a surface chemistry (12). A third property examined by LC is the kinetics of the unfolding of proteins on surfaces (13, 14).

Measuring the capacity of a sorbent for protein by LC involves monitoring the breakthrough during continuous loading (11). A family of data points can be generated in this manner in which either the temperature or eluent concentration is varied. A large amount of information can be gathered this way, but care must be taken to quantify the more and less permanently bound protein, since losses for some proteins is a time-dependent phenomena (13, 16). Other phenomena for protein losses to surfaces may be concentration and temperature dependent (10).

Relative retention of proteins is a typical measurement in LC, but its relevance to sorption is often under-emphasized. For many, the purpose of LC is in the separation and purification of components, such as proteins. The sorbent properties are not the focus of the study, and become a secondary consideration. However, for the surface scientist, elution order and relative retention help define the binding properties of the protein for the sorbent. One example of the significance of this approach is in the testing of biomaterials for protein adsorption. An easy method for testing whether a protein will bind would be to pass it through a column containing particles of the biomaterial in the presence of physiological buffer. Those proteins which elute will not adsorb. Another more definitive approach is to use a gradient that passes through

the physiological strength buffer. Using this approach, the relative binding strength can be determined (13). To illustrate this, Figure 2 shows a chromatographic separation of the three major proteins in blood plasma. These are albumin, immunoglobulin G (IgG), and fibrinogen. The sorbent is a weak ion exchange material, DEAE. Approximately one out of every 100 or 200 available sites on the



*Figure 2. Typical Chromatographic Separation of Proteins from an Ion Exchange Support using a Linear Salt Gradient. In this chromatogram, the first two peaks (1 and 2) are IgG, the next peak (3) is albumin, and the final peak (4) is fibrinogen. The physiological salt concentration was surpassed in the linear gradient around 10 minutes, between the elution of IgG and albumin. Separation conditions included elution at 37°C. This figure was reprinted from *Journal of Chromatography*, Vol. 186, S. C. Goheen and J. L. Hilsenbeck, *High-performance ion-exchange chromatography and adsorption of plasma proteins*, pages 89-96, 1998 with permission from Elsevier Science.*

surface of this non-porous material contains the DEAE functionality. On this weak ionic material, albumin binds at the start of the gradient (5.0 mM Tris buffer, pH 7.4 at 37°C). IgG, however, does not. Both the remaining IgG and fibrinogen elute after 0.15 M NaCl has been reached, indicating they would have bound to this sorbent if it had been placed in the blood stream. Sorption of IgG and fibrinogen to this particular ion exchange material has not been confirmed, but this approach allows one to easily predict whether sorption will take place or not.

The third protein adsorption parameter that can be measured by LC is the surface mediated unfolding property. Normally, as a protein or other analyte is held in a LC column and the flow of solvent continues across the sorbent, the analyte drifts through as in isocratic separations. However, proteins are large molecules which are potentially much more flexible than smaller substances. This allows the proteins to adsorb more strongly as a function of time and to become delayed as they are held for longer periods of time on a LC column. Cytochrome c is a relatively small protein (MW 12,400) which nicely shows this drift effect. Figure 3 shows that for two different temperatures (0 and 85°C), proteins kept on the column for longer periods of time required an increase in solvent strength for elution. The data also shows that the flexibility of the protein was enhanced at higher temperatures. As the protein increased its flexibility, it bound more strongly to the sorbent, requiring a greater solvent strength for elution [see, for example, (16)].

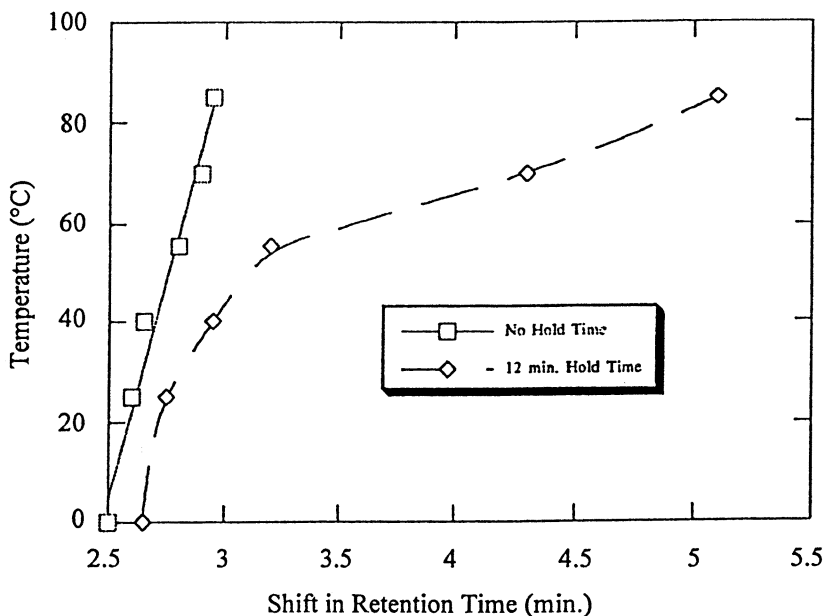


Figure 3. Drift in Retention Time with Temperature and Hold Time for Cytochrome C. The hold time was adjusted by delaying the start of the salt gradient [16].

Many proteins, when exposed to a sorbent material, will bind almost irreversibly. The surface-mediated unfolding of papain, soybean trypsin inhibitor, and lysozyme on reversed phase supports has been studied. In all cases, native and denatured peaks were widely separated from one another (14). However, even in weakly ionic supports there are losses due to protein unfolding. In some cases this is a time dependent process, as can be seen for cytochrome c in Table II. Cytochrome c was held on an anionic column surface for varying lengths of time and encouraged to desorb using a salt gradient, identical to the conditions used to generate Figure 3 (16). This figure demonstrates that after a few minutes, some of the cytochrome c unfolds so completely on the ionic sorbent that it does not desorb with the rest of the protein (using a salt gradient of up to 1 M NaCl). As the sorption time increased, so did the loss of the protein to the sorbent material. It is surprising that the process for protein unfolding is so slow. However, the kinetics for the unfolding of cytochrome c has been previously described as a potentially very slow process (17).

The surface-mediated unfolding of proteins as measured by LC can be better visualized in Figure 4. The three major plasma proteins were chromatographed using various gradients to accelerate or delay their time of desorption. The longer their desorption was delayed (at 37°C), the greater the loss, or the smaller the amount

Table II. Protein Losses with Retention Time for Cytochrome C at 85°C.

| <i>Retention Time (minutes)</i> | <i>% Loss</i> | <i>% Error</i> |
|-------------------------------------|---------------|----------------|
| 0 | 5 | 3 |
| 3 | 4 | 1 |
| 6 | 25 | 13 |
| 9.5 | 21 | 8 |
| 11.5 | 26 | 10 |
| 15 | 28 | 11 |
| 17 | 33 | 14 |

of eluted protein. Loss of protein in LC is probably due to surface-mediated unfolding (14), although for these specific examples unfolding of proteins has not been confirmed by spectroscopic techniques. Note that fibrinogen was lost more rapidly and more completely than albumin or IgG. From this data, it appears that the kinetics of unfolding are related to the molecular weight of the protein. The relationship shown in Figure 5 developed when we examined the amount of unfolding that took place at 37°C for these proteins, as well as for cytochrome c, on a weak ion exchange material. Although there are only four proteins in this graph, it appears that there is a trend for larger proteins to unfold more rapidly than smaller ones. While there are likely to be deviations from this relationship, we expect this trend to hold for most proteins.

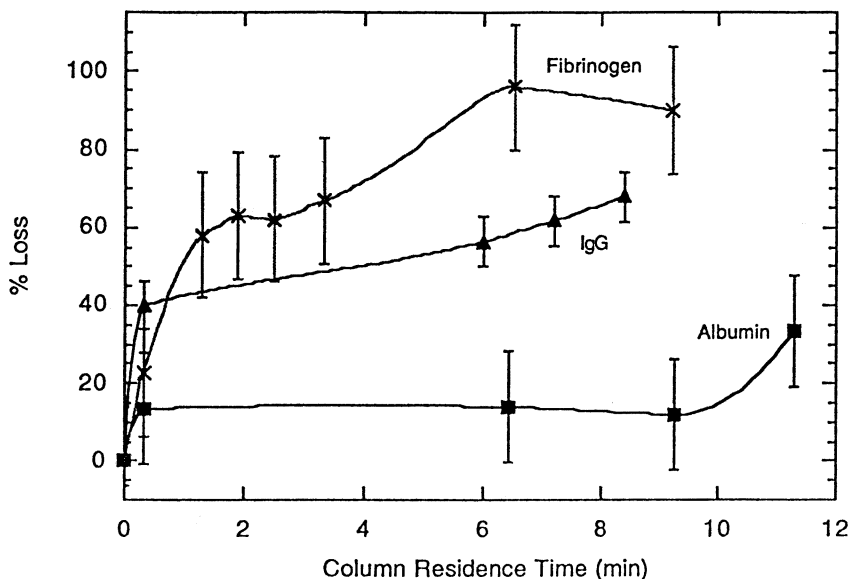


Figure 4. Protein Loss Kinetics on DEAE. Error bars indicate average standard deviation values for each protein. This figure was reproduced from Journal of Chromatography, Vol. 186, S. C. Goheen and J. L. Hilsenbeck, High-performance ion-exchange chromatography and adsorption of plasma proteins, pages 89-96, 1998 with permission from Elsevier Science.

PROTEIN ADSORPTION STUDIES ON COTTON

Cotton is a much weaker ion exchanger than the DEAE or SP supports described earlier. In its underivatized state, cotton is hydrophilic and nonionic. We have confirmed this by packing underivatized cotton into an empty HPLC column, then introducing proteins. Elastase did not bind, even under weak buffer conditions. However, when cotton was derivatized with an elastase-recognition-sequence, elastase was retained.

Elastase binds to the peptide Val-Pro-Val and it was anticipated that this peptide would sequester elastase if the peptide was bound to cotton. We attached Val-Pro-Val to cotton so that either the carboxy terminal or amino terminal end would be exposed to the solution. In testing these two peptides, the COO-terminal peptide bound more elastase than the NH₂ terminal form. Finding the correct orientation of the Val-Pro-Val peptide on the solid may help elucidate the biochemical process by which the inhibitory Val-Pro-Val sequence binds and reduces elastase activity.

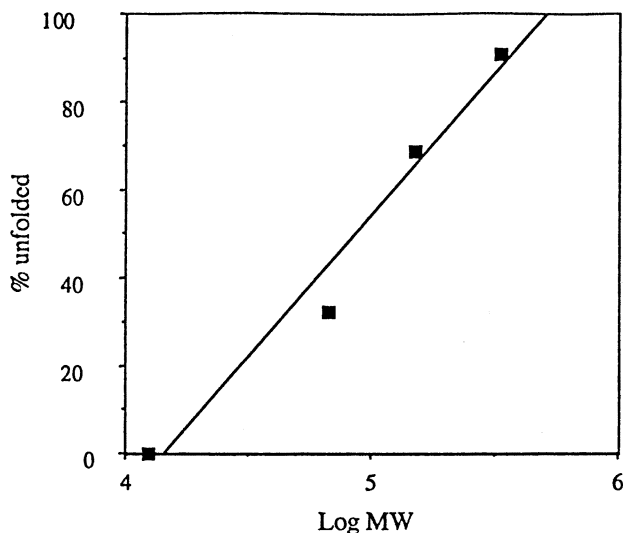


Figure 5. Extent of Protein Unfolding with MW. The unfolding kinetics are depicted here at 9 minutes of unfolding at room temperature for each of 4 proteins: cytochrome c, bovine serum albumin, immunoglobulin G, and fibrinogen. The correlation coefficient (R^2) for this curve was 0.97.

It was difficult to determine whether elastase underwent simple adhesion or unfolding in these experiments. Elastase was either retained or unretained. Since our application was to develop a new wound treatment, it was only necessary to determine when the elastase would be removed from a solution, not whether structural changes occurred.

For these studies, cotton was desized, scoured, and bleached before use. The fabric was also mercerized prior to synthesis and pulverized in a Wiley Mill for chromatographic analysis. Packed column dimensions were approximately 1 x 1 cm.

The reason we studied the binding of elastase to various derivatives of cotton was to develop an improved wound dressing (18). Elastase is a proteolytic enzyme with a molecular weight (25,000) between that of albumin (35,000) and cytochrome c (12,400). In chronic wounds, elastase digests neutral aliphatic residues such as those in fibronectin and important growth factors. It is released excessively by neutrophils in the healing process of some chronic non-healing wounds. When elastase levels are excessive in these wounds, they are believed to interfere with healing. The sequestration of elastase in bandages may accelerate healing for these patients with chronic non-healing wounds.

In these studies, we packed cotton into empty columns and eluted the elastase over the cotton derivatives with buffers of physiological strength. Much of the elastase did not bind to the cotton sorbent, but that which did was not easily desorbed. Elastase did not desorb even when 1.0 M of NaCl was used as an eluent. Residual elastase was desorbed using 6 M urea (18). As more elastase was passed over the sorbent, less and less binding occurred, but binding continued to take place as long as

the total capacity of the sorbent was not met. In this case, the LC technique allowed us to screen several different materials for their ability to sequester elastase. The carboxymethylated conjugate with the Val-Pro-Val-Ome sequence bound the most elastase (nearly 60%), with others such as Val-Pro-Val-Gly-cellulose, or carboxymethylated cellulose, binding much less. This approach has allowed us to identify a potential candidate for bandage materials for chronic wounds. The binding properties of other proteins on cotton can be easily tested by similar methods.

Cotton, being almost pure cellulose, behaves as a gel permeation support (19). It is non-ionic, though polar, with little or no hydrophobic characteristics. These properties have been tested but could also be quantified by chromatography using NaCl and ammonium sulfate gradients with proteins known to bind to ionic and/or hydrophobic supports.

CONCLUSION

We have discussed a number of characteristics that proteins possess as they adsorb to solid materials. Many of these can be examined by LC, as well as a variety of other techniques. LC allows us to measure recoveries and retention times, and may some day be able to provide quantitative values on how strongly a protein adsorbs. This is not currently the case, but from elution conditions, it seems feasible to be able to determine quantitative binding strengths. Certainly, the order of elution can tell us relative binding strengths. Through simple calibration processes this seems feasible. We can also measure whether the binding is ionic or hydrophobic simply by changing the eluent. LC can also be employed to measure the protein capacity of the sorbent. This tool can also be used to study the kinetics of surface-mediated unfolding. LC can also study the flexibility of the protein near the binding region at various temperatures. Finally, we have some preliminary evidence that LC can help us probe some structural features of active regions of proteins (such as the binding site of elastase). Overall, LC is a powerful tool for investigating surface-protein interactions.

The interactions we have observed for proteins on surfaces can be divided into two categories: sorption and unfolding. During our studies of cytochrome c, we observed that unfolding on a surface occurred at a lower temperature than it does in solution. We were not able to determine whether the conformational change on the sorbent was the same as it was in solution at a slightly higher temperature. However, there was a lowering of the kinetic energy requirement for unfolding to occur. We can express this relationship graphically in Figure 6, where both the activation energy and the energy of the product are lowered by the presence of a surface. Proteins that have unfolded on a surface are at lower energy than similar unfolded states in solution, as evidenced by the lowering of the unfolding temperature. Furthermore, we suppose that the activation energy barrier is also lowered, as is commonly the case for catalyzed reactions. Therefore surfaces act like catalysts for protein unfolding. Once we have a better understanding of protein adsorption, we can learn how to control it. With better understanding, we may be able to better control our design of adhesives, regulate fouling, and enhance biocompatibility.

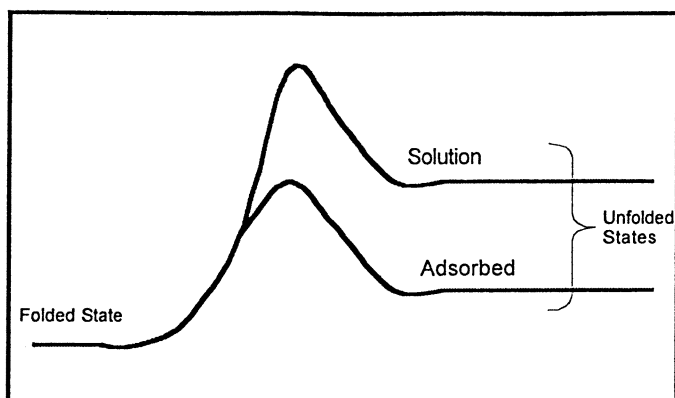


Figure 6. Influence of surfaces on energy requirements for protein unfolding. The y-axis is the free energy of the protein. The folded state in solution is depicted at the left. The unfolded state can be more favorable on a solid support than in solution, with the activation energy requirements lowered as well.

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Chapter 3

A Novel, Enzyme-Based Method for the Wound-Surface Removal and Decontamination of Organophosphorus Nerve Agents

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Organophosphorus hydrolase (OPH, EC 3.1.8.1) enzymes were covalently bound to chemically-modified cotton fabrics to produce a wipe that rapidly hydrolyzed four different organophosphate neurotoxins including a Sarin analogue, diisopropyl fluorophosphonate (DFP), and a VX analogue, demetonS. The immobilization was performed with both organic and aqueous-phase strategies. The fabrics were stable over time, retaining up to 70% of their starting activity after two months of storage at 4°C and at ambient temperature. OPH immobilized onto cotton provides an effective countermeasure against acts of domestic terrorism in which chemical threat agents are dispersed on solid surfaces, and could be an integral tool for emergency response teams to remove and decontaminate nerve agents from skin, wounds, and other sensitive surfaces.

Introduction

The widespread use and extreme toxicity of organophosphorus (OP) neurotoxins pose an environmental challenge for remediation and detoxification. The organophosphorus-degrading gene of *Pseudomonas diminuta* encodes the dimeric, metalloenzyme organophosphorus hydrolase (OPH), which catalyzes the breakdown of OP neurotoxins including the phosphorus-fluorine bond of Soman and Sarin, and the phosphothioates such as VX (1-3). Catalytic rates (k_{cat}) for these compounds have been shown to range from rates that are diffusion limited (e.g., paraoxon, P---O bond,

$k_{\text{cat}} = 15,000 \text{ s}^{-1}$) to rates that are several orders of magnitude lower (e.g., acephate, P--S bond, $k_{\text{cat}} = 0.258 \text{ s}^{-1}$) (1-4). This broad substrate specificity and the high catalytic turnover rates for OP neurotoxins make OPH a promising candidate for ameliorating exposure to these compounds.

The genes for other OP-hydrolyzing enzymes have been cloned and their products purified (5,6); however, OPH has been the most extensively studied enzyme. It is the only enzyme known to hydrolyze the P--S bond at significant rates (7,8). The catalytic mechanism of OPH proceeds via a S_N2 process in which an activated water molecule attacks the phosphoryl center of the substrate (Figure 1) (9). X-ray crystallography studies have described OPH as a dimeric metalloenzyme that contains two equivalents of zinc per monomer in its native form (10-12). The overall folding pattern of the monomer consists of an α/β barrel with eight strands of a parallel β sheet. Each active site has a binuclear metal center, and the native Zn^{2+} can be replaced by several metals, including Co^{2+} , Mn^{2+} , Cd^{2+} , or Ni^{2+} , with varying effects on rates of paraoxon hydrolysis (13-14). The functional catalytic role of the metal centers have also been investigated by ^{113}Cd NMR (15) and site-directed mutagenesis (16-18).

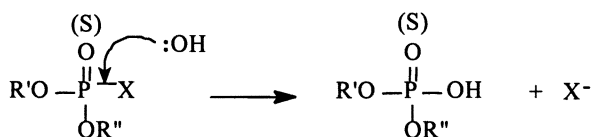


Figure 1. General scheme of hydrolysis for OP compounds. A hydroxyl radical attacks the phosphorus center of the substrate, displacing the leaving group, X. R is any alkyl group.

OPH has also been shown to be a very stable enzyme with a conformational stability estimated to be 40 kcal/mol, the highest stability reported for a dimeric protein (19).

The effects of substitutions near the active site of OPH on enzymatic function were characterized in detail (20). The original histidinyl residue at position 254, when changed to an arginyl residue (H254R) was found to be responsible for the improvement of the catalytic activity and specificity of OPH four fold against demeton-S, the analog for the chemical warfare agent VX (20). Similarly, the original histidinyl residue at position 257, when changed to a leucinyl residue (H257L) improved the catalytic activity and specificity of OPH more than eleven fold against NPPMP, the analog for the chemical warfare agent soman. The enzyme with both changes incorporated, H254R/H257L, showed greater than sixteen-fold and thirty-fold improvement of both catalytic activity and specificity, respectively, for demeton-S as compared with the wild type enzyme. These results demonstrated the ability to significantly enhance the specificity of OPH for various substrates by site-directed modifications.

Since the enzyme is capable of hydrolyzing a broad spectrum of OP compounds and is extremely stable, it has achieved considerable attention as a bioremediation agent for the decomposition of stockpiled and non-stockpiled OP nerve agents. This has prompted considerable research into the immobilization of OPH onto several solid supports to be used as solid-phase, flow-through reactors for nerve agent detoxification and disposal. These supports include nylon, trityl agarose, and polyurethane (21-24). The enzyme has also been successively cross-linked into polyurethane sponges and foams for nerve agent cleanup on structural surfaces (25-27). The present work describes the preparation of cotton towelettes to which both the wild type and the H254R/H257L enzyme have been covalently bound. These OPH fabrics rapidly hydrolyzed four different OP compounds including a Sarin analog (DFP) and demeton-S.

Materials and Methods

Materials

All chemicals used in coupling OPH to cotton were purchased from Aldrich-Sigma and were used without further purification. Paraoxon and DFP were obtained from Sigma (St. Louis, MO). Paraoxon was further purified as described (20). Demeton-S (97% pure) was purchased from ChemService (Westchester, PA). Dr. J. Vincent Edwards at the Cotton Textile Chemistry Research Unit, Southern Regional Research Center, Agricultural Research Service, New Orleans, Louisiana provided glycylic- and bisGlycyl-cotton.

Protein purification

OPH enzymes were purified in the presence of cobalt chloride as described (20). OPH fractions pooled after the first chromatography step (SP-Sepharose) were designated as "impure" OPH. The purity of OPH was determined by SDS-PAGE stained with Coomassie brilliant blue. The concentration of pure protein was estimated using the molar extinction coefficient for OPH ($\epsilon_{278} = 58,000 \text{ M}^{-1}\text{cm}^{-1}$) and concentrations of impure samples were determined using the Bradford method of protein determination (28).

Immobilization of OPH onto cotton

Organic Phase Coupling

OPH (2.6 mg) in 400 microliters of 10 mM phosphate buffer, pH 7.0, was lyophilized and reconstituted in 1 ml of anhydrous *N,N*-Dimethylformamide (DMF). The solution was kept on ice and 50 mg of 1,1'-carbonyldiimidazole was added and the

reaction was allowed to proceed for two hours. 200 mg of glycyl-cotton pre-swollen in DMF was introduced into the mixture. The reaction was allowed to continue overnight at 4°C. A control experiment was also conducted in which the coupling agent, 1,1'-carbonyldiimidazole, was excluded from the reaction sequence. Cotton fabrics were washed extensively in 10 mM phosphate buffer, pH 7.0, and used directly in enzymatic assays.

Aqueous Phase Coupling

Samples of cotton were weighed and soaked in sterilized water for 24 hours. Excess glutaraldehyde (25% aqueous) was added on ice at 0.5 ml per 60 mg of cotton. The samples were shaken for one hour followed by refrigeration for two to three days. Cotton samples were washed thoroughly with 10 mM phosphate buffer, pH 7.0. 250 micrograms of OPH in 0.5 ml were added to approximately 60 mg of cotton. A control cotton sample (i.e. not treated with glutaraldehyde) was also incubated with OPH under similar conditions. Samples were shaken for one hour in an ice bath followed by refrigeration for two to three days. Cotton was washed with buffer solution extensively to remove unbound enzyme. All washings were saved for analysis of recovered protein. The fabric was then stored at 4°C.

Determination of the amount of OPH immobilized on cotton

The amount of OPH bound to the fabric was quantitated to yield micrograms of OPH per square centimeter of fabric using the colorimetric Bradford method (28). This was an indirect method achieved by quantitating the amount of protein that was recovered in the solution from the coupling and subsequent washing steps, and subtracting this value from the initial amount of protein used in the reaction.

Measuring paraoxon hydrolysis

The hydrolysis of paraoxon (a P-O bond breakage) was monitored by incubating each cotton sample in five milliliters of 1 mM paraoxon in 20 mM CHES, pH 9.0, at 25°C with constant shaking at 150 rpm on a Lab-Line Junior Orbit Shaker. Aliquots were removed at various time points and the amount of p -nitrophenol released as a cleavage product was quantitated at 400 nm and converted to concentration using Beer's law and an extinction coefficient of $17,000 \text{ M}^{-1}\text{cm}^{-1}$.

Measuring demeton-S hydrolysis

The same cotton samples used in the paraoxon assays were washed extensively in phosphate buffer until paraoxon activity was undetectable and were then used to monitor demeton-S hydrolysis. When hydrolyzed, demeton-S (requiring P-S bond

breakage) yields a product that contains a free thiol group. This thiol group reacts with 2,2'-dithiodipyridine (2-TP) to form the corresponding 2-thiopyridone, which absorbs at 343 nm (29). The cotton samples were incubated in 5 milliliters of 1 mM 2,2'-dithiodipyridine (2-TP) in tripart buffer (50 mM MES, 25 mM N-ethyl morpholine, 25 mM diethanolamine), pH 8.0, at 25°C. Aliquots were removed at various time points and the enzymatic rates were determined based on the ultraviolet absorption formed using an extinction coefficient of 7060 M⁻¹cm¹ (29).

Measuring DFP hydrolysis

Assays with DFP (a P-F bond cleavage) were performed in a five milliliter reaction chamber with constant stirring at 25°C, in 500 mM KCl and 50 mM HEPES, pH 7.2. Release of fluoride was monitored with a fluoride-specific electrode from Orion Research, Inc. (Beverly, MA). The system was calibrated by measuring the signal from the electrode in standard fluoride solutions with concentrations ranging from 10 to 1000 μM fluoride. A Semi logarithmic plot of fluoride concentration versus signal in millivolts was fit to the following equation: $y = a + b (\log [F^-])$ in which [F⁻] is the concentration of fluoride in micromoles, a and b are constants, and y is the signal in millivolts. Rearranging to solve for [F⁻] gives a formula, which was used to convert recorded signal to fluoride concentration in the reaction at any time point. The reaction was initiated by adding the cotton samples into the chamber, and the resulting signal was recorded every four seconds and converted to concentration of fluoride released by fitting data to a standard curve. The slope of the line obtained by plotting fluoride concentration against time (dF/min) was employed in the calculation of reaction velocity.

Measuring the stability of the OPH fabric

The capability of the fabrics to retain catalytic activity was monitored with time. Assays were conducted on the specified days using paraoxon as the substrate and performed exactly as described above. However, time points were not taken and the reaction incubation time was constant, and in most cases was sixty minutes. The catalytic activities determined for each sample were then compared to the sixty-minute time point in the initial kinetic experiments, and the percentage of retained activity was then determined.

Stabilization of OPH fabric

OPH fabrics were prepared using aqueous chemistry as described above and were cut into small pieces. These pieces were separated into five different groups, with ten pieces each, and each group was prewashed with a sugar solution that

contained mannitol, sorbitol, trehalose or cellubiose. A control group was prepared that was prewashed with just PBS. The groups were lyophilized to dryness and stored at room temperature. At the time points indicated a piece of fabric was removed from each group and the activity was determined against paraoxon as described above.

Results and Discussion

The solid support: Glycyl-cotton

Cotton is a well-characterized fiber and commonly used by the medical community for various bandages and swabs. It is also extremely absorbent and can retain many times its own weight in water. The non-toxic characteristics make it very suitable for clinical applications and as a natural fiber is durable and completely biodegradable.

The reactivity of three hydroxyl groups at positions 2, 3, and 6 on the glucosyl unit of cellulose offers a variety of possibilities for making useful derivatives. Cellulose polymers with predetermined properties are made by appropriate derivatization. The desired properties are achieved by substituting on the average only one or two of the three hydroxyl hydrogens (30). Soluble derivatives of cellulose have been prepared like acetate (31) and carbamate (32). A variety of processes for incorporating amino groups in cotton have been investigated (32). Chemical modifications of the fiber to incorporate tertiary and quaternary amino alkyl groups leads to better dyeing properties (33).

Glycine derivatized cotton was selected as the solid support for our application because the primary amine functionality would provide many options for further derivatization. The incorporation is as high as 100 micrograms (approximately 2 micromoles) of glycine per gram of cotton twill as judged by amino acid analysis. Two types of modified cotton were utilized here. One is referred to as mono-gly indicating one glycine residue used in the derivatization process and the other is a bis-gly indicating two glycine residues in tandem used in the derivatization reaction. The term glycyl-cotton is a general term referring to both mono- and bis-gly cotton.

OPH immobilization on Glycyl-cotton fabric

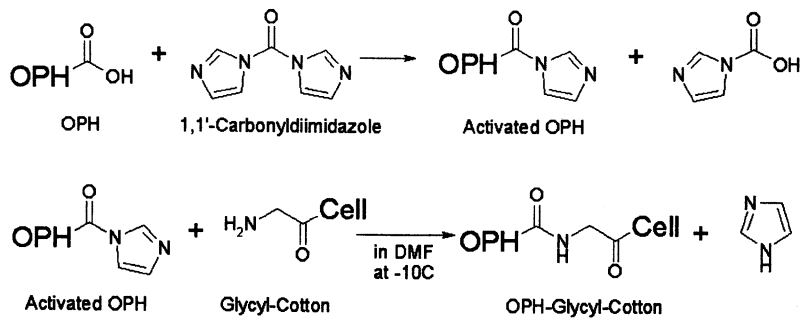
A purified and a crude, cell-free preparation of wild type (WT) OPH were used in the immobilization reactions. Only a purified preparation of the H254R/H257L

enzyme was immobilized because an impure preparation of this enzyme loses activity within a few hours as compared to weeks for the purified enzyme. Therefore, we did not expect the immobilization to be as effective under impure conditions for this particular enzyme.

An organic and an aqueous phase glutaraldehyde coupling of OPH to glycylicotton fabric were performed. The reactions are indicated in Figure 2. In the first step of organic phase coupling, an activated enzyme is prepared by forming an active ester on carboxyl groups of OPH with 1,1-carbonyldiimidazole. The second step involves the addition of the glycylicotton fabric to the activated enzyme. The amino group in the cotton displaces the imidazole of the active ester, forming a peptide bond between the enzyme and the cotton fabric. Both of these reactions are in anhydrous DMF at -10°C (Figure 2A). An aqueous, 2-step coupling procedure was also developed for mono-gly and bis-gly cotton fabric. The reactions consist of the pretreatment of the cotton with glutaraldehyde, which forms a Schiff base with the primary amine in the cotton (Figure 2B). The fabric is then extensively washed to remove unbound glutaraldehyde. The second step is the addition of OPH to the fabric. Coupling occurs when one of the many free amino groups of OPH reacts with the pendant aldehyde on the cotton, forming another Schiff base linkage. Both steps are performed in a phosphate buffer at pH 7 and 4°C , which is a considerably milder environment than the organic phase coupling procedure.

The amounts of OPH immobilized onto the fabric by either the organic or aqueous phase coupling method were determined and the results are presented in Table I. Eight times more WT OPH is immobilized by the aqueous method than the organic method. The two reactions involving aqueous chemistry and pure WT OPH yielded an average of $50\ \mu\text{g}$ OPH per cm^2 fabric while the organic coupling method and pure OPH yielded an average of only $6\ \mu\text{g}$ OPH per cm^2 fabric. It is not clear why the aqueous method is more effective at immobilizing OPH onto cotton; however, for all subsequent kinetic characterizations only cotton prepared by the aqueous method was utilized. Similarly, the immobilization of the H254R/H257L enzyme averaged $32\ \mu\text{g}$ OPH per cm^2 fabric. The organic method was not utilized with this enzyme since that coupling method was much less efficient. There is no significant difference between the mono- or bis-gly cotton regarding immobilization efficiency. The data also reveals, as expected, that when the cross-linking agent is omitted from the coupling reaction the amount of OPH immobilized drops considerably. The activity observed with these control samples most likely are due to non-specific interactions between OPH and the cotton fabric. In combination with the extensive literature of glutaraldehyde-based protein cross-linking (34), this result clearly suggests that OPH is attached covalently. In any case, any additional OPH adhering to the cotton fabric is certainly not detrimental.

A



B

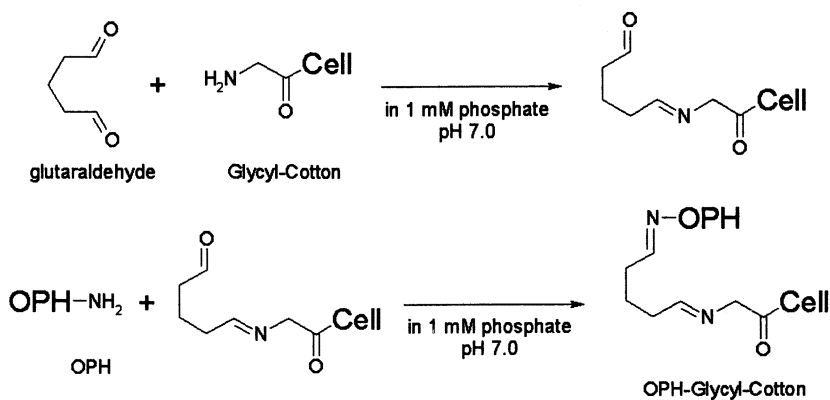


Figure 2. (A) Organic phase coupling of OPH to glycyl-cotton fabric (B) Aqueous phase glutaraldehyde coupling of OPH to glycyl-cotton fabric.

Table I. Coupling efficiency of immobilized OPH enzymes by organic phase and aqueous phase coupling methods.

| <i>Enzyme Preparation^a</i> | <i>Fabric</i> | <i>Coupling Method</i> | $\mu\text{g OPH}/\text{cm}^2\text{ fabric}^b$ | <i>Efficiency^c (%)</i> |
|---------------------------------------|---------------|------------------------|---|-----------------------------------|
| Pure OPH _{WT} | mono-gly | organic | 6 | ND |
| Pure OPH _{WT} | mono-gly | aqueous | 68 | 25 |
| Crude OPH _{WT} | mono-gly | aqueous | 49 | 25 |
| OPH _{WT} | mono-gly | aqueous ^d | 16 ^e | 14 |
| Pure OPH _{WT} | bis-gly | aqueous | 55 | 38 |
| Crude OPH _{WT} | bis-gly | aqueous | 49 | 59 |
| Pure OPH _{RL} | mono-gly | aqueous | 38 | 37 |
| OPH _{RL} | mono-gly | aqueous ^d | 19 | 17 |
| Pure OPH _{RL} | bis-gly | aqueous | 25 | 23 |
| OPH _{RL} | bis-gly | aqueous ^d | <2 | 2 |

^aHomogeneity of greater than 95% is considered to be pure and crude preparations are approximately 80% pure. WT, wild type OPH; RL, H254R/H257L enzyme. ^bThe amount of OPH bound was estimated as described in the text. ^cThe coupling efficiency is the amount of protein immobilized on the fabric expressed as a percent of the total amount exposed to the fabric. ^dAqueous control is a coupling step in which glutaraldehyde was omitted. ^eA similar value was obtained for the WT bis-gly control.

Capability of OPH/fabric to Detoxify Organophosphate Neurotoxins

Hydrolysis of Paraoxon

The capabilities of OPH cotton fabrics to hydrolyze the preferred substrate, paraoxon, were measured and these data are given in Table II. Fabrics prepared by the aqueous coupling method were 80 times better at paraoxon hydrolysis (39 $\mu\text{g}/\text{min}/\text{cm}^2$) than fabrics prepared by the organic method (0.47 $\mu\text{g}/\text{min}/\text{cm}^2$). Part of this effect may be the result of more enzyme bound per square centimeter on cotton prepared with aqueous chemistry (Table I) and because the aqueous method is less “damaging” to the active conformation of the enzyme. In general, the mono-gly cotton fabrics displayed slightly higher activity than the bis-gly cotton fabrics. Interestingly, fabric prepared with a crude sample of WT OPH is 3 times more active than when fabric is prepared using purified OPH (120 $\mu\text{g}/\text{min}/\text{cm}^2$ versus 0.47 $\mu\text{g}/\text{min}/\text{cm}^2$, respectively). Impurities in the crude enzyme preparation may prevent an unfavorable enzyme conformational change upon immobilization or prevent unfavorable intermolecular interactions. Nevertheless, these data demonstrate that the activity of the fabric has been improved 80 to 250-fold by using aqueous chemistry and that an impure preparation of WT OPH would be sufficient for detoxification. Cotton fabrics prepared with the H254R/H257L enzyme were also active against paraoxon (21 $\mu\text{g}/\text{min}/\text{cm}^2$ fabric for mono-gly cotton and 3.6 $\mu\text{g}/\text{min}/\text{cm}^2$ for bis-gly cotton). The

overall activity was slightly lower than the immobilized WT samples. The soluble H254R/H257L enzyme has a 10-fold lower k_{cat} for paraoxon than WT OPH does so this result was not surprising; however, in contrast, the relative activities for paraoxon of both immobilized enzymes are strikingly similar.

Table II. Kinetic Parameters of Immobilized OPH Enzymes for Paraoxon Hydrolysis.

| <i>Enzyme Preparation^a</i> | <i>Fabric</i> | <i>Coupling Method</i> | $\mu\text{g hydrolyzed per min/cm}^2$ |
|---------------------------------------|---------------|--------------------------------|---------------------------------------|
| Pure OPH _{WT} | mono-gly | organic | 0.47 |
| Pure OPH _{WT} | mono-gly | aqueous | 39.00 |
| Crude OPH _{WT} | mono-gly | aqueous | 120.00 |
| OPH _{WT} | mono-gly | aqueous (control) ^b | 2.80 ^c |
| Pure OPH _{WT} | bis-gly | aqueous | 11.00 |
| Crude OPH _{WT} | bis-gly | aqueous | 83.00 |
| Pure OPH _{RL} | mono-gly | aqueous | 21.00 |
| Pure OPH _{RL} | mono-gly | aqueous (control) | ND |
| Pure OPH _{RL} | bis-gly | aqueous | 3.60 |
| Pure OPH _{RL} | bis-gly | aqueous (control) | ND |

^aPurity >95% is considered as pure and crude preparations are approximately 80% pure. WT stands for wild type OPH and RL represents the H254R/H257L enzyme. ^bAqueous control is a coupling step in which glutaraldehyde was omitted and reflects the amount of enzyme that non-covalently binds to the fabric. ^cA similar value was obtained for the WT bis-gly control.

Hydrolysis of DFP and demeton-S

The capabilities of the OPH fabrics to catalyze the hydrolysis of DFP, the substrate analogue for Sarin, and demeton-S, the substrate analogue for VX, were also determined. The data were gathered with the aqueous coupling method since this method had been shown to be superior to organic coupling for the native enzyme. The activity of the immobilized WT OPH toward DFP was 0.45 $\mu\text{g/min/cm}^2$ fabric for mono-gly cotton (Table III) compared to the absence of DFP hydrolytic activity with the immobilized H254R/H257L enzyme (data not shown). However, the opposite was true for demeton-S hydrolysis. The activity of the immobilized H254R/H257L enzyme toward demeton-S was approximately 5 $\mu\text{g/min/cm}^2$ fabric and no activity was observed using this substrate with WT OPH. The parallel observations for the soluble enzymes are striking. The soluble H254R/H257L enzyme is 11 times more active against demeton-S than WT OPH and has very low activity for DFP. The specificity constant for soluble, WT OPH and DFP is $7.8 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ and this same constant for soluble H254R/H257L is $4 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ (20). The

corresponding values for demeton-S are $8.7 \times 10^2 \text{ M}^{-1}\text{s}^{-1}$ and 2.7×10^4 , respectively. We have proposed that in the modified OPH enzymes site-directed changes near the active site resulted in an expanded binding pocket, providing easier access for larger substrates, such as soman and demeton-S (20). Consequently, the catalytic efficiency for smaller substrates, such as DFP, is decreased. The catalytic flexibility of immobilized OPH enzymes suggests some attractive possibilities for bioremediation of neurotoxic agents. As catalytic characteristics are further improved, a cocktail or mixture of enzymes can be immobilized with an even greater potential for bioremediation technologies. The kinetic data gathered here also suggest that the inherent substrate specificities of immobilized OPH enzymes have been remarkably maintained.

Table III. Kinetic parameters of immobilized OPH enzymes for DFP and demeton-S hydrolysis.^a

| <i>Enzyme Preparation^b</i> | <i>Fabric</i> | <i>Coupling Method</i> | <i>μg hydrolyzed/minute/cm² fabric</i> |
|---------------------------------------|---------------|------------------------|---|
| Pure OPH _{WT} | DFP | mono-gly | 0.45 |
| Pure OPH _{WT} | DFP | bis-gly | 0.17 |
| Pure OPH _{RL} | demeton S | mono-gly | 5.2 |
| Pure OPH _{RL} | demeton S | bis-gly | 4.5 |

^aData represents immobilization using aqueous chemistry only. The same cotton samples used in paraoxon assays were used for DFP and demeton-S hydrolysis after extensive washing.

^bPurity >95%. WT stands for wild type OPH and RL represents the H254R/H257L enzyme.

Stability of OPH/fabric

Stability at 4°C

The ability of the fabrics to retain catalytic activity against paraoxon was measured with time. Fabrics were stored at 4°C in 10 mM phosphate buffer, pH 7, and the activity of the fabric was expressed as a relative percentage of the initial activity. The immobilization of the enzyme onto cotton stabilizes the activity considerably (Figure 3A). Soluble WT OPH retains only 7% of its starting activity after seven weeks while the immobilized WT OPH retains greater than 40%. The H254R/H257L immobilized enzyme retains 70% of its activity after seven weeks. This is quite significant, because the activity of the soluble form of this enzyme is much less stable than the soluble WT enzyme losing 50-70% of the initial activity after just a few hours. Thus, in addition to maintaining its substrate specificity after immobilization, the overall catalytic stability of the H254R/H257L enzyme has been dramatically improved.

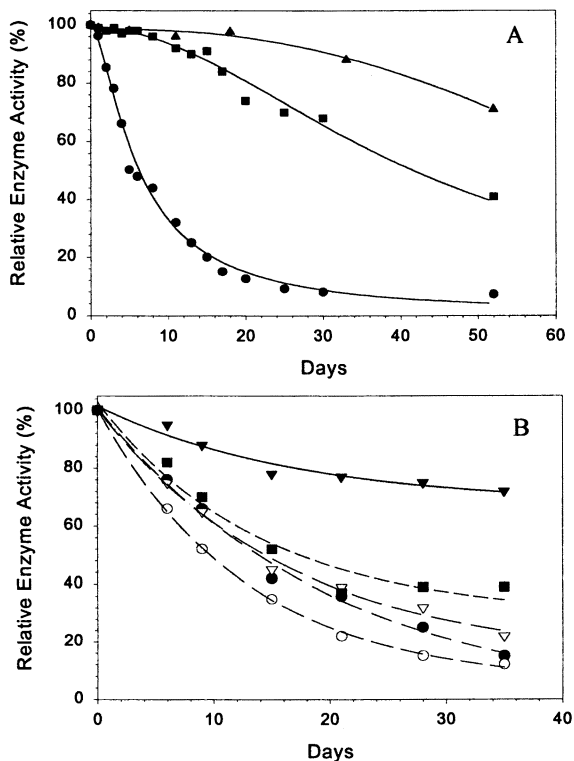


Figure 3. Stability of OPH/Fabric. Assays were performed using paraoxon as substrate and as described in the text. (A) Stability of immobilized and soluble OPH at 4° C. (▲) RL immobilized OPH; (■) WT immobilized OPH; (●) Soluble WT OPH. (B) Stability of WT OPH/fabric at room temperature in the presence of stabilizing sugars. Preparations were performed as described in the text. (▼) trehalose; (■) cellubiose; (▽) sorbitol; (●) no additive; (○) mannitol.

Stability at Room Temperature

OPH fabrics must be stable at higher temperatures, where long-term storage life is important, for them to be useful in realistic conditions. In addition, a dry fabric would reduce water weight and provide appropriate permeability for nerve agent absorption. We have evaluated several approved pharmaceutical additives, specifically stabilizing sugars, for their usefulness in the stabilization of the immobilized WT OPH fabric at room temperature. The activity of trehalose-treated fabric remained fairly constant throughout the evaluation period, maintaining greater than 70% of the initial activity at day 35 (Figure 3B). This is an improvement compared to the same fabric at 4°C. Thus, it is clear that these fabrics are amenable to further improvements in stability.

Practical application

The capability of the OPH fabric to hydrolyze a variety of organophosphate neurotoxins has been clearly demonstrated. These towelettes hydrolyze practical quantities of neurotoxic pesticides and chemical nerve agents. An OPH towelette that is 10 cm x 10 cm and four layers thick would yield a total of 400 cm². Data from Table II shows that crude OPH coupled to mono-gly cotton can hydrolyze 0.12 mg paraoxon/min/cm² of fabric. Therefore, a decontamination towelette, of the above dimensions, could hydrolyze 48 mg of paraoxon per minute. Even modest optimization of the towelette would likely produce a fabric that can decontaminate gram quantities of agent in just a few minutes. Based on absorbency data for cotton (not shown), a flannel towelette of the above dimensions could absorb up to 35 milliliters of fluid.

These studies demonstrate that OPH towelettes are capable of destroying battlefield relevant quantities of nerve agent analogues rapidly. Attachment to other medical materials such as gauze, swabs, bandages and wound dressings would most likely be successful based on the simplicity of OPH immobilization. This technology could easily become part of an effective chemical threat agent decontamination kit such that OPH fabrics can be removed from an airtight wrapping and immediately used for decontamination when needed. The high surface area and absorbency of cotton ensure rapid transfer of nerve agents from a skin surface into the towelette where OPH rapidly degrades the nerve agent.

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Chapter 4

Understanding Dressing Composition: A Biomaterial Point of View

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The evolution in our understanding and management of the wound healing process has been tediously slow over the past several thousand years, particularly in comparison to the progress made since the late 1960's. Even more rapid developments in our knowledge of wound physiology during the past decade have shown us much, but have also resulted in a lack of consensus regarding solutions for optimal treatment of all wounds under all conditions. Many factors affecting wound healing remain unquantified including: overall patient health, psychological status, and nutritional status; cellular nutrition and metabolism (including oxygen availability); the role of growth factors and how these might be artificially mediated; and the control of bacterial protease. Efficacy of simplistic vehicles to monitor wound status and healing relative to wound physiology is difficult to demonstrate. Claims of wound healing efficacy are difficult to prove due to a very significant placebo effect, but this does not diminish the need for products that can truly heal wounds faster and better. This chapter will begin with an overview of the state of the art including wound categories; currently accepted optimum conditions for wound healing; standard wound dressing and wound management categories; and dressing materials and construction. Issues not addressed by existing materials and technologies are summarized, and emerging technologies will be described briefly.

An Overview of Wound Categories

Wounds can be subdivided into three broad categories. These are acute wounds, burns, and chronic wounds. Acute wounds are caused by trauma or surgery and usually require limited local care. Burns are classed into three subcategories based upon the extent of skin damage -- third degree burns are the most extreme with full thickness destruction of the skin. Chronic wounds require longer than usual to heal as a result of poor health, local pressure, diabetes, carcinoma, poor circulation, poor nutritional state, immunodeficiencies, infection, or advanced age.

Normal Wound Healing

Both acute wounds and burns heal normally: the body's repair and protection systems institute three phases of healing starting with an inflammatory phase, followed by a proliferative phase and concluding with a maturation phase. In the inflammatory phase peripheral vasculature contracts to stem blood flow. Blood platelets are activated upon exposure to extravascular collagen and initiate coagulation and vasoconstriction. Platelets express the cytokine platelet-derived growth factor (PDGF) which is mitogenic and chemotactic for fibroblasts, some endothelial cells, chondroblasts and osteoblasts, and smooth muscle cells, among others. Neutrophils enter the wound early to consume bacteria and debris. Within three to four days vascular monocytes migrate into the wound and differentiate into an inflammatory or responsive macrophage. The latter may be central to regulating the proliferative phase. The inflammatory phase normally lasts a week or less. During the subsequent proliferative phase, which can require one to three weeks, fibroblasts begin to generate a network of collagen fibers to provide the scaffold for new tissue cells. Ultimately granulation tissue fills the wound, myofibroblasts facilitate wound contraction and a new skin surface forms during epithelialization. The maturation phase lasts from three months to two years. The collagen network is continuously reorganized to strengthen the tissue. Here the macrophage regulates collagen remodeling by elaborating one or more cytokines collectively known as macrophage-derived growth factors (MDGF), which stimulate fibroblasts to produce new collagen, while the existing fibers are reduced by enzymatic lysis. Further detail about these phases of normal healing are provided by Clark.¹ These processes are continuous, often much overlap occurs among them; and a host of growth factors, enzymes, as well as neutrophils, eosinophils, basophils, fibroblasts, etc. work in concert throughout the process to orchestrate healing.

Chronic Wounds

The issue with chronic wounds is that, once formed, the normal cascade is interrupted. Often, this is due to the unhealthy state of the patient and chronic wounds have been classified based upon the underlying disease state.

Venous ulcers form when the valve system in lower extremity vasculature fails. These valves function similar to locks in a canal. Blood is pumped upward through an open valve when then closes to retain the blood in place. When the valve fails, it loses strength, becomes flacid, and its ability to hold blood against gravity is impaired. The normal calf-pump system loses efficiency in returning blood to the heart, blood pools in the lower leg and edema results. This fluid is damaging to tissue outside the vessal and the ulcer occurs, seemingly, spontaneously.

Diabetic ulcers form as a result of various symptoms of the disease ranging from poor circulation, and its concomitant tissue deoxygenation, to degradation of the patient's sensation (neuropathy). Healthy sensory response alerts one to the potential for tissue damage. A neuropathic diabetic may have a stone in the shoe without their knowldege for hours which can result in a pressure sore. Stable glucose levels are essential for normal healing. The high levels seen in diabetics can adversely affect leukocyte function increasing the potential for infection.

Arterial ulcers, also known as ischemic ulcers, normally present in the lower extremities as a result of vascular disease. Vasoconstriction leading to reduced blood flow can instigate tissue necrosis when cell nutrients and oxygen are reduced. Ischemic ulcers can be associated with diabetes.

Pressure sores are common in geriatric patients when pressure or shear is applied to the skin for a protracted period. Barrier integrity is lost as tissue degrades under this constant pressure. These ulcers can be very large, particularly in the sacral area.

Recalcitrant wounds are a general class of ulcer resulting from bioburden, poor nutritional status, hypovolemia (inadequate vascular volume), hypoxemia (reduced blood oxygen), or hypoxia (reduced tissue oxygenation). Aside from the potential for infection, bioburden can increase protease levels in the wound bed. These enzymes inhibit wound healing in a number of ways, not the least of which is degradation of the collagen network. Recalcitrant wounds may initiate from trauma or surgery, but then healing is arrested and the wound fails to close or increases in size.

A number of co-factors have been identified that can lead to impaired healing. In addition to the patient's age, insufficient tissue oxygenation, malnutrition, bacterial contamination or infection, skin pressure, shear or friction, psychological stress, and disease state can singly or in any combination either cause or inhibit the healing of a chronic wound. However, cellular and molecular mechanisms that result in either healing or non-healing have not been adequately documented. This background is provided in more detail in Clark,¹ Krasner and Kane,² Cohen, Diegelmann and Lindblad,³ Mani, et al.,⁴ and Morison, et al.⁵

Optimum Conditions for Chronic Wound Healing

Currently accepted optimum conditions include keeping the wound bed: (1) moist, (2) free from excess exudate, (3) free from necrotic tissue, (4) free from trauma, (5) warm, (6) protected from bacterial infection, (7) acidic (sometimes recommended for bactereostasis), and (8) appropriately oxygenated, based upon the stage of wound healing.^{6,7} The apparent simplicity of this state of the art is due in part

to the goal of addressing as wide an array of wounds as possible. It is also due to a lack of consensus among thought leaders and researchers about how wounds should be actively managed for optimum and speedy wound closure. One thing seems clear: selection of management protocol should be decided on a case-by-case basis after full evaluation of the patient's overall health and psychological status and the conditions of the wound.

A moist wound bed increases the rate of re-epithelialization by providing epithelial, endothelial and fibroblast cells with an environment in which they can survive and migrate.^{8,9} This environment also allows better nutrient access than one which is dry or crusty. Neutrophils have been found in functionally active concentrations in a moist wound environment. On the other hand, wounds can exude fluids in significant quantities. Excess fluid can macerate and degrade healthy tissue in the wound periphery, so it is necessary to manage this condition without overly desiccating the wound surface. Many current wound management materials and products are designed to accomplish these two conditions simultaneously.

Necrotic tissue in or peripheral to the wound bed can allow bacterial proliferation and retard tissue cell growth by blocking access to nutrients and oxygen. This dead tissue can be removed by allowing the moist environment to soften and debride it. Enzymatic solutions have also been used with success. On the other hand, surgical debridement of the necrotic tissue is favored by some clinicians for wounds such as diabetic ulcers because it exposes the wound surface to healthy tissue and vasculature.

Wound Dressing Categories

Before proceeding with descriptions of wound dressing categories, it is appropriate to step back and discuss biomaterials in general. What is a biomaterial? Three definitions have been proposed:

"...a systemically and pharmacologically inert substance designed for implantation within or incorporation with living systems;"¹⁰

"...a nonviable material used in a medical device, intended to interact with biological systems;"¹¹

"...any substance (other than drugs) or combination of substances synthetic or natural in origin, which can be used for any period of time, as a whole or as a part of a system which treats, augments, or replaces any tissue, organ, or function of the body."¹²

The more common dressing categories used by clinicians are listed in Table I. Composition and function will be discussed in more detail in the next section. Other than a few exceptions, these materials are used to control moisture and exudate levels in wounds. Alginate, collagen, and foam dressings, as well as amorphous hydrogels, can be packed into deep cavity wounds or used to cover shallow wounds. By nature of their flat, somewhat rigid construction, hydrocolloid, hydrogel, and composite dressings are used for full thickness, but not deep cavity, wounds.

While most dressings are designed to maintain a moist environment, manage wound exudate, and cover and protect the wound, they are differentiated on the basis

of their physical properties, ease of use and cost to the clinician. Over the past several decades commercial offerings derived from these materials have proliferated with several thousand different products on the market today. Several of the references cited, particularly that used to derive Table I, give detailed descriptions of these products, their approved uses and sources.

Table I. Established Dressing Categories¹³

| Dressing Material | Indications For Use | Contraindications |
|----------------------------------|--|------------------------------------|
| Alginate | Moderate to heavily exuding, partial to full thickness wounds; Autolytic debridement; Hemostasis | |
| Collagen | Moderate to heavily exuding wounds | |
| Hydrocolloid | Light to moderately exuding, partial to full thickness wounds; Leg ulcers; Donor sites; Burns; Decubitus ulcers | |
| Hydrogel | Burns; Abrasions; Light to moderately exuding chronic wounds | Heavily exuding wounds |
| Transparent Film | Dry to lightly exuding, partial thickness wounds; Catheter insertion sites | Moderate to heavily exuding wounds |
| Foam | Light to heavily exuding wounds; Autolytic debridement | Wounds with dry eschar |
| Specialty Absorptive & Composite | Light to heavily exuding, partial to full thickness wounds depending upon specific construction | See Foam Dressings |

Dressing Materials and Construction

Alginate Dressings

Alginate dressings are composed of soft, nonwoven, polysaccharide fiber ropes, ribbons or pads produced from seaweed-derived calcium alginate. They are offered in the various configurations for tailoring to specific wound shapes from deep cavities and sinuses to partial thickness skin wounds.

Alginic acid, from which the calcium salt is derived, is a polymer containing mannuronic and guluronic acid residues. When in contact with wound fluids containing sodium ions, insoluble calcium alginate is partially converted (through ion exchange) to hydrophilic sodium alginate gel. Alginates rich in mannuronic acid form soft, flexible gels. Guluronic acid rich alginates are firmer gels. In addition to its

moisture retention property, the gel reduces disturbance of granulation tissue during dressing change. Release of Ca^{2+} adds hemostatic character, since this is an important blood coagulation factor.

In clinical and laboratory studies¹⁴⁻¹⁶ a comparison of four alginate dressings did not show significant differences in their effect on re-epithelialization. In some instances alginate dressings have been found to stimulate inflammatory reactions in the wound which resolve as the wound heals. This is not surprising since alginates are saccharide polymers. The body's immune system recognizes foreign saccharides as antigenic and may respond accordingly to their presence in the blood stream. Significant differences have been observed in ease of handling, lateral spread of wound fluid, adherence, and amount of dressing residue left in the wound.

Collagen Dressings

Collagen is the fibrous insoluble protein found in all connective tissues including skin, muscle, bone, ligaments and cartilage. In mammals it represents up to 60 percent of total body protein – it is the major component of the extracellular matrix forming the organized scaffold that supports cells. Almost one-third of the protein is glycine and an additional 25 percent is proline and hydroxyproline. Polar side group interactions between adjacent polymers are largely responsible for fiber structure. Ten or more types have been identified with Types I and III collagen being prevalent in humans. Collagen dressings are packaged in a variety of forms from powders and gels to strips, ribbons and pads. The material is sourced primarily from bovine or porcine hides and after extensive purification, it is epichlorohydrin-crosslinked, lyophilized and cut into fibrous sponges of desired shape.

Collagen is believed to contribute to wound repair by debridement, binding blood clotting factors XII and XIII, and attraction of granulocytes and fibroblasts. It may reduce wound contraction and enhance fibroblast deposition of oriented, organized collagen fibers.⁷ Some of these are speculative comments and cannot be used to promote commercial sales of a particular product brand. On the other hand, animal collagen is recognized as a foreign protein by the body's immune system, with the potential to stimulate an inflammatory response, and naturally occurring collagenase responds to and lyses the peptide as it does the body's own collagen. Collagen dressings have been shown to absorb wound fluid and since the material digests by collagenase lysis, there is reduced clinical concern about residue remaining upon dressing removal. Crosslink density affects the rate of digestion.

Hydrocolloid Dressings

Introduced in the early 1980's, hydrocolloids are a suspension of various natural or synthetic polymer granules in an adhesive matrix. Gelatin or pectin are common granular materials used. The granules are in a semihydrated state and are hydrophilic while the adhesive (e.g., synthetic rubber) is hydrophobic. Hydrocolloids absorb fluid

slowly, leading to a change in the physical state of the dressing, forming a gel. These dressings are offered in a variety of shapes and their fluid absorption capacity depends upon this and their specific composition. In the past their pitfall may have been that they left residue in the wound, but many producers have overcome this limitation.

Hydrogels

Hydrogels are available as amorphous gels, sheets and impregnated gauze. They are composed of acrylics, polyvinylpyrrolidone, polyethyleneoxide, carboxymethylcellulose, hydroxyethylcellulose, or possibly combinations of these, in water. They are typically utilized to keep a wound moist and cool, but by suitable choice of polymer, and partial substitution of glycerin for water, these dressings can be made to absorb some wound fluid. Hydrogels may include preservative and pharmaceutical agents.

Transparent Films

Transparent film dressings are adhesive-coated polyurethane films. Though waterproof, they do transmit gasses and moisture, but not bacteria. A desirable feature of these dressings from the clinician's perspective is that the wound can be viewed without removal of the dressing. Thus, wound status is assessed without trauma or increase risk of bacterial infection. Adhesives used are polyethers or polyacrylates with well-established biocompatibility. The moisture vapor transmission rate (MVTR) can be controlled within broad limits. Low MVTR films are designed to reduce eschar, erythema, and pain and to enhance granulation tissue quality. Re-epithialization has been found to occur 2-3 times faster than air exposed wounds.^{8,9} High MVTR films are more breathable and this reduces maceration of tissue peripheral to the wound. These latter films are used over intact skin to secure catheters and protect the insertion site.

Foams

Foam dressings are typically composed of polyurethane, but can also be constructed from rubber, acrylic, polyethylene, neoprene, etc. The value of urethane foams is derived from their well-established biocompatibility. Further to this, by suitable choice of diisocyanate and polyol combination, they can be tailored to be hydrophilic or hydrophobic. The former is useful in exudate reduction, while the latter may have lower adherence to the wound surface. These foams can be impregnated with pharmaceuticals, such as antibiotics, or activated carbon to reduce odor.

Specialty Absorptive and Composite Dressings

Specialty absorptive and composite dressings are composed of various combinations of foams, gels and absorbent fibers such as cellulose, cotton or rayon. They may be laminates with or without an adhesive border, and may be covered with a moisture permeable, bacterial barrier. Constructions are designed for management of wound fluid level and enhanced ease of use.

Dermal and Epidermal Substitutes

Synthetic skin has been proposed as a modality for the management of wounds. Constructions of selected skin substitutes are outlined in Table II. These are classed into three categories based upon tissue source. Autogenic materials are constructed using cells removed from the patient and re-applied back to the same patient. Allogenic materials are constructed from cells of one person and applied to a second. Xenogenic refers to use of materials in humans that were originally derived from another species.

Table II. Selected Skin Substitute Constructs

| | | | | | | |
|---------------------------------------|---|-------------------------------|--|---|--|-----------------------------|
| Source | Organo- genesis Inc. | Integra Life Sciences | Advanced Tissue Sciences | LifeCell Corp. | HC Implants | Genzyme Tissue Repair |
| Brand | Appligraf | Integra Artificial Skin | Derma- graft | | | |
| Category | Xenogen. Allogen. | Xenogen | Allogen. | Autogen. | Synthetic | Autogen. |
| Barrier Material | Stratum Corneum | Silastic Mem- brane | | | Porous bilayer of polyether polyester | |
| EK Used | X | | | | | X |
| DF Used | X | | X | | | |
| Extra- cellular Matrix | Bovine collagen lattice w/ cell- produced matrix | Bovine collagen sponge | Cell- produced matrix w/ resorbable Vicryl mesh | Cryo- preserved extra- cellular cadaver skin | | |

Note: EK = Epidermal keratinocytes; DF = Dermal fibroblasts

Some of these materials suffer from issues pertaining to cryopreservation, limited shelf life, sterilization, high manufacturing waste in terms of cell cultivation and collagen production, and immune response (non-autogenic materials).¹⁷ However, at least one has been shown to result in reduced scarring in burn victims, reduced pain by allowing partial vs. full thickness skin grafts. With further clinical demonstration, some may be indicated for management of additional wound categories.

Limitations of Existing Materials

Many of the current products and materials available today provide significant benefits to the patient and clinician in terms of pain reduction, enhanced lifestyle, ease of use and lower cost of care. What cannot be packaged and sterilized is this: the care and attention provided by clinicians. This is can be a significant factor in wound healing. Frankly, many chronic wounds are due to neglect. Having them cleaned and dressed, together with the positive psychological impact this has on the patient, helps wounds to heal. A comprehensive, critical analysis of existing clinical studies will show, after weeding out the numerous anecdotal reports, that the majority of the benefit is not clinical: use of a particular dressing does not heal wounds better or faster than a standard moist dressing protocol. This is not to say, however, that one dressing or another will not have a positive economic benefit to the patient or clinician. Some dressings are easier to use, require less time to apply, and need less maintenance than others. With proper clinical study design this can be translated to reduced cost. Though the economic benefit is more difficult to define, patients prefer some dressings more because they are more comfortable, less noticeable and help manage odor.

Emerging Technologies

The limitations of existing commercial offerings should point to the fact that the benefits of moist wound healing have been realized, and that if more is to be accomplished to enhance healing rates and recovered tissue quality, a better understanding of the underlying physiology is necessary. Though there may not be a universal solution that applies to all wounds and all patients, continued investment into and exploration of the basic science of wounds will lead to better outcomes. At least three significant goals can be conceived today. First, the wound could be manipulated, through mediation of cells, nutrients, metabolites, growth factors, and enzymes, to reach a significant clinical endpoint (e.g., faster wound closure, reduced pain, reduced scarring) by application of physiologically relevant, active agents. Second, the accurate, simple diagnosis of stage and quality of wound healing could reduce management protocol variation and clinician error, thereby improving the

overall quality of clinical care. Third, reducing recurrence of chronic wounds would lead to improved patient comfort and wellbeing and have a strong impact on healthcare costs in an aging population. These three areas will be discussed in more detail in this section. Current and potential future paradigms are contrasted in Figure 1.

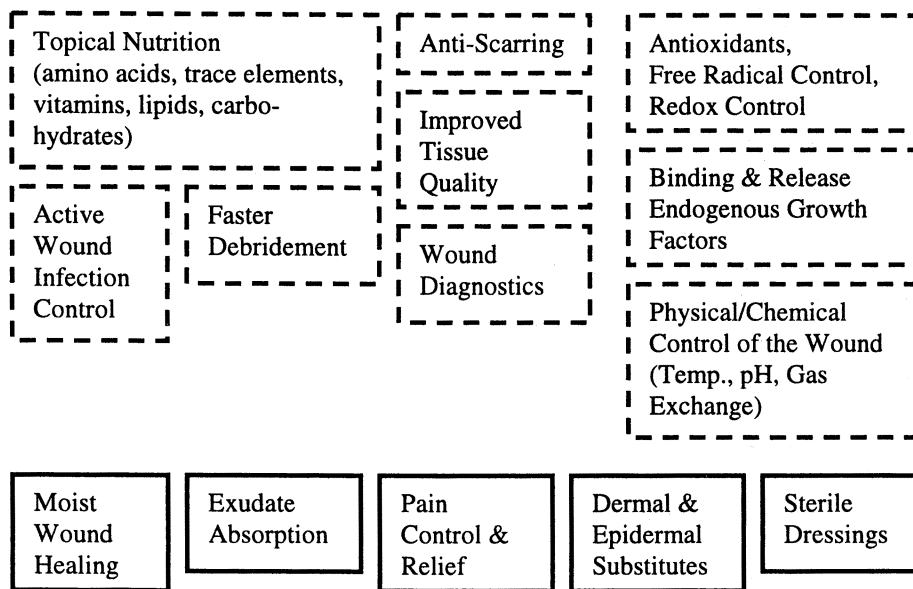


Figure 1. Current and future paradigms in wound healing. Currently available technologies and practices are given in solid boxes. Future possibilities are in dashed boxes.

Active Agents

Active agents are substances that promote wound healing. Cell nutrients and cytokines are attractive candidates as active agents because much is known about their roles in tissue function and their incorporation into dressing materials is technically feasible.

Nutrients

Cellular nutrients include all of the usual ones: carbohydrates, proteins, lipids, vitamins and trace elements. Carbohydrates are essential for energy production. Protein and amino acids are important in neovascularization, collagen synthesis, fibroblast proliferation, lymphocyte formation and phagocytosis. Lipids and triglycerides are used in energy production and the construction of cell membranes. Iron assists in collagen metabolism and, of course, is essential in oxygen transport. Zinc is important in building a healthy immune response and is central to many metabolic enzymes. Magnesium and copper are instrumental in peptide synthesis,

collagen crosslinking and erythrocyte formation. Vitamin A assists in the inflammatory response and in fibroblast cell differentiation. Vitamin B assists in fibroblast function, Vitamin D in bone formation and calcium homeostasis. Vitamins C and E are antioxidants and assist in collagen synthesis. Vitamin K is important in normal blood coagulation.

The role of balanced nutrition and good diet have been understood for quite some time as having an overall positive impact on wound healing. In people with good dietary habits, who do not smoke, wounds heal well. However, for many patients with chronic wounds, who are geriatric or have an underlying disease, nutritional levels are not optimal. Further, since vasculature in the neighborhood of a wound is often inadequate to meet the needs of tissue in the wound, these nutrients do not get to the cells that need them. While they might survive this condition, cells do not perform well and proliferation is retarded. This raises the question: what if the clinician could actively control the levels of these nutrients in a wound by applying them topically in a physiologically appropriate way? Clearly, there are barriers to this, not the least of which is sterility and stability of the material, and it is not evident what constitutes an appropriate combination of nutrients.

Growth Factors

Growth factors are cytokines and low molecular weight peptides that act locally, having a specific high affinity for cell surface receptors and the ability to stimulate or inhibit cell proliferation and differentiation. Over thirty growth factors have been identified falling into three groups: proliferative (causing cell replication), migratory (chemoattractants stimulating cell movement), and transforming (producing phenotypic alteration). A few of these are listed in Table III. Growth factors have condition-dependent activity and may interact with one another. Clinical treatment is not simply a question of sprinkling autologous growth factors in a wound and waiting for it to heal. Too high or low a concentration of one relative to others at the wrong stage may have a deleterious effect on cell function and healing. Proper concentration modulation and sequencing of several may be optimal. While the biochemistry of growth factors in wound healing is not adequately understood at this point, this is a promising area and further productive attention will be paid to it.

Wound Diagnostics

Current diagnostic practices include evaluation of wound appearance (i.e., presence or absence of granulation tissue, epithelialization, slough, black or brown eschar, size, depth, location etc.); exudate amount, color and odor; severity, frequency and location of pain; appearance of the skin peripheral to the wound (color, erythema, edema); presence or absence of infection; and, the underlying disease state (carcinoma, diabetes, arterial or vascular disease, etc.). Without significant experience in the field and detailed familiarity with a patient's condition, the causes of wounds are inherently difficult to diagnose placing proper management of the wound at risk. Also, these practices do not tell the clinician much about wound physiology: activity of growth factors, vitality of fibroblasts, endothelial and epithelial cells, the presence of metallo-proteases to name a few.

Table III. Example Growth Factors And Their Role in Wound Healing

| Growth Factor | Role |
|---|---------------------------------|
| Epidermal Growth Factor (EGF) | Epithelialization |
| Endothelin – 1 (En-1) | Contraction |
| Fibroblast Growth Factor (FGF) | Angiogenesis |
| Granulocyte Monocyte Colony Stimulating Factor (GM-CSF) | Monocyte Migration & Maturation |
| Interferon – γ (IFN – γ) | Maturation |
| Interleukin – 4 (IL-4) | Fibroplasia |
| Insulin-like Growth Factor – 1 (ILG – 1) | Fibroplasia |
| Monocyte Colony Stimulating Factor (M – CSF) | Monocyte Migration & Maturation |
| Platelet Derived Growth Factor (PDGF) | Angiogenesis & Fibroplasia |
| Transforming Growth Factor – α (TGF – α) | Epithelialization |
| Transforming Growth Factor – β (TGF – β) | Matrix Synthesis |
| Vasoactive Endothelial Growth Factor (VEGF) | Angiogenesis |

Imagine how much more confidently clinicians could make choices about treatment, if they knew the states of these parameters on the spot. With a relatively inexpensive, hand held instrument, diabetics routinely measure their blood glucose level and self-administer insulin several times each day. Suppose clinicians had a similar instrument that could quantitatively report levels of vital and degenerative components within a wound from a single swab and suggest a preferred management direction for the wound. Such a device does not exist today. However, quantitative analyses of fungi and bacteria as well as many healthful constituents from growth factors to fibroblasts are possible, although not necessarily at the convenience, or within the cost constraints, of the clinician.

Before launching into a program to develop such an instrument or series of instruments, it will be necessary to thoroughly understand how all of the components

in a wound can be measured and correlated to the health of the wound and the stage of healing. How do the “ingredients” in a wound compare when it is chronic, non-healing to when granulation tissue is forming to when progress toward closure is evident?

Tissue Quality

Once covered with epidermis, the skin is not free from risk of ulcer recurrence. Chances are the underlying disease, environmental condition or nutritional and health status of the patient have not changed. Continued clinical attention and improvement in the patient's health will ameliorate the risk. However, these incur additional medical costs which are, in this time of aging populations, likely to be significant. By paying particular attention to the quality of healed skin and identifying treatments that help to improve this quality, the chances of recurrence will be reduced without additional significant medical costs.

Parameters that can be related to skin quality are, but not limited to, its flexibility (modulus), tensile, tear, and shear strength, and skin thickness. These are measurable physical properties. In healthy individuals the tensile strength of healed skin is on the order of 70% the original value prior to the wound. With topical use of Vitamin A derivatives, this can increase due to skin's thickening response to these compounds. Emollients can improve flexibility. This is a field that has been studied for many years by the cosmetics industry. Their discoveries certainly have merit relative to improving the mechanical properties of healed skin and should be studied further in this light.

Conclusion

All of the areas covered briefly here can be positively impacted by support from chemical technologists. The materials discussed here comprise, albiet sometimes complex, chemical compounds, associations of compounds, and polymers. Indeed, chemists have already been involved in many of these developments for years. It has been the intention of this author to introduce the new comer to this area of medicine and provide sufficient background and reference resources that she or he can begin to fathom the true issues quickly and begin to make a contribution.

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Chapter 5

The Non-Healing Wound: Mechanisms and Dressings

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Chronic dermal ulcers are a major cause of disability and have a significant socioeconomic impact. Annual health care expenditures for the treatment of chronic dermal ulcers exceeds \$3 billion in the United States alone. The development of improved treatment strategies should proceed from a better understanding of the pathophysiology of these problem wounds. A consistent feature of chronic leg and pressure ulcers is the presence of large numbers of activated neutrophils and perhaps other inflammatory cells. These cells, especially neutrophils, generate large amounts of degradative enzymes and oxygen metabolites that may overwhelm endogenous controls leading to tissue damage. This article summarizes studies that have focused on characterizing the proteolytic environment of chronic dermal ulcers and the implications that this may have for current and future treatment strategies of chronic nonhealing wounds.

Introduction

The skin is the body's largest and most complex organ. It performs numerous functions including acting as a barrier against infection, maintaining fluid balance, and providing thermoregulation. Insults that result in a disruption of the continuity of the skin permit entry of microorganisms and are potentially life threatening. As a result, we have evolved the ability to make rapid and somewhat less than perfect patches or scar tissue to fill these breaches. This process of skin wound healing or repair consists of an extraordinarily complex cascade of highly regulated biochemical and cellular events. It involves many types of cells that not only interact with one and another but must also respond in a coordinated fashion with the extracellular matrix and soluble factors such as cytokines and growth factors.

The wound healing cascade can be perceived as four overlapping phases. An initial or hemostasis phase, an early or inflammatory phase, an intermediate or fibroplasia phase, and the late or remodeling phase. Each phase is characterized by distinct biologic processes. The hemostasis phase consists of vasoconstriction and fibrin clot formation. This serves to stop further hemorrhage and to place a temporary plug in the gap of the skin. This plug serves dual functions in it inhibits the invasion of microorganisms and serves as a provisional scaffold upon which cells involved in the subsequent phases use for a pathway to migrate into the wound site. During the inflammatory stage, phagocytic leukocytes consisting of neutrophils followed by macrophages ingest invading microorganisms, foreign bodies and devitalized tissue. These leukocytes also produce soluble factors that appear to attract and coordinate the activities of the cells involved in the fibroplasia phase. During the fibroplasia phase, reepithelialization, angiogenesis (ingrowth of new blood vessels), and connective tissue deposition occur. By far, collagen is the predominant protein of connective tissues (25% of all body protein and more than 50% of the protein in scar tissue)(1). Although peak levels of collagen occur relatively early during repair this new collagen provides little functional strength to the wound. During the remodeling phase, there is a continual rearrangement and cross-linking of collagen that ultimately leads to a mature scar with a strength approaching but not equaling that found in normal skin. An important feature of this rearrangement is an ongoing equilibrium of collagen degradation and synthesis.

However, wound healing does not always occur in this predictable fashion. Many local and systemic factors possess the capability to influence the pattern and rate of healing. A chronic wound is one that fails to heal in a timely fashion because of the existence of one or more pathologic conditions. These include infection, hypoxia, radiation damage, venous insufficiency, toxins, malnutrition, diabetes, pharmacological agents (e.g., steroids), and age. Chronic dermal ulcers represent a major perplexing and costly clinical problem, a problem that will only worsen as our population grows older. Therefore, there has been a great deal of interest in developing new strategies for treating these problem wounds.

A consistent feature of arterial, stasis, and pressure ulcers (which make up the majority of chronic wounds) is a chronic, prolonged inflammatory phase (2,3). An example of the degree of inflammation seen in many chronic wounds is shown in Figure 1. To facilitate their egress from the circulation, penetrate the extracellular matrix, and degrade and digest devitalized tissue and pathogens, neutrophils possess an armamentarium of proteolytic and free radical generating enzymes (4). Unlike other cells that primarily express and secrete proteases on demand, the proteases of neutrophils are formed during myelopoietic development and stored within a variety of cytoplasmic granules and secretory vesicles (5). Although neutrophils have obvious important positive roles in host defense and debridement of damaged tissues, these cells with their free radical generating enzymes and proteases have been implicated in mediating much of the tissue damage associated with chronic inflammatory diseases such as periodontal disease, rheumatoid arthritis, adult respiratory distress syndrome, and cystic fibrosis (6-12). We have thus proposed that a similar over exuberant neutrophil response may likewise contribute to a significant extent in the pathophysiology of chronic wounds (13,14).

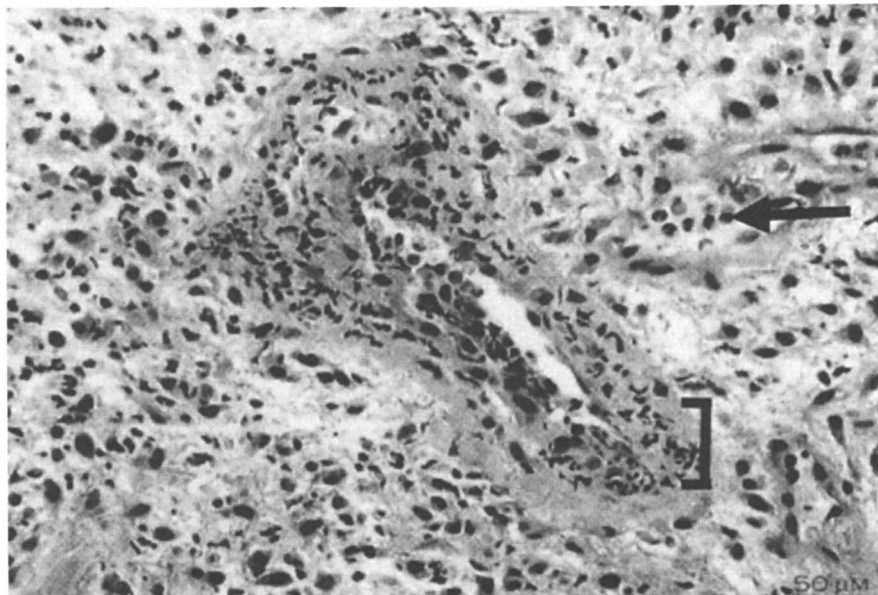


Figure 1. Inflammatory nature of chronic wounds. Representative H&E staining of a pressure ulcer granulation tissue. Large numbers of neutrophils are distributed throughout the granulation tissue as well as with edematous (bracket) blood vessels. Lumens of vessels (arrow) contain an abundance of migrating neutrophils. Also note apparent widespread fragmentation of collagen extracellular matrix. Photograph courtesy of Brad Garrett.

Results & Discussion

Neutrophil Proteases in Chronic Wounds

The matrix metalloproteases (matrixins, MMPs) are a family of approximately 20 mammalian neutral pH proteases that collectively, can degrade virtually all the protein components of the extracellular matrix (15). The MMPs are expressed as zymogens that require cleavage of an amino-terminal domain to become active. One subgroup of MMPs, the interstitial collagenases (MMP-1 or collagenase-1, MMP-8 or collagenase-2, and MMP-13 or collagenase-3), are the only mammalian enzymes capable of initiating the degradation of the triple helix of native fibrillar collagens (including types I, II, III, VII, and X) (16). Thus, it is the actions of these enzymes that represent the rate limiting step in the turnover of the major protein component of the extracellular matrix. Because it is made almost exclusively by neutrophils, MMP-8 is often called neutrophil collagenase. MMP-8 is sequestered in the specific or secondary granules of neutrophils. ELISA analysis reveals that on a molar basis,

MMP-8 is by far the most abundant collagenase in both healing wounds and in chronic wounds (Table I) (17). Chronic wound fluids contain significantly higher levels of collagenolytic activity than is found in fluids from surgical wounds, healing open dermal wounds, or split thickness wounds (Table I) (17,18). Based on substrate preference studies, the active collagenase found in chronic wounds is composed almost entirely of MMP-8 (Figure 2).

Substrate gel electrophoretic techniques have been used to indicate that levels of the gelatinase, MMP-9, are higher in chronic wound fluids compared to fluids from healing wounds (18-21). When performed under quantitative conditions, levels of MMP-9 have been found to be 25-fold higher in chronic wound fluids than surgical wound fluids (Table I). Although not exclusively expressed by neutrophils, MMP-9 is a major constituent of the small storage or tertiary granules stromelysins, MMP-3 and MMP-10, in chronic wounds with most of the expression associated with basal keratinocytes.

Table I. Levels of Proteases and Protease Inhibitors in Wounds

| | <i>Plasma</i> | <i>Surgical Drainage Fluids</i> | <i>Open Dermal Wound Fluids</i> | <i>Chronic Wound Fluids</i> |
|--|-------------------|---------------------------------|---------------------------------|-----------------------------|
| <i>Collagenolytic Activity^a</i> | N.D. [†] | 20.6±9.2 | - | 462±31 |
| <i>MMP-8^b</i> | N.D. | 3.0±3.0 | 40±15 | 60±7.5 |
| <i>MMP-9^c</i> | N.D. | 72±19 | - | 1,863±591 |
| <i>Elastase Activity^d</i> | N.D. | 0.087±0.08 | - | 1.10±1.2 |
| <i>TIMP^b</i> | 125±20 | 5,000±25 | - | 130±25 |
| <i>MMP/TIMP^b</i> | - | 16±10 | - | 51±25 |
| <i>α₂-Macroglobulin^e</i> | 53.6±20 | 23.8±15 | - | 6.8±5 |

^aMean levels of where one unit cleaves 1 μg of type I collagen in 60 minutes at 30°C.

^bMean levels in micrograms MMP-8 ±SEM per milligram total protein . Determined by sandwich ELISA.

^cMean levels in nanograms ±SEM per milligram total protein . Determined by substrate gel electrophoresis.

^dMean levels in milliunits ±SD per milligram total protein. Determined by proteolysis of methoxysuccinyl-ala-ala-pro-val-p-nitroanilide.

^eMean levels in micrograms per milligram protein ±SD. Determined by quantitative immunoblotting

Neutrophils also contain several cationic serine proteases (5). The enzyme, elastase, is particularly abundant. The amount of elastase contained in the granules of a single neutrophil is in excess of one picogram (4) Several groups have used specific inhibitors as well as antisera to demonstrate that elastase or an elastase-like protease is abundant in chronic wound fluids (13,22-24). Mean levels of elastase activity is approximately 12-fold higher in fluids of chronic dermal ulcers compared to surgical

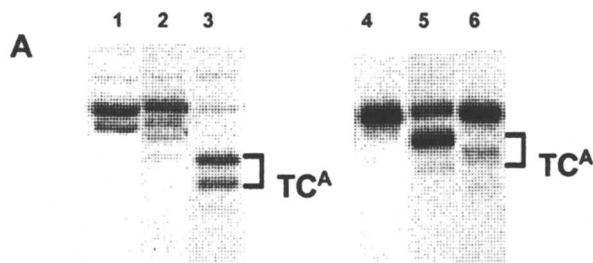


Figure 2. *MMP-8 is the active collagenase in chronic wounds. Panel A. MMP-1 and MMP-8 exhibit substrate preference in assays using soluble collagen as substrate. In lanes 1-3, type I atelocollagen was incubated with 1) buffer, 2) MMP-1, or 3) MMP-8. In lanes 4-6, type III atelocollagen was incubated with 1) buffer, 2) MMP-1, or 3) MMP-8. Type I collagen serves as a preferential substrate for MMP-8 whereas, type III collagen is the preferred substrate for MMP-1. Panel B. Two representative fluids from chronic wounds were incubated with type I collagen (lanes 1-4) or type III collagen (5-8) and the products resolved by SDS-gel electrophoresis. Odd numbered lanes represent wound fluid incubated with collagen in the presence of EDTA (specific inhibitor of MMPs). Both chronic wound fluids generated TC^A products from type I collagen substrate (lanes 2 and 4) with little corresponding activity directed towards type III collagen (lanes 6 and 8). This is consistent with the majority of the collagenolytic activity in chronic wounds being due to neutrophil (MMP-8) collagenase.*

wound fluids (Table I). Elastase possesses a broad substrate specificity, preferentially cleaving bonds that are carboxy-terminal to valine and to a lesser extent alanine (25). Because of this, elastase has an extremely broad substrate specificity. This enzyme has been demonstrated to be responsible for degrading a number of extracellular matrix proteins including fibrin, fibronectin, tenascin, and vitronectin (2,22-24,26,27).

The extracellular matrix may not be the only significant target for elastase in chronic wounds. As shown in Figure 3, elastase present in chronic wounds is capable of degrading critical soluble factors such as platelet-derived growth factor and transforming growth factor β_1 (13,28). Although it has not been examined within the milieu of chronic dermal ulcers, neutrophil elastase has also been shown to degrade cell surface receptor proteins (Tierney and Yager, unpublished results) (29-31).

Additional serine proteases have been found in chronic wounds and neutrophils probably are also the primary source of these enzymes. These include cathepsin G, another cationic serine protease with broad substrate specificity, urokinase-type plasminogen activator (uPA), and protease 3 (3,32-35). In all probability, when looked for, elevated levels of additional neutrophil proteases will be found in chronic dermal ulcers.

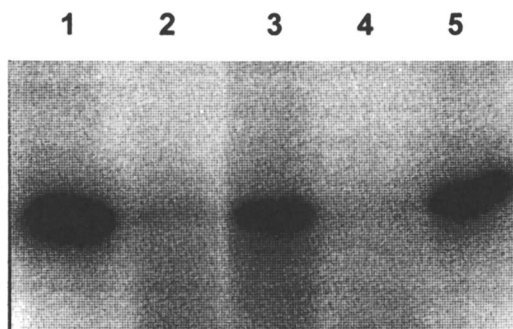


Figure 3. Neutrophil elastase in chronic wound fluids is capable of degrading peptide growth factors. ^{125}I -Transforming growth factor- β_1 was incubated alone (lane 1), with purified neutrophil elastase (lanes 2 and 3), and with 100 μg of chronic wound fluid (lanes 4 and 5) at 37°C for 90 minutes. The presence of intact growth factor was assessed by gel electrophoresis and autoradiography. A specific inhibitor of neutrophil elastase (10 μM *N*-methoxysuccinyl-ala-ala-proval-chloromethyl ketone) was included in lanes 3 and 5.

Antiprotease Screen

The tissue-destructive potential of neutrophil-derived enzymes are countered by powerful plasma antiproteases. These include α_1 -proteinase inhibitor, α_2 -macroglobulin, leukoproteinase inhibitor, plasminogen activator inhibitor (PAI-1), and tissue inhibitor of metalloproteinases (TIMPs) (4). Together, these inhibitors create an antiprotease shield in both plasma and in interstitial fluids. There is growing evidence that this antiprotease shield can be overwhelmed by the over exuberant neutrophil response in chronic wounds.

MMPs are specifically inhibited by TIMPs, a family of small proteins (36). TIMPs inhibit active MMPs by forming tight non-covalent 1:1 complexes (37). ELISA analysis indicates that TIMP-1 levels are lower in fluids from leg and pressure ulcers than are found at peak levels in fluids from healing surgical wounds (Table I) (17). Similarly, ELISA analysis has also demonstrated the existence of elevated levels of MMP-1/TIMP-1 complexes in fluids from pressure ulcers (18).

α_2 -Macroglobulin, is an abundant (2.5 mg/ml) plasma protein that is a potent nonspecific protease inhibitor (38). In healing wounds, levels of this proteinase inhibitor approach that of plasma (13,22,39). In chronic dermal ulcer fluids, proteolytic activity has been shown to correlate with a decrease in the level of intact α_2 -macroglobulin monomers or with the appearance of the protease cleaved forms (Table I) (13,22,39,40). Complexes of α_2 -macroglobulin and MMP-1 have also been observed in stasis ulcer fluids (40).

Another abundant plasma protease inhibitor, α_1 -antiprotease (α_1 -antitrypsin), irreversibly inhibits neutrophil elastase by forming an enzyme-inhibitor complex (38). Levels of complexed forms of α_1 -antiprotease are significantly increased in chronic wound fluids and at least some of these complexes contain elastase (13,22).

Plasma levels for α_2 -macroglobulin and α_1 -antiprotease are micromolar and similar levels are found in fluids of healing surgical or open dermal wounds (13,22). In healing wounds, TIMP-1 levels increase more than ten-fold to approximately 1,500 ng/ml (18,19). This raises the question of how this seemingly substantial and extensive antiprotease shield can be overwhelmed in chronic wounds? In all probability, the continual influx of large numbers of neutrophils into the chronic wound site places great strain on the antiprotease shield. As discussed above, neutrophils contain enormous amounts of proteases. In addition, these proteases may cooperate in a synergistic fashion to reduce the amounts of endogenous protease inhibitors within the wound site. TIMPs are substrates for neutrophil elastase, whereas α_1 -antiprotease itself is a substrate for neutrophil collagenase (MMP-8) (41,42).

Another important neutrophil function that can influence the protease-antiprotease balance is the generation of hypochlorous acid (HOCl) and N-chloramines via the myeloperoxidase-H₂O₂-halide system. HOCl and N-chloramines efficiently oxidize or chlorinate a wide number of molecules including both α_2 -macroglobulin and α_1 -antiprotease (43,44). Up to 2500 to 5000 nmol of HOCl can be generated by as few as 25×10^6 neutrophils (45). Furthermore, the ability of HOCl and N-chloramines to activate latent MMPs may shift the protease-antiprotease equilibrium further towards degradation (46,47).

Treatment of Chronic Dermal Ulcers

The implications of an over exuberant neutrophil response within the milieu of a wound are various and significant. The net deposition of extracellular matrix is difficult to achieve in face of overwhelming proteolytic environment. Extracellular matrix fragments generated by the action of the neutrophil proteases can act as chemotactic peptides that attract additional waves of neutrophils (48-50). Soluble growth factors and their receptors are both likely to be degraded further reducing the ability of wounds to heal or to respond to the application of exogenous growth factors. Thus, the accumulating evidence indicating the ability of the neutrophil to mediate damage to normal tissue would seem to indicate a need to develop therapeutic interventions that can ameliorate these unwanted destructive activities.

Potential strategies could target neutrophil functions, the activity of specific neutrophil products or by augmentation and protection of the antiprotease shield. Anti-inflammatory agents that block or inhibit one or more neutrophil functions such as chemotaxis, adherence, infiltration, or degranulation may be worth exploring (51-58). Topical application of a chemically modified tetracycline to full-thickness open wounds in streptozotocin treated rats results in decreased collagenase and gelatinase levels and increased granulation tissue formation (59). Protection of protease inhibitors from oxidative damage is another potential approach. A methionine at

position 358 is the primary target for oxidative inactivation of α_1 -antiprotease (60). Free methionine protects this site from oxidative damage (61). General free radical scavengers might also be employed to not only protect serine protease inhibitors but to also reduce levels of oxidant-activated MMPs. Likewise, inhibitors of enzymes involved in generating reactive oxygen metabolites might be utilized to modulate the chronic wound environment (61-63). Augmentation of the antiprotease shield could be produced by inducing the expression of endogenous antiproteases or by topical application. Alternatively, natural or synthetic exogenous protease inhibitors could be used (64-66).

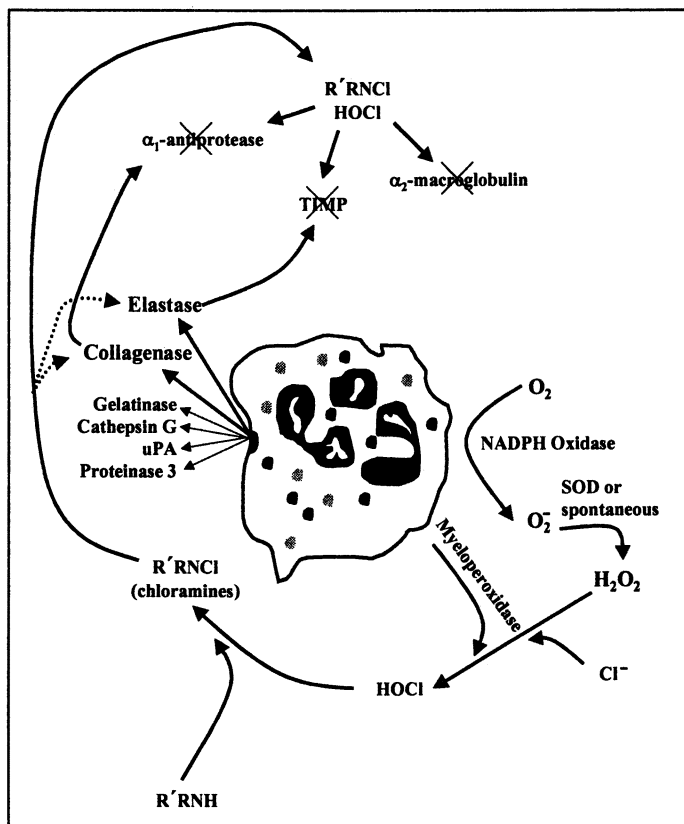


Figure 4. Schematic of interactions of neutrophil enzymatic and oxidative products and endogenous protease inhibitors. Short-lived and long-lived oxidants generated by the neutrophil NADPH oxidase/myeloperoxidase system can activate latent proteases (MMP-8 and MMP-9) as well as inactivate major protease inhibitors such as TIMP, α_2 -macroglobulin, and α_1 -protease inhibitor. Furthermore, neutrophil proteases can also interact in a synergistic fashion as evidenced by the ability of MMP-8 to degrade α_1 -protease inhibitor and elastase to degrade TIMP.

A growing body of evidence supports a role for neutrophils in the pathophysiology of chronic dermal ulcers. There is a growing appreciation that the extensive proteolytic and oxidative armamentarium of the neutrophil can subvert the extensive and overlapping barriers that have been erected to protect host tissues from injury. A better understanding of that role and of the functions of neutrophils will provide opportunities for novel treatment strategies of these problem wounds. The development and use of inhibitors directed against neutrophil functions and neutrophil products may be useful in attenuating inflammatory damage. These reagents may be effective by themselves or as adjuncts to other treatment strategies. One obvious potential use would be to alter the environment of a chronic dermal ulcer so as to make therapies involving peptide growth factors more effective.

There is a bewildering array of dressing products for treating chronic dermal ulcers on the market. With few exceptions, the functions of these dressings are very limited that basically, do one or more of three things: provide protection, facilitate wound debridement, or regulate wound moisture. Perhaps with our increased understanding of the pathophysiology of chronic wounds the stage has been set for the next generation of chronic wound dressings. These "smart dressings" would modulate the biochemical environment of chronic wounds add or remove enzymes or oxidants. This might be achieved by relatively nonspecific approaches which could include using resins that act as ion exchange supports or by modifying the dressing materials themselves. Alternatively, therapeutic agents could be incorporated into dressing materials. These could include specific inhibitors of proteases, antioxidants, and agents that interfere with neutrophil functions. There would be many advantages to these approaches. Treatment would be topical rather than systemic. By varying the composition of the dressing materials, the dynamic nature of wound repair could be taken into account.

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Chapter 6

Design, Preparation, and Activity of Cotton Gauze for Use in Chronic Wound Research

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We consider the rational design and chemical modification of cotton gauze, which is used widely in chronic wounds, to improve wound dressing fibers for application to chronic wound healing. Cotton gauze may be tailored to more effectively enhance the biochemistry of wound healing. The presence of elevated levels of elastase in non-healing wounds has been associated with the degradation of important growth factors and fibronectin necessary for wound healing. In the healing wound a balance of elastase and antiproteases precludes degradation of beneficial proteins from taking place. Cotton gauze modified to release elastase inhibitors or selectively functionalized to sequester elastase provides a dressing that decreases high levels of destructive elastase in the chronic wound. Three approaches have been taken to explore the potential of protease fiber-inhibitors useful in chronic wounds: 1) formulation of inhibitors on the dressing; 2) synthesis of elastase recognition sequences on cotton cellulose; and 3) carboxymethylation, and oxidizing textile finishes of cotton gauze to remove elastase from the wound.

Since antiquity cotton-based wound dressings have provided an important starting point in wound care. An example of improvement to cotton gauze was the discovery of semielastic cotton gauze bandages in 1945 (1). The invention of semielastic gauze arose from an unmet need in wound and orthopedic care and improvement in cotton gauze based on cotton's shrinkage upon immersion in concentrated sodium hydroxide. Cotton is used both as a primary (direct contact with the wound) and

secondary (placed over primary dressing for protection, absorptive capacity, compression and occlusion) wound dressing. Cotton is manufactured for use as bandages, sponges, tubular bandages, stockings, and gauzes. The world market for woven and non-woven cotton medical textiles has grown steadily over the last ten years and is estimated to be well over \$500 million .

Absorbent cotton gauze is inexpensive and it is utilized widely in packing deep wounds that tend to pool wound fluid. Chronic, non-healing wounds are highly exudative and require frequent changing of the dressing. Wounds are often treated with cotton gauze as part of routine long-term wound care. Wet, saline-soaked gauze is used in debridement and cleaning and provides a moist environment enhancing granulation tissue growth. Bacteria and wound fluids are wicked into the interstices of the gauze as drying occurs.

Chronic wounds are a worldwide health problem and one of the most costly unsolved problems in health care today. Approximately 15 percent of the diabetes patients develop skin ulcers, which result in over 60,000 amputations per year. Medical costs are as much as \$36,000 per patient and overall costs, including loss in productivity, is as high as \$20 billion per year. There are 175,000 to 250,000 spinal cord injury patients in the United States with 25 to 85 percent developing pressure ulcers. When elderly nursing home care is added to the spinal cord injury population it is conservatively estimated that 2 million people in the United States alone suffer from pressure ulcers each year (2). Thus, the costs associated with care of all pressure ulcers is very significant and advances in chronic wound research and fiber dressing design for accelerated healing are currently needed.

Continued advances in the science of wound healing will lead to new wound care products. Understanding how destructive enzymes play a role in preventing healing in the chronic wound signals a potential new product area of wound dressings. The elucidation of the destructive role of elastase (3,4) and metalloproteases (5) in the breakdown of important growth factors and fibronectin involved in wound healing has lead us to design wound dressings that function to inhibit elastase. Elastase proteolysis in pathogenic diseases such as arthritis and emphysema has been well documented and numerous classes of both protein and small molecule inhibitors have been developed and placed in clinical trials. However, with the recent implication of excessive concentrations of destructive proteases causing dysfunction in tissue repair, efforts to understand how dressings can be tailored to inhibit or sequester chronic wound proteases will become a priority in the development of wound dressings.

Cotton cellulose may be chemically modified in a variety of ways to retain the basic properties of a wound dressing while incorporating novel functional modifications. For this reason we have asked the question: How may cotton gauze be modified to improve its role in accelerating the healing process of chronic wounds? This paper addresses two approaches for modifying cotton cellulose fibers in gauze for research on inhibition of destructive proteases that keep wounds from healing.

The process we have taken to develop a cotton-based wound dressing containing a fiber-inhibitor is outlined in Figure 1. The goal of this process is integration of a chemical modification with the cotton fiber textile design compatible with the wound environment. *In vitro* assessment of fiber-inhibitor formulations is necessary to demonstrate the relative inhibition kinetics on the textile fiber. The dose response profile of the protease fiber-inhibitor may then be utilized as a benchmark for

incorporating fabric design with inhibitor efficacy. The overall effect on absorbency, wicking, pH, strength/elongation, air permeability, and elasticity are properties that require careful consideration when chemically modified gauze is used in wound management. Consideration of the effect of sterilization on the modified gauze is a key issue because high temperature sterilization may alter chemically modified gauze. The lack of good animal models for chronic wounds prompts a need early in wound dressing development for human clinical trials to assess the *in-vivo* efficacy of a fiber-inhibitor. Demonstrated protection of growth factors, fibronectin and collagen from degradation is the goal of the mechanism based approach.

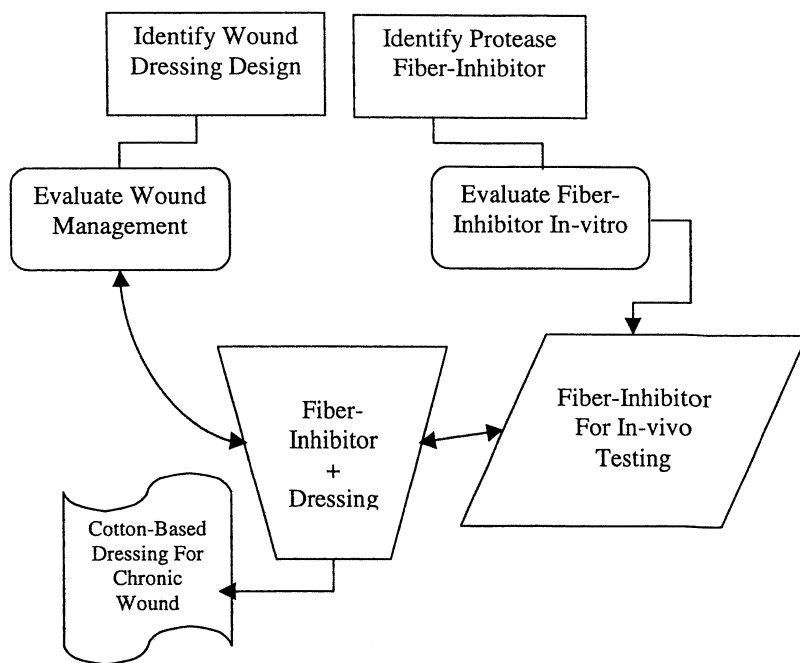


Figure 1: Discovery process for cotton-based dressing containing fiber-protease inhibitors for application to chronic wounds where proteases such as elastase and metalloproteases are elevated.

Approaches For Inhibiting or Sequestering Proteases

Two approaches of controlled release and sequestration to inhibit elastase activity in the chronic wound are considered here. The mechanism of activity for these two approaches, which is depicted on cotton cellulose in Figure 2, is based on electrophilic attack of the active site Ser-195 on aldehyde and ketone-based inhibitors. 1) Controlled release of an inhibitor from a wound dressing matrix requires formulation of a protease inhibitor in the matrix of the fiber such that it would be released into the environment of the wound and act to maintain a balance of proteolytic enzymes and antiproteases. A moist primary phase, such as a hydrogel or hydroactive alginate in contact with the fiber-inhibitor, would facilitate delayed diffusion into the wound bed versus bolus release. 2) Selective sequestration of harmful proteases into the fibers of the wound dressing implies a dressing design that selectively recognizes or permits passage of the proteases into the fiber where they are immobilized and prevented from returning to the wound bed. Selective uptake precludes beneficial growth factors such as platelet derived growth factor from being extracted by the dressing. Platelet derived growth factor is thought to promote healing of chronic wounds.

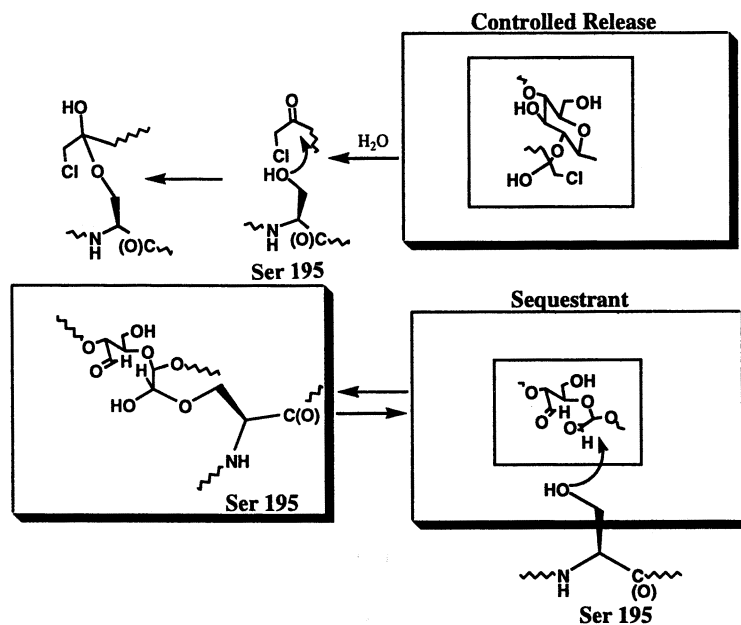


Figure 2: Molecular mechanism of controlled release inhibitors and sequestration on cotton fibers of serine protease (active site serine-195) elevated in the chronic wound. Diagram of molecular structure of cellulose contained in cotton gauze bandages as the primary wound dressing is represented by a modified anhydroglucose unit of cellulose.

Controlled Release of Inhibitors

For controlled release of an inhibitor from the fibers of a wound dressing to be effective the following criteria are required: 1) a selective elastase inhibitor formulated in a wound dressing matrix; 2) release of the inhibitor into the wound environment with duration of effect and; 3) adequate clearance from the wound site. Proteinaceous inhibitors, such as human alpha-1-protease inhibitors, would be effective and turned over in the wound site. However, there are a number of potent peptide-based elastase inhibitors, some of which have been considered for oral therapy. These types of inhibitors might be utilized as a fiber- released supplement to new growth factor treatment modalities for diabetic skin ulcers. Controlled release of agents linked with important roles in wound healing includes growth factors (6), antibiotics (7), and trace elements (8). The use of the enzyme inhibitor aprotinin for treatment of corneal ulcers was reported (9), however, there have been few reports of treatment methods on the release of elastase inhibitors into wounds to maintain proteolytic balance.

A key issue with inhibitors released into the wound is the kinetic profile upon delivery of inhibitor from the attached fiber. The pharmacokinetics of the inhibitor is dependent on both the type of fiber and the condition of the wound. Inhibitors may be formulated as an ion-pair with the textile fiber, through physical entrapment in fiber pores, or in a gel or resin on the surface of the fiber. To evaluate the kinetics and release profile of inhibitors from fibers we have developed a fiber-inhibitor assay utilizing cotton-bound inhibitors formulated for *in vitro* evaluation (10). Development of a wound dressing fiber design for inhibiting serine proteases necessitates screening of fiber-inhibitor formulations in an *in vitro* assay to distinguish the differences in inhibitory activity between fiber-bound and freely dissolved inhibitor. Evaluation of the kinetics of a slow-binding inhibitor may be done by assessing the first order rate constants upon release of the inhibitor from the fiber and comparing with the first order rates constants of the freely dissolved inhibitor under pH and saline conditions similar to the wound. Inhibitor release is measured by quantitative chromatographic quantification techniques. Inhibition kinetics is characterized for the fiber-inhibitor complex by determination of initial velocities for both the fiber-inhibitor and the freely dissolved inhibitor. A comparative measurement of the inhibitory activity of the fiber-inhibitor with freely dissolved inhibitor may be made with the I_{50} , which is 50% inhibition at the total inhibitor concentration. The concentration of tight-binding inhibitor required to give 50% inhibition as measured by initial velocity is based on the concentration of the enzyme. Inhibitor concentration may be measured by using high performance chromatography analysis of inhibitor (10).

Selective Sequestration

Selective sequestration of proteases by modified fibers to reduce excessive protease concentrations in the chronic wound provides an approach that is less likely to be complicated by potential drug reactions associated with a synthetic inhibitor or

antigenicity arising from protein inhibitors. A variety of wound dressings have been developed over the last thirty years since George Winter's early 1960 published results demonstrated that moist wounds heal faster than air exposed wounds (11). These new products include films, gels, foams, and various polysaccharide materials that have improved case management of chronic wounds. However, there is no evidence that any of these occlusive dressings actually accelerate healing of chronic wounds. Nonetheless, research on the uptake of harmful proteases by chronic wound dressings, such as hydrocolloids, hydroactive alginates, collagen, hydrogel polymers and cotton gauze, may further the improvement of these wound dressings as protease sequestrants. For example, recent work with hydroactive dressings has demonstrated decreased interleukin-1-alpha (an inflammatory cytokine) and collagenase levels following dressing treatment of chronic wounds (16).

Elastase recognition sequences have been placed on cotton cellulose as a model to determine the properties of a synthetic wound dressing required to selectively sequester the enzyme from the fluid of the chronic wound (Figure 3). Using this approach the tripeptide sequence Val-Pro-Val-OMe (Figure 4) was synthesized on both cotton cellulose and carboxymethylated cellulose cotton (CMC) and subsequently formulated as ground fibers and prepared as chromatography columns to study elastase retention. The sequence Val-Pro-Val-OMe was terminally anchored to carboxymethylated cotton with an amino terminal valine and demonstrated retention of 58% of elastase. Selective functional group finishes have also been incorporated on cotton gauze dressings with the aim of making cotton gauze a more effective sequestrant of destructive enzymes in the environment of the wound (19). Both approaches constitute a rational design for inhibiting elastase with biologically-based methods.

We have recently shown that oxidized, phosphorylated, and sulfonated gauze decrease elastase activity in solution and in human wound fluid. The cotton gauze finishes were designed for either active site uptake or by negatively charged counterion binding to the guanidinium groups on elastase-containing arginine (Figure 4). To prepare oxidized cotton gauze designed as an active site enzyme uptake as shown in Figure 2, cotton gauze is converted to dialdehyde cotton by treatment with sodium periodate (Figure 5).

Preparation of Carboxymethylated and Dialdehyde Cotton Gauze

Both carboxymethylated cellulose (CMC) and oxidized cellulose (oxycellulose) have been utilized in wound management either as a constituent of hydrocolloid wound dressings (14) which produce high absorbency or as a preferred hemostatic agent in surgery (15), respectively. We have investigated both carboxymethylated and dialdehyde cotton gauze in order to assess their effect on elastase sequestration.

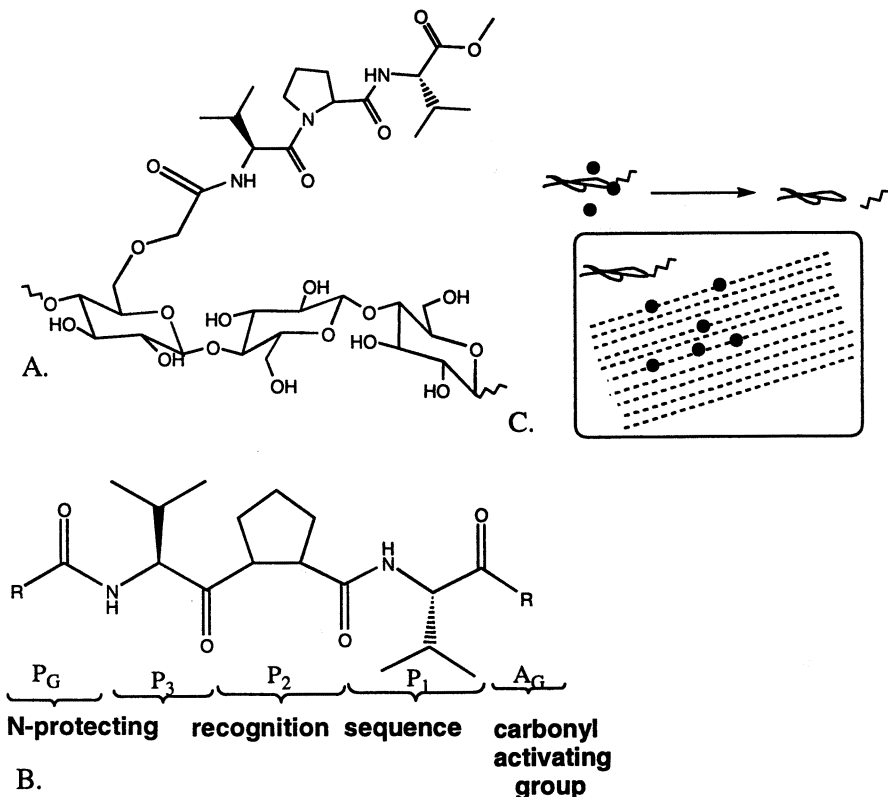


Figure 3: (A) Design of cellulose conjugates of elastase inhibitors based on the tripeptide recognition sequence (B) of elastase. Representative structure of the peptide on cellulose. (C) Illustration of modified cotton gauze to selectively bind elastase preventing degradation of growth factors and fibronectin.

Preparation of Dialdehyde Cotton Gauze

Cotton gauze (12 ply- 4in. X 4in.), USP type VII, were treated under three different reaction conditions in lots of 50 gauze sponges as follows; Treatment #1: A 0.07 M solution of sodium periodate for one hour at 45 C with a solution pH of 4.2. Treatment #2: A 0.2 M solution of sodium periodate for 1.5 hours at 45 C with a solution pH of 4.5. Treatment #3: A 0.2 M solution of sodium periodate for 3 hours at 45 C with a solution pH of 4.5. Following the treatment excess periodate was removed by rinsing the gauze through a screen under running tap water. Following the rinse cycle the gauze samples were passed through a conventional ringer to remove excess moisture. The samples were then separated and placed on a wire rack to air dry overnight. Upon drying the cotton gauzes were packaged in chex-all II

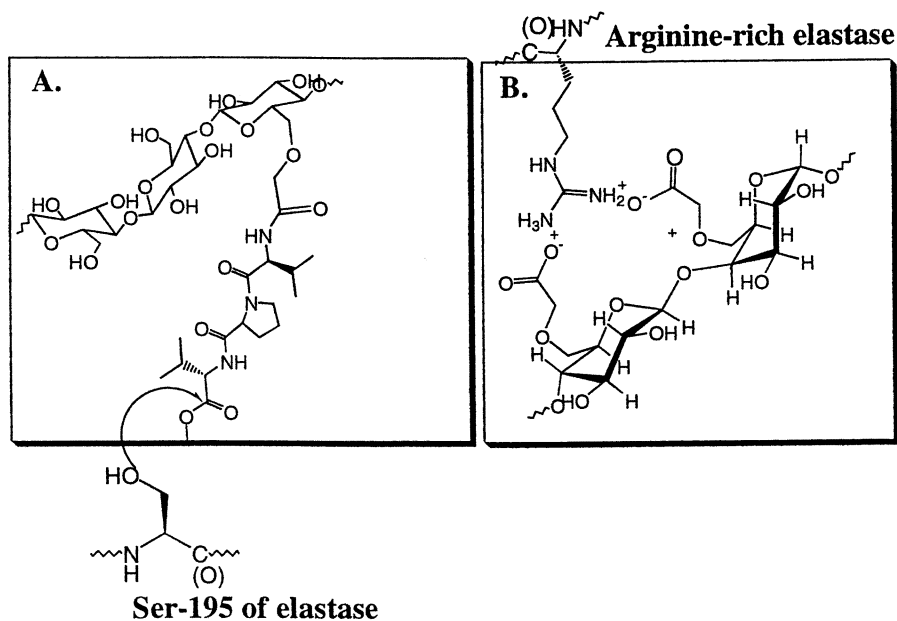


Figure 4A: Structure of peptido-cellulose conjugate oriented with the amino-terminal portion of the sequence anchored to carboxymethylcellulose allowing solution solvation for binding of the elastase recognition sequence; illustrating active site uptake of elastase. **B:** Structure depicting arginine salt bridge from the protein surface with carboxylate group of carboxymethylcellulose.

instant sealing pouches (5 in. X 10 in.). The packaged gauzes were ethylene oxide sterilized by Micro-Test Laboratories, Wagonum MA.

Preparation of Carboxymethylated Cotton Gauze

Carboxymethylation was completed as outlined previously (8). A solution was made by mixing 24 parts of dichloroacetic acid with 24 parts of water, and while cooling in an ice bath, stirring in 75 parts of sodium hydroxide solution. This solution was used to pad a sample of cotton gauze to a wet pickup of 135%. The wet sample was then placed in an oven at 100 C, and dried/cured for 10 minutes.

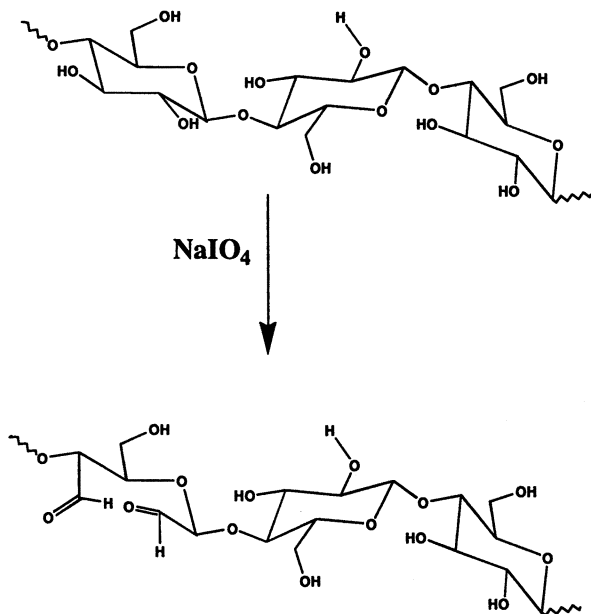


Figure 5: Reaction for conversion of cellulose to dialdehyde cellulose

Determination of Dialdehyde content and Degree of Substitution of carboxymethylcellulose

To determine the dialdehyde content the following procedure was employed (17). Weigh accurately a 200-milligram sample of dialdehyde cotton gauze and place it in a 125 mL Erlenmeyer flask. To the flask is added 10 mL of standardized 0.1 N sodium hydroxide and the flask is swirled and placed in a 70 C water bath for 60 minutes. The flask is cooled under running tap water. The residual sodium hydroxide is then titrated with standardized 0.1 N hydrochloric acid to the phenolphthalein endpoint. The percent dicarbonyl units is calculated with the following equation:

$$\frac{\text{Total meq. Base} - \text{total meq. Acid}}{\text{Dry sample wt., mg.}} \times 100 = \% \text{ dicarbonyl units}$$

161

where 161 is the molecular weight of the anhydroglucose repeating unit in cellulose, 50% of which has been converted to dialdehyde units. The degree of substitution for the carboxymethylcellulose gauze was determined by the procedure previously reported (18).

Assay of Treated Gauze For Elastase Activity

Treated and untreated gauze samples were submerged in 1 milliliter of buffer containing 1 unit/ mL of human neutrophil elastase. The samples were allowed to incubate for one hour at room temperature after which the gauze samples were removed and placed in an Autovial press filter (Whatman,) to drain unbound buffer and enzyme. The unbound elastase fractions were combined and assayed for elastase activity as described below.

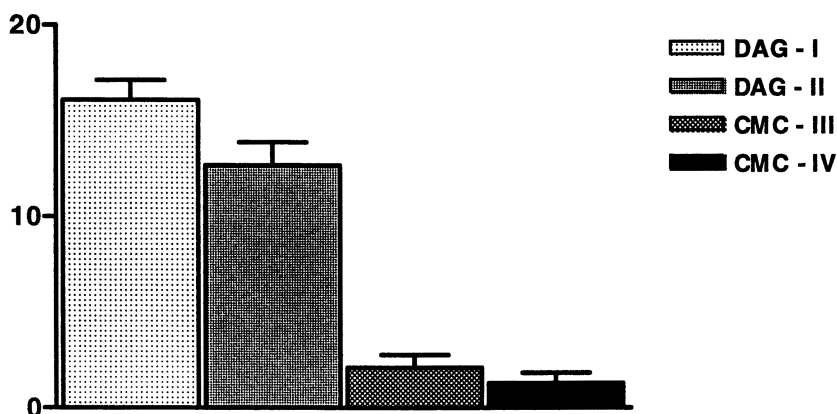


Figure 6: Percent levels of dicarbonyls in dialdehyde cotton gauze (DAG I and II) and carboxylates (CMC III and IV) on carboxymethylated cellulose as determined by titration on modified cotton fibers. Data are mean \pm SE of triplicate determinations.

Enzyme Assays

Enzyme assays of the solutions containing unbound human neutrophil elastase were conducted in pH 7.6 buffer composed of 0.1M sodium phosphate, 0.5 M NaCl, and 3.3 % DMSO and subjected to spectrophotometric measurement of the release of p-nitroaniline at 410 nm from the enzymatic hydrolysis of MeOSuc-Ala-Ala-Pro-Val-pNA (Sigma) (9). The spectrophotometric kinetic assays were performed in a Bio-Rad Microplate Reader (Hercules, CA) with a 96-well format. Two hundred microliter aliquots of a elastase solution (0.2 units) were assayed per well, and 20 microliters of a 475 micromolar substrate solution was added to initiate the enzyme reaction.

Results

The gauze finishes employed in this study were prepared to assess the effect of both sterilization and variation of the sodium periodate finishing conditions on the activity of dialdehyde cotton gauze in reducing elastase activity. The reaction for

conversion of the cotton cellulose to dialdehyde cotton is shown in Figure 5. Previously we have shown that elastase activity is reduced in solution in a dose-dependent manner with increased amounts of dialdehyde cotton gauze when compared with treated gauze (19). Here we have assessed the effect of varying periodate treatment conditions upon the elastase-reducing activity of dialdehyde cotton gauze. As shown in Figure 7 variation of the oxidation conditions, and hence percent aldehyde incorporation, effects elastase-lowering activity of the dialdehyde cotton gauze. The results of these studies suggest that treatment #1 is optimal for retaining efficacy of the dialdehyde cotton gauze. Prolonged exposure and higher periodate concentration decreases the efficacy of the gauze in reducing elastase activity in solution, which is correlated with fewer dicarbonyl units in the cotton cellulose.

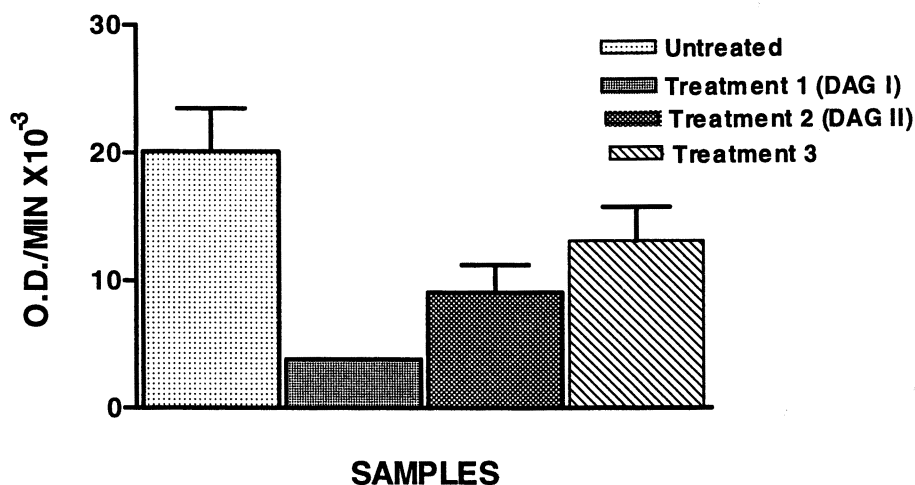


Figure 7: Assessment of reduction in human neutrophil elastase activity from several oxidized cotton gauze samples. Untreated cotton gauze was employed as a control. Treatment #1; Treatment #2; Treatment #3. Elastase activities are compared in terms of initial velocities for solutions taken from dialdehyde cotton gauze samples. Data are mean \pm SE of triplicate determinations.

Previously we have shown that elastase activity may be reduced in solution by carboxymethylated cotton gauze (19). Two different degree of substitution (D.S.) levels of carboxymethylated cotton cellulose were compared to further assess this. As shown in Figure 8 higher substitution levels of carboxylate on cotton resulted in an equivalent reduction in elastase activity in solution.

We have compared two types of cotton gauze modifications, the resulting elastase activity, and the relationship of activity to levels of oxidation and carboxymethylation on the cellulose fiber. As seen in Figure 6 levels of carboxymethylation were relatively low. However, lower levels of carboxymethylation on cotton gauze may be preferable in precluding non-specific binding of favorable proteinaceous growth factors. Nonetheless, enzyme activity levels shown in Figure 8 were consistent with

percent levels of CMC, and a higher percent carboxymethylation corresponded to lower activity. With regard to the oxidized cotton gauze, as much as sixteen per cent of the cellulose was modified with aldehyde dicarbonyls and a similar relation of activity (Figure 6) to titratable aldehyde was observed. Correlation of decreased enzyme activity with number of carboxylate or aldehyde sites on cellulose observed within a narrow range of enzyme rates of activity suggests the cotton derivatized aldehyde and carboxylates bind elastase into readily assessable binding sites in the modified cotton fiber of the gauze.

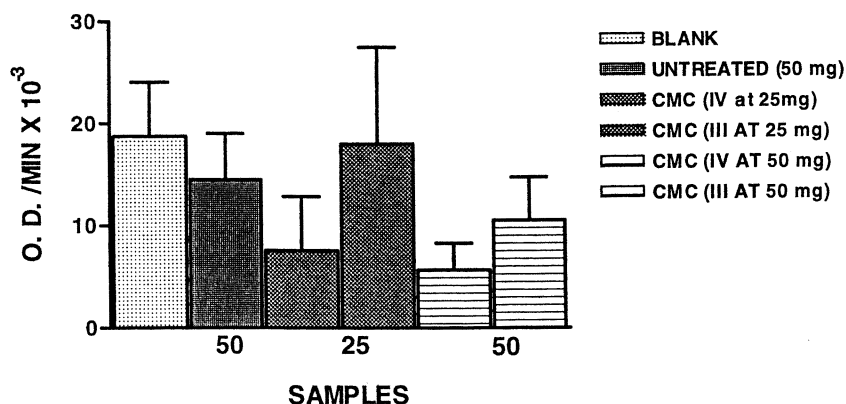


Figure 8: Assessment of reduction in elastase activity from carboxymethylated cotton gauze samples. Cotton gauze was carboxymethylated (CMC) as previously reported (13). Two carboxymethylated cotton gauze samples (III and IV) were assayed. Data are mean \pm SE of triplicate determinations.

Summary

The future development of chronic wound dressings technologically aligned with the biochemistry of wound healing will be grounded in a mechanism-based dressing design included with an effective treatment modality for chronic wounds. A mechanism-based approach to designing chronic wound dressings considers the molecular interaction of the target enzyme or receptor with the fibers and composition of the wound dressing. Development of a wound dressing that promotes selective uptake of destructive proteases is a challenge in chronic wound research. Chronic wounds constitute a complex pathology and a 'magic bullet' cure is not expected. However, we have focused on a component in the pathology of chronic wounds that constitutes a major interruption in the healing process: the proteolytic degradation of growth factors and fibronectin. Polysaccharide fibers such as cellulose may be modified chemically for targeting protease inhibition on the cellulose cotton fibers of

cotton gauze dressings or in carboxymethylated cotton gauze, composites of hydrocolloid dressings and gels. We have specifically targeted the serine protease elastase, by incorporating inhibitory compounds either directly into the cellulose chain of the cotton fiber or as a carrier molecule that will inhibit upon release from the fiber. Other approaches that might be considered mechanism-based but are not treated here include immunological and physical sequestration of undesirable factors in the wound. An immunological approach implies using antibodies to the targeted protease or inflammatory cytokine as the mode of action, whereas physical removal may be possible by selective passage or permeation of the protease or inflammatory cytokine into the wound dressing. Since cotton is a naturally occurring fiber the future use of a variety of chemical and biochemical based strategies for controlling wound biochemistry appear promising.

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Chapter 7

A Hybrid Bioabsorbable Wound Dressing

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A wound, whether it is the result of an incision during surgery, or an injury, or a burn, requires a dressing to protect it from infection and help it heal. Many products are available for doctors and hospital staff to choose from; however, for wounds that are complex, such as those resulting from severe burns, there is a need for an improved dressing. In this paper, we provide a detailed review of the earlier and currently available dressings, and the results of a study on a dressing involving a new design concept. The paper is divided into three parts. In the first, we give a general introduction to the wound healing process, a definition of an ideal dressing, and a brief description of structural factors known to affect wound dressing performance. In the second, we present a brief review of both ancient and current dressing concepts and arts. In the last part, we discuss the design concept of a new dressing that we believe holds promise for use in the future. The results of preliminary studies we have carried out are included in this final section.

General Introduction

The Healing Process

The body can heal a wound if left to its own accord. To begin this process, the body cleanses the wound by releasing extra blood that contains white blood cells. These cells fight bacteria and consume and destroy other debris. Fibroblasts multiply during the cleansing process and fill the wound gap with new fibers and a jelly-like substance. If no infection is present, blood vessels grow into the region which promote further tissue building that results in a scab. The scab is formed by

connective granulation tissues. These also close the wound by a process called wound contraction, much like a muscle contracting.

If infection does set in, the bacteria destroy the newly formed cells and tissues. They also cause pus to form by overproduction of polymorphonuclear granulocytes (polys). As the polys die, they produce chemicals that digest the dead tissues, but they also kill the fibroblasts. A salve or covering can protect the wound from infection. Until all the infection is depleted, healing cannot be considered completed (1).

Factors Affecting Performance

Environment

Until recently, wounds were thought to heal quicker under drier conditions. Now a moist environment is considered ideal. Assuming that exudate production has already ceased, a dry dressing will lead to a scab, which may anchor some of the dressing fibers and cause pain and reopen the wound when the dressing is removed. This scab also prevents the migrating epidermal cells from reforming as fibrous layers of skin and, therefore, lengthens the healing time. An occlusive dressing, which keeps the wound moist, does not allow a scab to form, thus the cells are free to move through the exudate at the wound-dressing interface. A totally occlusive or semi-permeable dressing also prevents secondary damage due to dehydration. Loss of moisture can lead to the drying out of epidermal cells in the hair follicles and sweat glands located in the skin's base layer and is a determining factor for the time of healing. This moisture loss produces formation of a hard eschar or scab (excess plasma combined with necrotic cells). Allowing exudate to pass through the dressing while keeping cellular material at the wound surface increases the healing rate, provided that the fluid transfer rate of exudate through the dressing is approximately equal to the rate of exudate production (2).

Permeability and pH

The partial pressure of oxygen (pO_2) at the wound plays an important role. By reducing pO_2 , an increase in fibroblast growth and production of angiogenic factors from tissues on macrophages occurs *in vitro*. On the other hand, with an increase in pO_2 , the growth of epidermal cells increases. Accordingly, a pO_2 balance is needed to promote healing at an optimum rate. A healthy, non-infected wound needs a more hypoxic environment (low pO_2) that favors angiogenesis and formation of granulation tissue. Once the wound has stopped exuding, a more permeable dressing should be used to increase epithelial growth and discourage production of excess granulation tissue.³ The conclusion of most studies conducted on the effects of pH on healing is that a consistent, chemically mild, acidic environment at the wound will lead to an increase in healing rate (3).

Adherence

The problems caused by a wound contacting a dressing have been examined since 1913 and continue to be studied. The formation of a granuloma caused by fibers entering the body is of great concern (3). Fibers from dressings, disposable

paper gowns, gauze, and polyurethane foams can become part of the eschar or even the granulation tissue. Blood or exudate seeping into the dressing hardens and forms a scab. This causes the dressing to become stiffened and part of the scab, which can disrupt normal healing and lead to keloids, wound dehiscence, incisional hernias, intestinal obstructions, and bed sores. A foam can cause hypertrophy of the epidermis and inflammatory cell reaction of connective tissues which can result in infection. Removing the dressing can reopen the wound, disturbing the formation of new epithelium, and cause pain (4). The dressing, accordingly, should have little or no adherence to a wound and should not lint. For a non-adherent dressing to function, however, it should have small pores in contact with the tissues so that the exudate will pass through and not collect on the wound surface causing inflammation.

Functions of an Ideal Dressing

The main purpose of a dressing is to protect the wound from further damage as well as to alleviate the pain, absorb exudate, and curb bleeding. An ideal dressing, in order to accelerate healing, would keep the wound at an optimum temperature and pH level, moist, free of infection and excessive slough, free of toxic chemicals, particles, or fibers that could be released from a dressing, and undisturbed from dressing changes (2).

Overview of Earlier and Currently Available Wound Dressings

A review of the wound dressing literature and available products was conducted to assess past developments and their efficacy, as well as to understand the structure and performance of the currently available materials.

Historical Review

Wound dressings have been used since the dawn of time. Dressings have progressed from herbs combined with leaves or rags used by primitive man to a complex product involving synthesized polymeric materials compounded with an engineered multi-layer textile structure.

The earliest dressings were held in place on wounds by what is now commonly referred to as a bandage. A dressing rests directly against the wound, allowing the medicinal compound to make contact while absorbing the exudate. Lints were the first such dressings. At first they were comprised of the fluff from old linen, but later produced by a complicated process in which the fluff was soaked in oil of roses and yolk of eggs. This, so called "patent lint", was actually inserted into the wound (5). Patent lint was used often in the treatment of gun shot wounds. By 1963, the lint was treated with a mild boric acid, but was only occasionally used (6).

Early people used the materials available in their natural surrounding. Natural adhesive was used as early as 4000 years ago; resins were applied to rags for

"stickiness" (7). Herbs boiled in water or wine and applied directly to wounds, or wool based materials soaked with wine, vinegar, eggs, honey, powdered earthworms or pig dung are mentioned (8). Medieval healers used many of the same products, including ones soaked in beer. Their dressings for burns consisted of egg whites, fats, herbs, and sometimes goat droppings (9). Minerals with salts or oxides of copper and lead were also thought to aid in healing and were used (10). The occurrence of gunpowder wounds during the Renaissance necessitated the invention of new dressings. Renaissance doctors used eggs, oil of roses, and turpentine. These mild agents were found to promote healing better than boiling oil. For burned skin, other types of dressings were developed (11).

For the next several centuries, dressings did not progress far beyond the traditional rag and herb structure. It wasn't until after World War II that medical science began to investigate other procedures and materials for wound care. One major development in wound treatment was combating infections frequently caused by poorly laundered hospital cloths (12). In the 1950's, synthetic materials were first used to help promote healing.

Types of Dressings Available

The base of most dressings, the part that has actual contact with the wound, is commonly made of bleached cotton or a cotton/viscose blend gauze with differing amounts of a soft paraffin to prevent adherence. This is known as Tulle gras. The Tulle dressing can also be medicated to promote healing and defend against infection. Although some of these dressings cause microbial infections due to fiber influence into the wound, companies continue to research and produce this line of products (13).

Polymeric films or bonded non-woven materials have now become the norm for the base of the dressing because they are generally smoother and have no loose fibers to lint and infect the wound. There are several different general types of these: alginates, semi-permeable films, foam sheets, hydrocolloids, hydrogels, and dressings containing medicines.

Aliginates

Alginates, a substance found in certain species of brown seaweed, have been used since the early 1880's in many products, including thickeners for food products. Passé and Blaine (14) and Oliver and Blaine (15) have conducted studies of alginates formed into sheets as wound dressings. The sodium and calcium salts of alginic acid were used and found to be non-toxic and caused no histological changes. Bromium salts were not fully effective in the absorption of exudate and fluid. Alginate dressings absorb exudate and keep the wounds from drying out. The material reacts with the exudate and form a hydrophilic gel over the wound surface and creates a moist environment (16). The gel becomes a part of the wound and is eventually absorbed. An alginate dressing is not considered good for dry wounds and may interfere with the healing of burn wounds due to excess slough.

Foams

Foams were first made of marine sponges, like those found in the Mediterranean Sea. They were hard to sterilize and they adhered easily to wounds. Synthetic foams were available but continued to be used in only limited amounts until the 1970's, when Dow Corning foams were introduced. They appeared to have ideal characteristics for a dressing, i.e. they kept the wound moist and free of contamination, they are gas permeable and non-adherent to the skin (17). Since then, several companies have gone into the foam market. Polymers are mixed with a catalyst in a 100:6 ratio, allowed to set and expand (for about 3 minutes), and then poured directly on or injected by syringe into the granulating wound; thereby conforming to the shape of the wound. The dressing allows the exudate to penetrate into the structure. Foams are useful in heavily exuding wounds and have been used for burns, but are not ideal for this type of injury. Because they must be inserted into a wound, removed, cleaned, and then reinserted, the questions of adhesion and pain become issues.

Hydrocolloids

Hydrocolloids are usually based on carboxymethylcellulose (CMC) , but can include other polysaccharides and proteins as well. These dressings are produced in the form of either a flexible foam or a film containing a layer of hydrocolloid in granule or paste form. Hydrocolloids were first used as treatment for oral lesions. They can adhere to moist surfaces, absorb water and form a protective gel. Coated on polyethylene sheet, they were used in gastro-intestinal surgical procedures, such as involved in ostomy appliance attachment. Without the plastic backing, they were used for treatment of ulcers. They increase granulation tissue formation, which can be overproduced if not watched, but they do not promote epithelialization (18).

Leg ulcer dressings are still the major uses for hydrocolloids, but they can also be used in high pressure areas as they promote faster healing. According to Thomas, "hydrocolloid dressing(s) are best suited for application to non-infected wounds that do not produce excessive quantities of exudate" (18). Odor and leaking fluid are the primary disadvantages of these dressings. The large amounts of exudate and slough of skin cells expected in burn wounds make hydrocolloid dressings unsuitable for their treatment. Leaking fluid could cause infection due to fungal growth and also result in drying out of the burn wounds.

Hydrogels

Hydrogels were first developed as contact lenses or optical implants, and are usually composed of glycol methacrylates. Gels consist of insoluble polymers with aqueous, interactive, hydrophilic sites. The two basic types are (1) a flexible sheet, comprised of a 3-dimensional macro structure, that does not change physical form as fluid is absorbed but swells until equilibrium is reached, and (2) a non-fixed amorphous gel whose viscosity decreases as fluid is absorbed until the gel assumes the shape of the wound. In the latter case, the fluid continues to be absorbed until all cohesive properties are lost and the gel becomes a dispersion of the polymer in water (19). Several types of gels are available on the market but the properties are similar whether the product is in the form of a sheet or a gel. Hydrogels could be excellent

medicinal carriers. Also, they can be easily rehydrated from dry sheets to 50-60 % of their total fluid capacity in 30 min. However, there are many limitations to the use of these dressings. They are hard to apply unless used with a secondary fabric, are suitable only for low exuding wounds, and must be changed frequently. If there is too little exudate, the dressing can dry out, and if there is too much exudate, maceration can occur (19). The major problems with the gels are their need for frequent removal and their inability to hold moisture, which increases the possibility of the dressing drying out and adhering to the wound.

Films

The 18th century saw the development of many medical devices including the first film dressings. It was made of isinglass, which is comprised of the dried swim bladder of certain species of fish (20). Cellophane was the next type of film used. It was used on severe burn victims in studies by Bloom (21). All wounds inspected, including the more infected ones, healed normally and quickly and the amount of pain experienced was less with the film than with other forms of dressings.

Today many types of films are on the market. Most are composed of polyurethane (PU), or PU composites. The latter includes biodegradable films grafted onto PU (European patent 0 371 736) (22), PU foams with the film (US Patent 4 730 611) (23), and polyurethane/polyurea copolymer films and gels (US Patent 4 786 657) (24). The PU film is permeable to water vapor and oxygen but impermeable to water and microorganisms (25). Films appear ideally suited for the care of wounds. When constructing films the properties to consider are thickness and weight, extensibility, moisture vapor and gas permeability, and tissue compatibility. Thickness determines the permeability, elasticity and flexibility of the dressing. Water vapor can permeate through the film quite easily, which can be a tremendous advantage. Large amounts of exudate can collect underneath the film, as with most dressings, but a haemostatic agent can lessen this effect. Since infection can occur if this exudate continues to collect, the moisture vapor permeability of the film is very important (20).

As stated earlier, a dressing's oxygen permeability is also an important property. High oxygen permeability of a dressing will increase epithelialization, according to Winter (26). However, a dressing should not be too permeable to carbon dioxide emitted from the wound, as losses of this gas may cause the wound to become highly alkaline and, therefore, retard healing. Films are smooth, thin and flexible and most only need to be changed infrequently. A problem posed by the film dressing is in the sensitivity of the patient to the adhesive part of the dressing. Vinyl-based and acrylic adhesives are the ones most likely to cause allergic reactions. Among many applications of the film dressings are the treatment of burns, ulcers, surgical wounds, and the protection against catheter wounds. Some products include both a medicated and a plain version.

Summary

The main problems with current dressings are odor control, medication release, adhesion and pain, and ease of use. As previously stated, alginates have problems with dry wounds as the healing rate depends on gel formation from a reaction

between the alginates and the exudate. Foams have pain and application problems, as they must be inserted directly into the wound to absorb exudate. This could be a problem especially for burn wounds. Hydrocolloids emit an odor and only absorb a limited amount of exudate. Hydrogel dressings must be frequently changed and, they do not keep the wound moist as they do not hold moisture. Films have an adhesion problem and must provide an adequate barrier to bacteria, as well as maintain a moist environment.

A New Composite Dressing

Introduction

Different manufacturers have tried to combat wound-dressing problems in different ways, but with only limited success. One approach that has not been investigated is an absorbable dressing. By its very nature, the dressing would not need to be removed; therefore, no pain due to it should be involved. Because of its inherent properties, i.e., flexibility, permeability, and low adherence, a polymeric film seems to be the best candidate for this type of dressing. The flexibility of the dressing should provide the needed comfort to the patient as well as make the dressing easy to apply. Depending on the type of polymer used, a medication can be incorporated into the structure, and the permeability of the polymer can be engineered to exclude bacteria while maintaining a moist environment.

In summary, the characteristics visualized in such a dressing are : low weight and flexibility, permeability to exudate and water vapor, impermeability to microorganisms, transparent for wound examination, and biodegradable and absorbable into the skin.

Proposed Structure

Many new polymers that have emerged recently for use in bioabsorbent sutures can also be used in constructing a dressing. Most sutures are absorbed within 2-6 weeks allowing for adequate healing to occur. A flexible transparent polymer film made of one or more of these polymers could provide the ideal material needed. From the standpoint of design of the products, a three layer composite dressing appears attractive. The first layer is proposed to consist of chitosan, which has been studied in medical applications and has been shown to aid in healing by preventing the penetration of microorganisms and permitting fibrin to grow in wounds (27). The material has a weblike structure (28) which acts as a scaffold for epithelialization. At the contact interface with the wound, the film should easily adhere and allow exudate, air and water vapor to pass through. Its structure allows fibroblastic cells to integrate into the wound. This film should break down rather early in the healing process. However, as this occurs, bacteria would then gain access to the wound. Therefore, the second layer resting on top of the first, should act as a barrier, prevent adhesion of a gauze bandage, absorb the exudate, and protect the wound. The second layer is

proposed to be composed of poly (ϵ -caprolactone) or poly (D, L-lactic acid). Both the chitosan layer and polymer layer will biodegrade, the latter more slowly than the former, and be incorporated into the newly formed skin. Because these layers do not need to be removed, the wound is spared new trauma, and also because the proposed polymer films are transparent, the wound can be viewed from time to time to note the progress of healing.

The third and top most layer is proposed to be an average gauze dressing of cotton or cotton/viscose that absorbs the exudate. This is the only layer that would need to be removed and changed. Each component of the dressing would play its own role in wound healing and must be carefully engineered. Figure 1 shows a schematic of the proposed dressing.

The objectives of this research were to determine the viability and efficacy of a composite wound dressing, mentioned above, utilizing *in vitro* and *in vivo* studies. We desired to evaluate these dressings in the presence of biological tissues in order to determine their effectiveness in epithelialization and overall wound healing.

Materials and Methods

Three polymeric compounds, mentioned above, were utilized during the study: chitosan, poly D, L-lactic acid (PLA) and poly ϵ -caprolactone (PCL). Chitosan has been studied in medical applications, but PCL and PLA have only been studied as sutures or in film form as drug delivery systems. This study also employed the film form of these polymers. The focus of the study was degradation of the films, fibroblastic activity, and healing rate, both in *in-vitro* and *in-vivo* experiments.

Chitosan

Chitosan is the N-deacetylated form of chitin. The chemical formula of both is similar to that of cellulose and starch (see Figure 2). But unlike cellulose and chitin, chitosan is easily absorbed into the body and can be processed into many forms; fibers, paper, nonwovens, and films. A film was cast for use in this project. In general, the chitosan powder was dissolved in acetic acid, and the mixture diluted with distilled water. The gel that developed was filtered and cast on a polycarbonate plate with a doctor's knife. After setting and drying at room temperature for 48 hours, the films were removed, neutralized in a methanol and sodium hydroxide solution, and then washed in distilled water.

Poly ϵ -caprolactone and poly D,L Lactic acid

Poly ϵ -caprolactone (PCL) and poly D, L lactic acid (PLA) are both polymers which have been shown to degrade in biological systems. The L-form of lactic acid is the naturally occurring form, while the D-form is man-made (see Figures 3a-b). Their main uses include fabrication into capsules for drug delivery purposes and extrusion into filaments for sutures, but for this project they were cast as films.

Two samples of poly D, L-lactic acid (lot #931040201; L/D=50/50, Lanthanum catalyst and lot # 930500201, L/D=98/2, Tin catalyst) were procured from DuPont

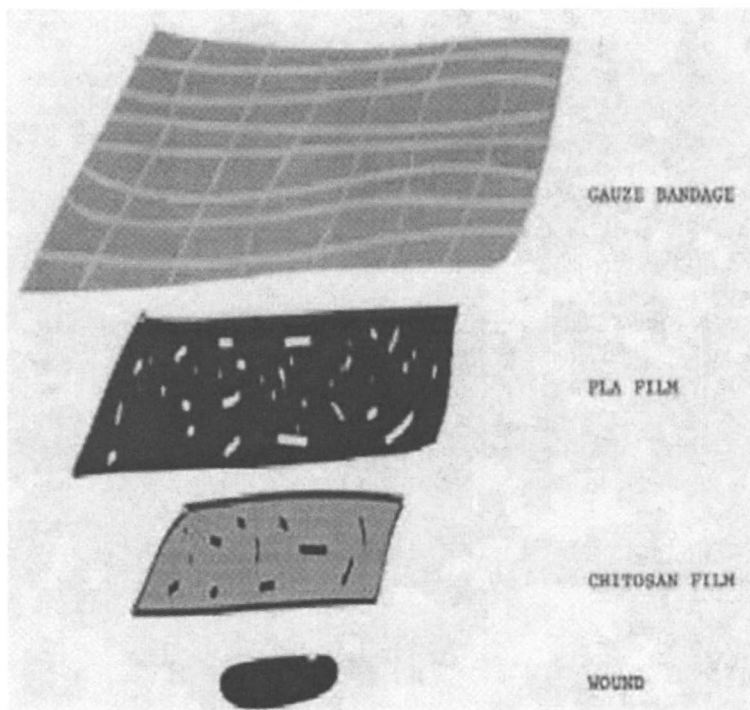


Figure 1. Schematic of proposed dressing

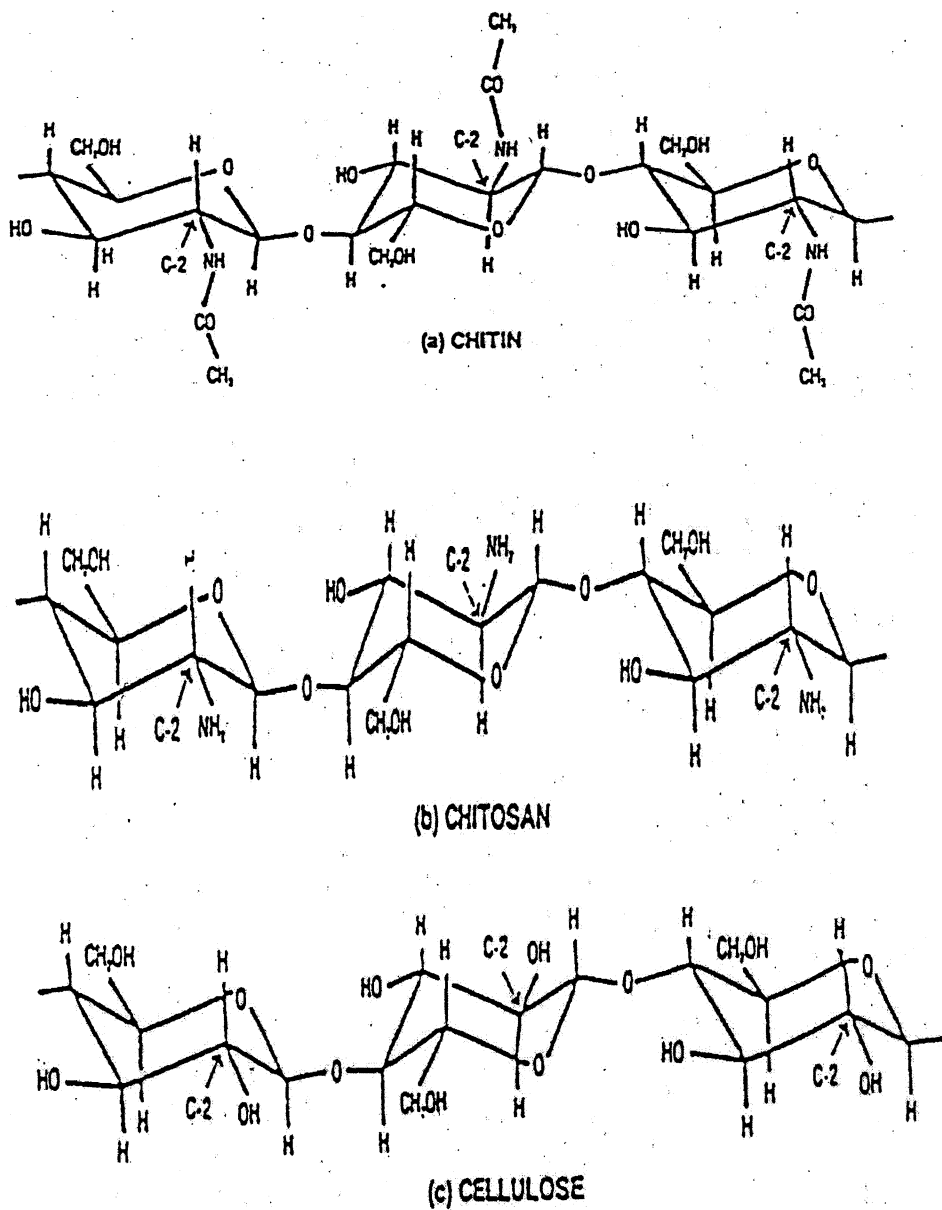


Figure 2 Structures of cellulose, chitin, and chitosan

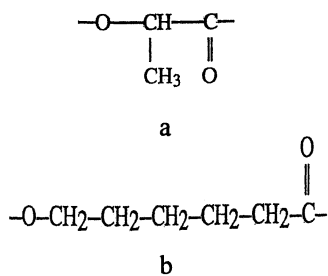


Figure 3. (a) poly L-lactic acid; (b) Poly ϵ -caprolactone

and studied. After experimenting with appropriate solvents for film casting, it was determined that the 50/50 films were too weak. After more work, it was concluded that the 98/2 sample was preferable and should be used, but that a more acceptable ratio for use in the future would be 70/30 for a dressing of this type. The increase of the D-lactic acid from 2% to 30% would decrease degradation times as well as decrease brittleness and increase pliability. In the present case, however, 0.75 g of each polymer (PLA L/D98/2, and PCL pellets) dissolved in 50 ml of methylene chloride was found to create the best films in terms of both handling and strength properties.

Results and Discussion

Physical Properties of Films

An indepth study of the *in vitro* properties was undertaken before an *in vivo* study involving animals was conducted. This preliminary study of the aforementioned materials was conducted in several phases, and involved assessment of a number of physical and biological properties.

Film Thickness

Film thickness affects many important properties, including vapor permeability, degradation, bacterial impermeability, comfort, and ease of application. A thick film may not allow sufficient water vapor into or out of the wound site. If the rate of exudate in the form of vapor being released through the dressing is not comparable to the rate of exudate production, excess slough build-up or drying out of the wound could occur. A thick film will have a slower degradation rate, and will be stiffer and thus less comfortable for the patient. One definite benefit, however, is higher barrier protection against bacteria. A thin film, on the other hand, may degrade too quickly leaving the wound vulnerable to bacterial attack and further trauma. It could allow easy penetration of bacteria into the wound causing infection and prolonging the patient's need for care, and increasing the time and cost of treatment. However, the thin film would be more flexible and therefore more comfortable to the patient. One problem observed with the thinner films has been adhesion of the film to itself, which could cause problems in application to the wound.

Although the process used in casting films, described earlier, did not produce films of reproducible and exact thickness, the values obtained (Table 1) seemed to be adequate in meeting the requirements. The moisture vapor permeability, bacterial barrier and degradation studies, directly affected by the thickness, should provide a more effective design criteria for the size of the films.

Table 1. Comparison of Film Thickness

| Film | Average |
|----------|-----------|
| Chitosan | 0.0177 mm |
| PCL | 0.0144 mm |
| PLA | 0.0179 mm |

Degradation of Films

Polymers in nature degrade at different rates. Chitin degradation, for instance, depends on the molting cycle of the particular species and on the ratio of degradation to synthesis of cuticle (29). Enzymes are responsible for the degradation of the majority of biopolymers, and chitosan is no exception. The goal was to develop a biodegradable film dressing that would be absorbed into the biological system. An important parameter was the degradation time, as this should affect not only healing rates but also bacterial interference. The time should be relatively short (1-2 weeks) for the chitosan layer and longer (up to 2 months) for the middle polymeric layer.

The polymers used in this study are all broken down by enzymatic reactions of microorganisms, as are all other biodegradable polymers (30). The extent of degradation is determined by the access of the microorganisms to the active sites, which are governed by the flexibility of the polymer chain. The more crystalline or cross-linked are the polymer chains, the less flexible and, therefore, the longer are the degradation times. The hydrophilic nature of a polymer affects degradation time as well, so polymers exhibiting both hydrophobic and hydrophilic properties tend to be the best candidates.

Chitin endocuticles (the inner layers) are degraded by the Chitonase enzyme in nature. Almost the entire degraded cuticle is used to make a new shell that is formed underneath the old exocuticle and is sloughed off during the molting stage (31). The enzyme lysozyme is also able to degrade chitosan, but requires a certain sequence of N-acetylated residues for activation. A sample that is 70% deacetylated is considered the most responsive. Chitosan has also been shown to improve the healing rate of different tissues by promoting tissue growth and inhibiting fibroplasia (33).

In this study, a non-sterile saline solution was used at room temperature to determine degradability. The latter was characterized by the viscosity measurements. While chitosan film degraded rapidly, the flow times of the PLA and PCL increased, due possibly to cross-linking and swelling, like that occurs in hydrogels. The results obtained were, therefore, inconclusive.

Both the *in vivo* and *in vitro* studies were completed using intrinsic viscosity measurements in benzene and also gel permeation chromatography to determine film life. The general conclusions reached were that poly ϵ -caprolactone homopolymer would last around one year, while a copolymer of PLA and PCL had much shorter degradation times. For our purposes, however, both had too long a lifespan. Accordingly, additional studies are needed to develop films with the desired shorter degradation times.

Surface Morphology of Sterilized Films

This portion of the study was focused on examining the surfaces of the polymer films. Pictures were taken on a JEOL JSM-35C SEM at 15 kV. In order to increase the conductivity and improve the contrast of the materials, the samples were mounted on double-sided carbon tape, coated with osmium tetra oxide (OsO_4) vapor, sputter coated with gold palladium (AuPd), and grounded with silver colloidal paste.

It was thought that with SEM one could characterize the porous nature of the films. Also, if there was any effect of sterilization on the films, it might become apparent from the micrographs. The samples were sterilized using ethylene oxide (EO), at 100°C , and γ -irradiation. SEM studies were conducted before and after sterilization. From the smooth and continuous nature of the surfaces noted, it was concluded that no pinholes were present either before or after sterilization and that neither EO nor γ -sterilizations had affected the topography of the polymeric films.

Bacterial Permeability

Fighting bacterial infection is still a main goal of wound dressings. Many companies are investigating anti-bacterial finishes and their applications for dressings. Chitosan is one such material that has been shown to have inherent anti-bacterial property (34).

The question of bacterial permeability becomes particularly important when a biodegradable dressing is used. In the beginning, the dressing may be a good barrier to microorganisms, but as the time passes and the dressing becomes less integral, microbes gain access to and infect the wound. For this reason and because chitosan has a short lifespan, the sandwich layers were thought to be important. As the chitosan layer degrades into the wound, the middle layer of polymeric film should protect the wound from the anticipated microbe invasion.

The study was conducted at the North Carolina State University Department of Food Science. The films were inoculated with *Staphylococcus Aureus* and *Escherichia coli* separately as well as together as a sandwich. While no growth was detected underneath the films, the problem of the bacterial solution running off the film onto the agar was quite prevalent with some growth detected on the edges of the films except the PLA and the PLA/CH plates.

Moisture Vapor Permeability

As stated before, a dressing should have pores large enough to allow the exudate vapor through, but small enough to prevent bacterial penetration. There are acceptable rates for moisture vapor permeability (MVP).

The ASTM method E-96-92 was used as a basis to determine the MVP rates of the films both separately as well as together as a composite using silicon gel as the sealant. Vapor equilibrium was reached before the initial weight was measured and the final weight measurement was taken after a 24-hour period. A summary of results is given in Table 2. The MVP rates of the sandwiched and individual films were comparable to the rates found on the commercially available dressings. Accordingly, the permeability values of the dressings used in this study were acceptable.

Table 2. Comparison of moisture Vapor Permeabilities of Commercial Dressings and Materials of Proposed Dressing

| Dressing | MVP g/m ² /24hr | | Film | MVP g/m ² /24hr |
|-------------|-------------------------------|--|--------|-------------------------------|
| Opsite™ | 862 | | PLA | 170 |
| Bioclusive™ | 605 | | PCL | 523 |
| Tegaderm™ | 846 | | CH | 1152 |
| Ensure-it™ | 436 | | PLA/CH | 90 |
| Operaflex™ | 477 | | PCL/CH | 542 |
| Dermafilm™ | 472 | | | |

Summary

All data obtained from the physical properties tests were favorable. The dressings were thin, transparent, degradable, flexible and comfortable, had adequate MVP, bacterial barrier qualities, and were not noticeably affected by sterilization.

Aspects of the interactions of the dressing materials to the biological environment were examined next. The interactions studied included changes in the wound at the wound-dressing interface, toxicity of materials on cells, and the effects of degrading materials on tissues.

In vitro Study

A major phase of any study involving biological tissues is the toxic effect of the materials on the tissues. A second important objective of such a study is the effect of dressing on the rate of epithelialization. In order to spare unnecessary pain to test animals, the initial studies of this aspect are usually conducted outside of the said biological organism, i.e. *in vitro*. All biological work was completed at the NCSU College of Veterinary Medicine.

Toxicity

The advantage of degradation is that the film would not need to be removed, sparing pain and trauma. The disadvantages, though, are that bacteria can infect the wound as the dressing degrades, particulates of the degrading film could cause a granuloma (3) and the degradation process could have a toxic effect on the cells. The latter was examined through *in vitro* studies.

Individual films were left in a biological cell nutrient solution that was not a solvent for the films, and any toxic effect present was determined. This toxicity could have been due to the sterilization process (using ethylene oxide) or due to the innate nature of the films themselves as they degraded. After both the seventy-two hour and the three-week incubation periods, selected for our studies, the film/cell nutrient solutions were diluted to 100%, 50%, and 25% solutions and introduced into the cell

monolayers. Cells were then counted for viability statistics (33). The results found were mixed as the cells were quite viable in the PLA and PCL wells, but the CH wells seemed to show toxic effects as more cells died. After examining the γ -irradiated CH films, which had high viability counts, it was concluded that the EO sterilization had some detrimental residual effect on the CH films. Thus all three films seemed to have no inherent toxicity, especially the CH films, as they degraded the fastest. This observation is substantiated by the results of Albanese (34) in a study in which EO and steam-formaldehyde sterilized PUPA materials had exhibited a toxic response on the cells. They determined that the more hydrophilic a material is, the more particulates of steam-formaldehyde toxic gas it would retain and this gas would be released into the system.⁵⁰ This hypothesis could also apply to EO sterilized material and, therefore, all materials used for the *in vivo* study were only γ -irradiated.

Cell Growth

The ability of the films to support cell adhesion was evaluated by using the film as a substrate for cell growth. Films were placed into wells and coated with a cell rich solution. After seven days, all films were microscopically and histopathologically examined. Some of the results obtained are shown in Figures 4 a-d. Both the PLA and PCL films had complete monolayers on their surfaces comparable to the control. The CH film had either sparse, rounded cells or no cells present. The work by Albanese had determined that cells could not attach themselves to substrates such as EO-sterilized CH. This substrate changed the shape of the cells from the usual elongated cylindrical fibroblast form, to a rounded form and the cells then remained in suspension (34). The γ -irradiated chitosan film also showed the same behavior.

In vivo Study

This study incorporated all other phases into the final outcome to see if the dressing would (1) aid in the healing rate and (2) have an effect on the quality of tissues during healing. Mixed Yorkshire pigs were used. These were sedated and 1" square thin sections of tissues removed from the backs (Figure 5a). The films were individually placed over the wounds, using Telfa™ dressing as a control. Surface tension held the films onto the wound surfaces. At 48 and 68-hour intervals, the wound site tissues were harvested and histopathologically studied (35).

There was no observed inflammation or irritation at the wound site or in the surrounding areas (Figure 5b). The films allowed excess exudate from the wounds to pass through, but also seemingly caused excessive drying of the wound sites. In light of this observation, it was concluded that the dressing should be modified to keep a moist environment, possibly by using an outer bandage soaked in saline solution.

Histopathological tests, procedures which check for inflamed cells and determine types of infected cells, and histomorphometrical tests, which allow measurements of cell counts, were inconclusive due to the physical movement of the dressings during healing, and due to the difficulties encountered in the preparation of histological sections of the wound tissues. It is proposed that a more effective method be used in the future to keep the dressing in position during the *in vivo* studies.

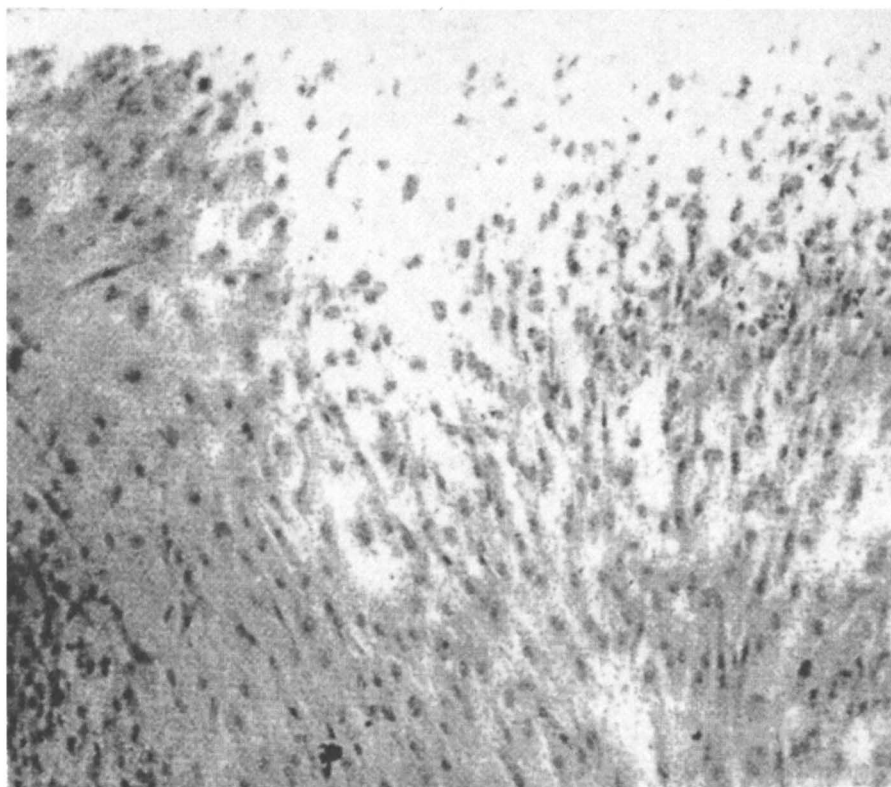


Figure 4a. Cell growth – control

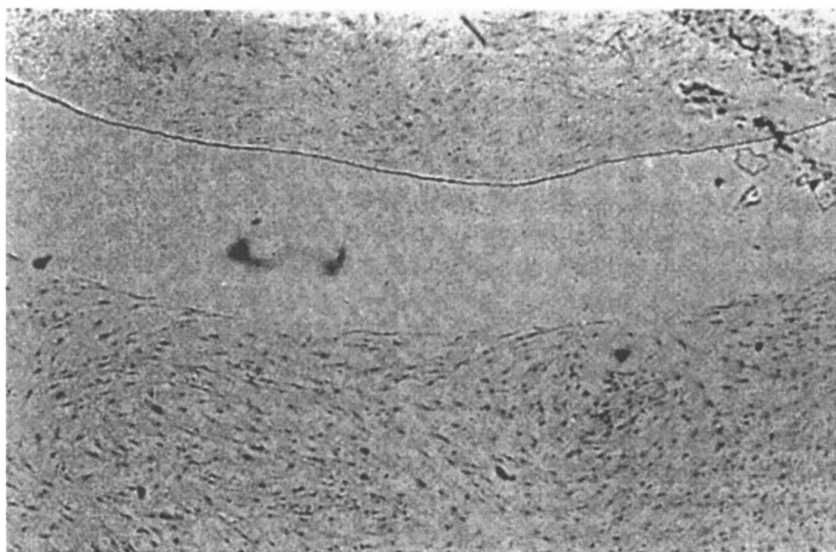
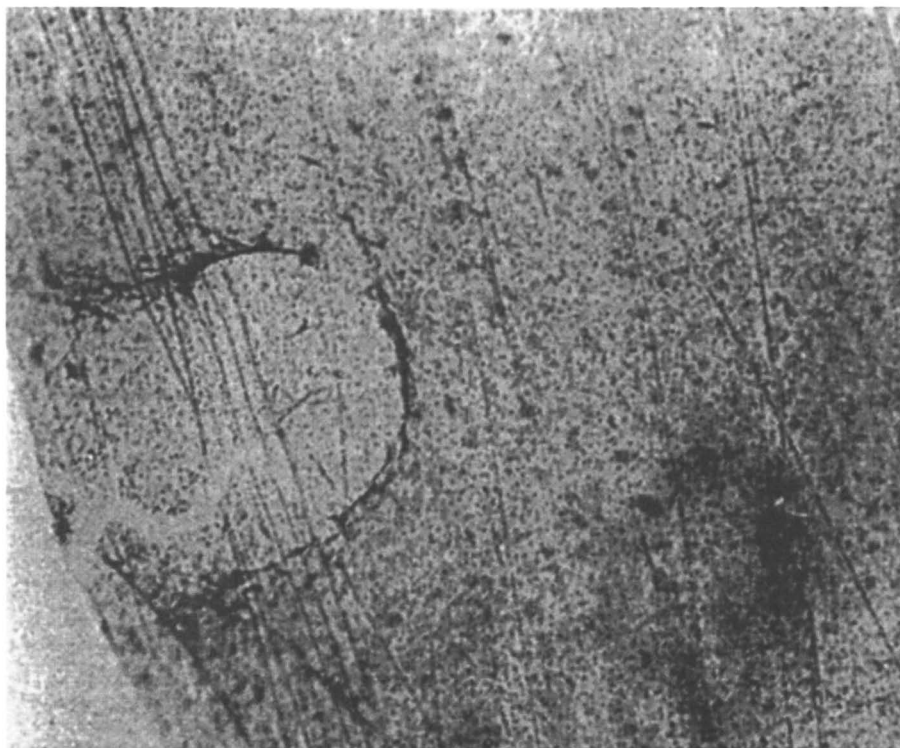


Figure 4b. Cell growth – poly D L-lactic acid film



4c. Cell growth – poly ϵ -caprolactone film

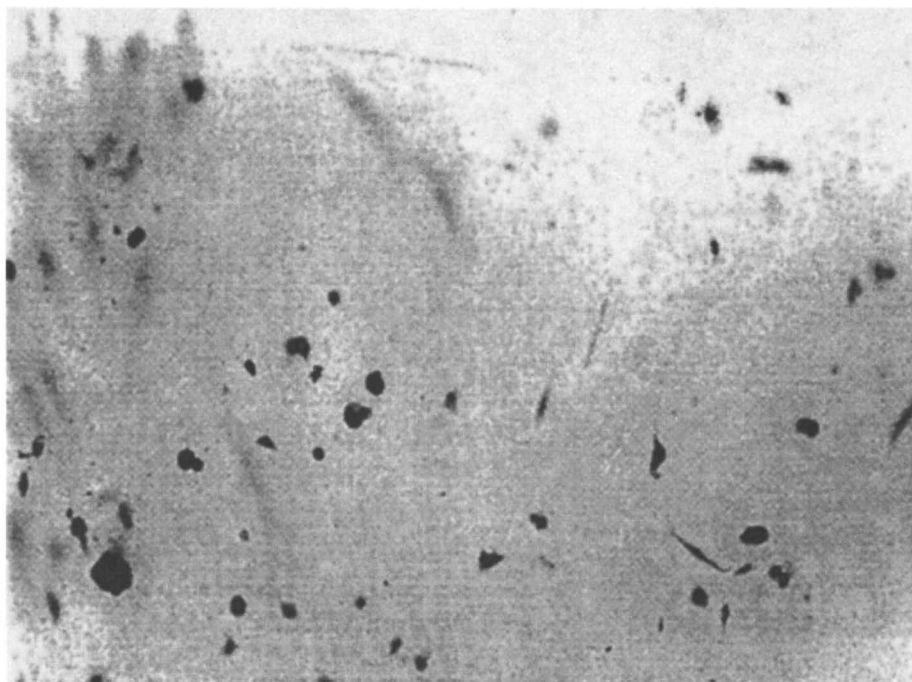


Figure 4d. Cell growth – chitosan film

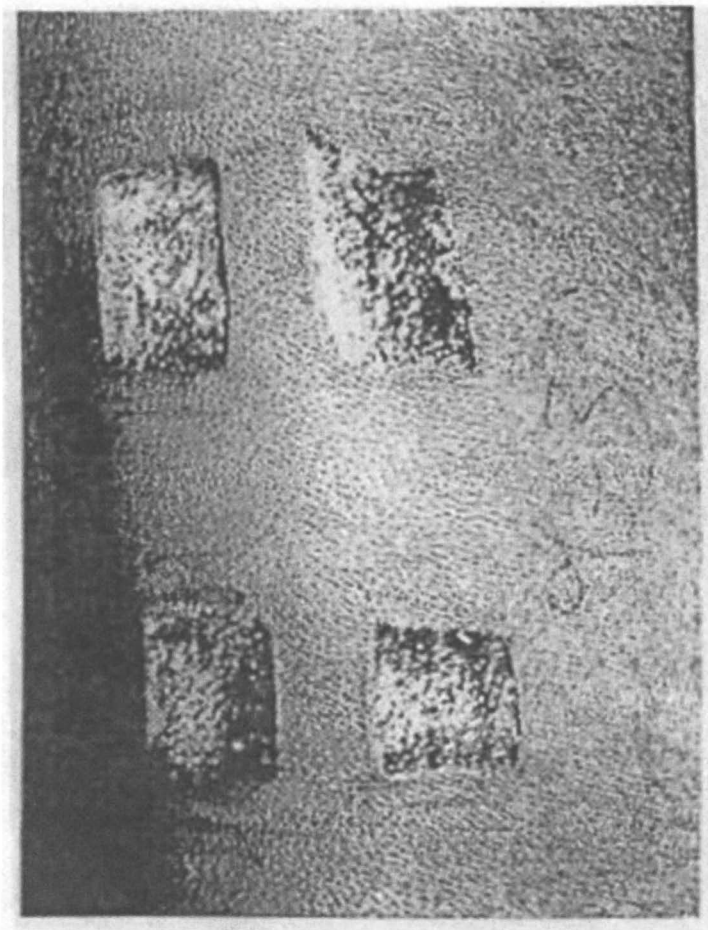


Figure 5a. *In vivo* study – close view of wounds

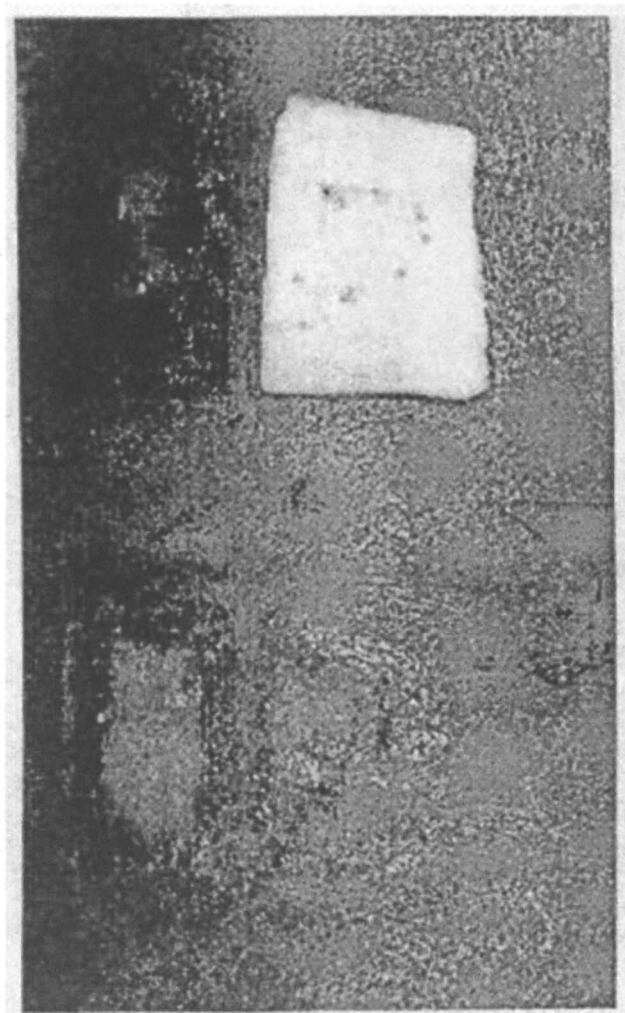


Figure 5b. *In vivo* study – close view of dressing on wound

Conclusions

The study showed that the concept of the dressing proposed is viable and the configuration suggested is sound. The films were adequately permeable to moisture vapor, but impermeable to bacteria. Toxic effects on cells were only seen with the EO sterilized and gamma-irradiated chitosan, and the cells grew readily on the PCL and PLA films, but did not grow on the chitosan films. The *in vivo* study determined that the dressing was too permeable to moisture that resulted in the drying out of the wound. Thus a modification was needed to keep the wound environment moist.

Further studies should be completed utilizing different film thicknesses and compositions. Copolymers of PCL and PLA and films cast from a mixture of chitosan and PCL or PLA from a common solvent should be studied. Thickness irregularities formed during the film casting process should be dealt with, as well as film thickness appropriate for dressing should be identified. A PLA film of 70/30 ratio should be tried as its more amorphous structure would increase the flexibility of the film in comparison to the somewhat brittle nature of the 98/2 currently used. Topographical characterization of the wound surface should also be included during the *in vivo* trials.

Further biological work *in vitro* studies should be included to determine the reasons for the death of cells on the γ -irradiated chitosan. Additional *in vivo* work implementing the composite design and conducted over a longer time period should be considered. This would provide invaluable information on the effect the film characteristics have on the rate and the quality of wound healing.

An improved protocol must be developed for testing the efficacy of the composite wound dressings on animals, one that eliminates the movement of the dressing relative to the wound during healing. In addition, a procedure to obtain histological cross-sections of the wounds during and after healing, which are not partially obscured by film fragments from the dressing, needs to be developed.

An additional development from our laboratory that can have a significant impact on the performance of a dressing is worth noting. We have recently formed crystalline inclusion compounds (ICs) between cyclodextrin hosts and several antibiotic/antibacterial materials (36). When these high melting IC crystals were embedded into PCL and PLA films and fibers, they were rendered antibacterial against *E. coli*. An attractive adjunct to the composite wound dressing described here would be the incorporation of antibacterial materials in the protective PCL/PLA film layers. This would eliminate the need to produce pinhole-free films and eliminate the need for the subsequent sterilization step. It may also be possible to deliver other substances that promote wound healing in this manner (37), such as certain protease inhibitors (38).

Acknowledgements

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Chapter 8

Carboxymethylation–Cotton for Wound Care Management

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Chronic wounds are not easy to heal. Research in wound physiology has shown that healing is accelerated when the wounds are kept in a moist condition. Since 1989 moist wound dressings based on calcium alginates have been made commercially available. While alginate dressings produce accelerated healing they are very expensive. The present work is on developing less expensive dressings from chemically modified cotton, which would heal wounds under moist conditions. Chemical modification of cotton (carboxymethylation, CM-cotton) was carried out by treating cotton gauze or nonwoven with caustic and monochloroacetic acid in 80/20 ethanol/water media. The results have shown a promise of highly swellable, highly water retentive CM-cotton dressings that remain moist at 98.6°F. The CM-dressings have passed preliminary animal safety evaluations. A successful development of the CM-cotton dressings may prove possible, if such dressings are effective in tests on wound healing. Such dressings, if efficacious, should be economical to produce.

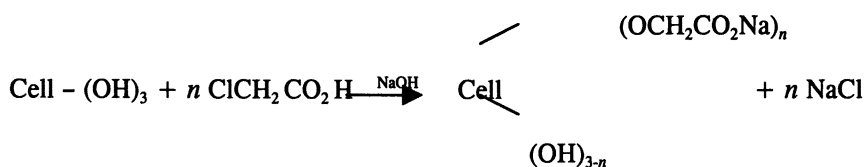
The principal function of a wound dressing is to provide an optimal healing environment for a wound, since the latter must be protected from infection and further damage. In general, infection impedes wound healing by damaging the tissue and promoting excessive inflammation. Traditionally, wounds have been kept dry by covering them with a dry dressing. However, recent research in wound physiology has shown that healing is accelerated when the wound is kept in a moist condition. This enhances migration of healthy skin cells. Since 1989 moist wound dressings based on calcium alginates have been made commercially available. While alginate dressings produce accelerated healing they are very expensive. The present work is on developing less expensive dressings from chemically modified cotton, which would heal wounds under moist conditions. Fresh wounds initially produce a large amount of fluid exudate, which provides a fertile environment for bacterial growth and infection. Thus, an efficient absorption of fluid exudate, while maintaining a sterile environment at the wound site, is preferable. Furthermore, an occlusive dressing (defined as the relative ability of a wound dressing to transmit gases and water vapor from a wound surface to the atmosphere.) appears to

function by limiting tissue desiccation and secondary damage. By maintaining a moist environment, the epidermal barrier function is rapidly restored. Interestingly, this effect may be noted by the faster rate of epithelialization in moist climates compared with that in an arid one [1,2]. An ideal moist wound dressing should perform the following functions:

1. Maintain a moist environment at the wound site to enhance healing but without maceration condition.
2. Absorb fluid exudate.
3. Protect the wound site from bacterial contamination by providing a barrier to bacteria.
4. Be non-adherent so that the wound is not traumatized when the dressing is changed. Easy to apply and easy to remove.

The moist environment is also conducive to migration of defensive and reparative cells such as macrophages. In an uncomplicated wound such as a donor site, occlusion may decrease the incidence of infection. It is suggested that the scar left by an occlusively dressed wound is more cosmetically acceptable than that left by an exposed wound [3,4].

An exploratory project was undertaken to develop cotton gauze and cotton nonwoven (NW) with high swelling, high water retention, and high total water uptake capabilities, with a view to developing a moist dressing. Our research approach was to chemically modify cotton gauze through a carboxymethylation [CM] process while still maintaining its gauze or nonwoven structure intact. Depending on the nature of the substituent and the degree of substitution (DS)*, cellulose ethers may be soluble in water. The solubility of cellulose ethers in water also depends to a great extent on the uniformity of substitution. Since both the DS and the uniform distribution of substituents affect absorbency properties and thus the end uses of the cellulose ethers, a proper control of these variables demands considerable attention in the manufacturing process. The final product and its quality depend also on factors which are inherent to the cellulose structure or to the reagents used in the preparation. Such factors are cellulose accessibility, hydroxyl group reactivity and reagent reactivity.



The conventional two-stage carboxymethylation in aqueous media, which consists of sequential treatment first with monochloroacetic acid [MCA], air drying, followed by a treatment with caustic and a final thorough washing [5,6,7], was tried first, however, it did not preserve the swelling and gelling characteristics of CM-cotton. Apparently, the

* DS reflects the average number of carboxymethyl [-CH₂COONa] groups per anhydroglucose unit of cellulose.

process is suitable only for low DS gauze. With medium to high DS, the product itself gets partially or fully dissolved during the final washing. anhydroglucose unit of cellulose.

Single-Stage Development

A single-stage CM process was developed in 80/20 ethanol/water that preserves swelling/gelling capabilities until the gauze/nonwoven is wetted in water or until used as a wound dressing. The major merits of the single-stage development were in:

1. Preserving the fibrous form of gauze/nonwoven while retaining its strength.
2. Custom etherification to produce low-to-high DS cotton, and
3. Imparting desired properties of improved moisture regain, high bound water, high total water uptake, and swellability.

Table 1 shows the superiority of the single-stage development over the two-stage CM process.

Table I. Two-stage versus single-stage carboxymethylation

| <i>Two-stage Features</i> | <i>Single-stage Features</i> |
|---|--|
| <ul style="list-style-type: none"> • CM development in water • Good for low DS • Economical • Not suitable for medium to high DS cotton | <ul style="list-style-type: none"> • CM development in alcohol • Good for low-medium- and high-DS • Expensive • Preserves swelling |

Scale-Up: Laboratory Package Dyeing Machine

Bleached absorbent gauze or hydroentangled nonwoven (NW) cotton fabric was carboxymethylated in a yarn dyeing machine. The machine, which was built of stainless steel, had gaskets/packings of rubber. Rubber packings were affected by ethyl alcohol. They had to be replaced by Teflon packings. Teflon packings are stable up to 500°F and are inert to ethanol. A pump in the machine was capable of circulating solution in the package vessel so that the solution was in continuous flow

Table II Formulation (in gms) for Water Soluble CM-Gauze #104

| <i>Chemicals</i> | <i>Grams</i> |
|--|---------------|
| FIRST STEP: | |
| Caustic soda (5%, W/V) --50% Solution | 1,200 |
| Ethanol | 8,000 |
| SECOND STEP: | |
| Monochloroacetic acid (6% W/V) | 720 |
| Caustic soda (5%, W/V) --50% Solution | 480 |
| Ethanol | 1,600 |
| Total | 12,000 |

W/V= weight/volume

- Typically nine gauze rolls weighing 0.7 lbs. were packed in tub.
- Alcoholic 1200 g of 50% caustic solution was added.
- Temperature was raised from 80 °F to 100 °F while circulating the solution for 15 minutes.
- Second step solutions were added.
- Initial pH was 9.31, final pH was 9.41.
- Reaction at 150-155 °F was continued for five hours.
- Formulation was drained.
- Gauze rolls were washed with ethanol containing 350 mL HAC for 15 minutes at 110 °F.
- Two 90/20 ethanol/water washes, 15 minutes each were carried out.
- Gauze rolls were hydroextracted and dried.
- Recovery of ethanol is a must.

through the cotton gauze. This circulation helped in imparting a uniform degree of substitution to the cotton cellulose.

In the single-stage development, adding chemicals in two steps was found to be beneficial. Cotton gauze was first treated with alcoholic caustic soda for 15 minutes at 100° F [to swell cotton and increase accessible hydroxyl groups] before treating it in the same bath with alcoholic monochloroacetic acid for 4 to 5 hours at 155 +/- 3° F [refer to formulation in Table II]. Temperature and pH of the solution were continuously monitored. At the end of the reaction time, the bath was drained, the gauze quenched with acetic acid, and the CM-gauze was thoroughly washed in 90/10 ethanol/water to make it free from the residual alkalinity, unreacted chemicals, sodium chloride, and acetate byproducts. Purity of the CM-cellulose was ensured by giving an additional wash. The reaction parameters can be varied to produce CM-cellulose with different DS.

By suitably controlling DS, we were able to develop CM-products as shown in Tables III and IV.

Table III. CM-Cotton Products

| <i>Sample Code</i> | <i>DS</i> | <i>Description</i> |
|--------------------|----------------------------|--|
| #101 | Medium 0.150-0.180 | Surface Gelling of hydroentangled cotton nonwoven |
| #104 | Medium-High 0.287-0.310 | Water soluble cotton gauze rolls. Initially gelling and then subsequently dispersing in water |
| #109 | Medium 0.150-0.180 | Surface gelling cotton gauze rolls. |
| PTCM | | Post treated CM #104 with diethylene glycol or higher molecular weight glycols or with Kymene (A trade product, wet crosslinked from Hercules) will be highly water absorbent and with high wet gauze integrity. |

Table IV. Product Developments

| | |
|------------------------------|--|
| Low DS (0.002-0.003) | No surface gelling -Hygroscopic |
| Medium | Surface gelling -Yarn/gauze strength unaltered. |
| High DS | Highly gelling Subsequently disperses in H ₂ O |
| High DS after treated (PTCM) | Crosslinker Kymene R or PEG, DEG, or Glycerol |

Sterilization and Safety Evaluation Health

Since the CM-cotton developments were designed for healthcare applications, the CM-products were sterilized with ethylene oxide (EO). The products passed the cell culture, primary skin irritation and intracutaneous irritation tests. Safety evaluation on an animal model is yet to be completed. No adverse toxicological or environmental factors are reported for cellulose ethers in general. In fact, some purified carboxymethylcelluloses, methylcelluloses, and hydroxypropylcelluloses are approved as direct food additive [8]. A significant use of CM-cellulose is also found in pharmaceuticals for skin care products such as ointments, lotions, and creams [9].

Absorbency and Moisture Retentive Properties

Percent moisture regain, absorbency factor (water/gauze, g/g), and water retention (g/g) by a centrifuge test of various CM samples are shown in Table V. Sample # 104 has very high moisture regain (21.2%) and high water retention (10.99 g/g). The results suggest that a swellable CM-cotton may also be an absorbent for physiological liquids such as urine, blood, and perspiration.

Table VI shows the water retained in samples [Dunk-N-Drain test] at zero minute and after oven drying at 100° F at different intervals of time up to 150 minutes. Sample # 104 retained 54 g of water vs. 0.15 g in control gauze, after drying for 150 minutes. Since the healthy human body temperature is around 98.6° F, we used a drying temperature of 100° F. The test data have shown that the CM-dressings maintain a moist environment at 100° F.

Table V. Moisture Regain, Absorbency Factor, Water Retained after Centrifugation

| <i>Gauze Roll</i> | <i>% Moisture Regain</i> | <i>DS</i> | <i>Dunk-N-Drain Absorbency Factor, g/g</i> | <i>Centrifuge Absorbency Factor, g/g</i> |
|----------------------|--------------------------|-----------|--|--|
| Water soluble #104 | 21.2 | 0.287 | 19.0-24.0 | 10.99* |
| Surface Gelling #109 | 9.4 | 0.099 | 9.5 | 2.17 |
| Hygroscopic #106 | 7.9 | 0.002 | 10.2 | 1.52 |
| Untreated Control | 6.6 | | 7.5-9.0 | 1.10 |

*Part of the sample may be lost.

Table VI. Drying at 100°F. Dunk-n-Drain Samples

| <i>Sample code</i> | <i>Bone Dry Wt. (g)</i> | <i>Water, (g)</i> <i>-after drying for the specified time</i> | | | |
|--------------------|-------------------------|--|---------------|---------------|----------------|
| | | <i>0 Min</i> | <i>30 Min</i> | <i>60 Min</i> | <i>150 min</i> |
| Control Gauze | 3.94 | 9.27 | 0.20 | 0.18 | 0.15 |
| #104 | 4.88 | 75.95 | 67.41 | 63.82 | 54.02 |
| #109 | 3.91 | 27.54 | 22.34 | 19.19 | 12.02 |
| #106 | 3.32 | 17.41 | 13.66 | 10.15 | 0.15 |

Since a burn dressing has to be multifunctional, i.e., antimicrobial, highly absorbent, moist, and non-adherent, and since Silvadene and silver nitrate are used on burn patients for their antimicrobial property, we studied silver nitrate retention at 100 °F. Table V11 shows silver nitrate retained on CM samples subsequent to drying at 100 °F. Again, the data indicate possible suitability of CM samples for moist healing as well as for treating a burn patient whose immune system may be compromised. (No burn patients have actually used this dressing).

Table VII. Drying at 100°F. Dunk-n-Drain in 0.5% Silver Nitrate

| Sample code | Bone Dry Wt. (g) | Silver Nitrate, (g) -after drying for the specified time | | | | |
|---------------|------------------|---|-------|--------|--------|--------|
| | | 0 hr. | ½ hr. | 2 hrs. | 3 hrs. | 5 hrs. |
| Control Gauze | 3.64 | 15.17 | 10.84 | 1.62 | 0.19 | 0.14 |
| #104 | 3.29 | 40.26 | 37.61 | 31.26 | 27.98 | 22.32 |
| #109 | 3.85 | 25.66 | 27.40 | 17.61 | 15.30 | 11.11 |
| #106 | 3.63 | 22.31 | 19.18 | 14.56 | 11.91 | 6.31 |

Wicking and absorbency properties of the CM samples are shown in Table VIII. It is very interesting to note that the wicking height of sample # 104 is less than that of the untreated control. Hydration of the outer surface of CM-cotton, results in the formation of a viscous gel layer that inhibits further wetting or wicking. In other words, the phenomenon of gel-blocking occurs.

Calcium-CM-cotton: Future Directions

Since 1989 some of the moist wound dressings based on calcium alginates have become available to treat chronic wounds. Modern alginate dressings are a blend of calcium alginate and sodium alginate which, on contact with blood or exudate, release calcium ions in exchange for sodium, thereby increasing the local calcium ion concentration and thus stimulating both platelet activation and whole blood coagulation. In the process of ion exchange calcium ion is converted to soluble sodium ion (yield sodium alginate), producing a hydrogel that absorbs wound exudate, while providing a moist wound environment [10]. CM-cotton presently produced is a 100% sodium salt

Table VIII. Rates: Wicking and Water Absorbtion (7" x 1" strips)

| <i>Sample</i> | <i>Sample wt. (g)</i> | <i>Wicking Time (Sec.)</i> | <i>Height (in.)</i> | <i>Water Absorbed (g)</i> |
|---------------|-----------------------|----------------------------|---------------------|---------------------------|
| Control Gauze | 0.558 | 10 | 1.00 | 0.767 |
| | | 60 | 2.50 | |
| #104 | 1.289 | 10 | 0.25 | 5.498 |
| | | 60 | 2.00 | |
| #109 | 0.546 | 10 | 1.00 | 1.482 |
| | | 60 | 3.00 | |
| #106 | 6.681 | 10 | 2.50 | 1.324 |
| | | 60 | 4.25 | |

of CM-cotton. We partially converted sodium salt of CM-cotton into calcium-CM-cotton and obtained some preliminary samples. It is possible to produce dressings with varying ratios of calcium and sodium compositions. If these dressings (calcium/sodium-CM-cotton) are applied on dry non-exudating wounds no absorption takes place, and no gel is formed, and there is no benefit of the dressing. These dressings are useful mainly on exudating wounds. Additional research and extensive clinical evaluation will be required to fully exploit this development. However, it is easy to envisage that a successful development of such products (calcium/sodium-CM-products), may cost less and be competitive to alginate dressings.

Summary

A highly swellable, highly water retentive cotton fiber gauze or nonwoven may be obtained by partial carboxymethylation with a degree of substitution in which the fiber does not lose strength but absorbs fluid when wet. Such carboxymethylation is obtained by treating a cotton gauze or nonwoven roll in alcoholic caustic and monochloroacetic acid using 80/20 ethanol/water. The CM fabric may be sterilized by ethylene oxide. The CM-cotton thus produced has passed preliminary safety evaluation tests. Post treating CM-gauze with polyethylene glycol or crosslinking with a wet crosslinker imparts the desired wet integrity to the fabric, while maintaining high water retentivity. The CM-cotton appears to be a potential candidate for use as moist bandage and dressings especially on burn patients. Efficacy tests will be planned for these products.

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Chapter 9

Arterial Grafts as Biomedical Textiles

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Prosthetic arterial grafts as well as other implantable devices suffer complications such as infection, clot formation due to the interaction of blood and the foreign surface, and limited host cellular ingrowth. The alkali saponification of polyester grafts provides surface sites for the attachment of biologically active proteins via crosslinkers that block clot formation or encourage cellular ingrowth. Infection-resistance has been achieved using antibiotics as “dyes” applied to polyester grafts by a pad heat procedure. Antibiotic application to ionic polyurethane using an exhaust dyeing technique has generated similar sustained antibiotic release, allowing the correlation of chemical affinity with antibiotic release rates. These collaborative efforts between the medical and textile fields have produced a range of promising avenues.

Textiles have been used in artificial arteries for over 40 years, with polyethylene terephthalate (polyester) and polytetrafluoroethylene (PTFE) being most widely used. Artificial arteries are utilized when an autologous venous transplant (vessel explanted from the patient) cannot be obtained due to vein damage via disease progression (e.g. atherosclerosis, diabetes) or prior use of the vessel in other anatomic locations to block disease progression (e.g. heart bypass). Approximately 250,000 prosthetic grafts are implanted in the United States each year. While large diameter grafts with high blood flow, such as the abdominal aorta, work well, small diameter grafts (such as femoral arteries in the leg or dorsalis pedis arteries in the foot) are limited in use due to several significant complications such as:

- Clot formation as the body responds to “injury”, with subsequent thrombosis (blockage of blood flow via clotted blood)
- A lack of cellular ingrowth over time: the implant remains “foreign”, in turn making other problems worse such as uncontrolled cellular growth at the artery/graft interface (anastomotic intimal hyperplasia)
- Infection leading to graft failure/replacement and occasional mortality.

Each of these three problems demands a unique approach to its solution, and will be discussed in turn. As the author list suggests, the research reported represents the result of collaboration between disparate disciplines, whose borders merge here. Working to solve these problems has led to potential solutions that may apply eventually to medical materials more generally. The development of a bioactive arterial graft also has implications in the technology of sutures, wound dressings, catheters, heart valves and so on.

Modification of Graft Surface for Clot (Thrombus) Prevention

Thrombus formation and anastomotic intimal hyperplasia on the surface of polyester vascular grafts are major concerns in the clinical setting. Thrombin, a pivotal enzyme in the blood coagulation cascade, is primarily responsible for thrombus formation and smooth muscle cell activation (1,2). Many attempts have been made to create a thromboresistant surface that would “passivate” this acute enzymatic reaction. A majority of surface modification studies have focused on covalent or ionic binding of the anticoagulant heparin, either alone (3,4,5,6,7,8,9,10) in conjunction with other biologic compounds (11,12,13,14,15,16) or with spacer moieties (17,18,19,20,21). Alternative strategies involving surface modification via protein adhesion have included covalent linkage of thrombomodulin(22) and growth factors(23), non-specifically binding albumin to the surface followed by heat denaturation (24) and non-specifically crosslinking albumin (25,26,27). Other studies have focused on modifying the composition of the biomaterial surface by either increasing the hydrophilicity of the biomaterial via incorporation of polyethylene oxide groups (28) or by establishing an ionically charged surface (29).

There are several potential flaws with these types of surface modifications. Thrombin is not directly inhibited, thus remaining fibrinogen on the material surface permits platelet adhesion. Heparin-coated biomaterials may be subject to attack by heparitinases, potentially limiting the long-term use of these materials. Charge-based polymers may be “passified” by other blood proteins such that anticoagulant effects are reduced. Any treatment involving non-specifically bound materials relies on purely physical forces of attraction (i.e. hydrogen bonds, van der Waal’s forces). While these forces may be enhanced by physical modification of the graft surface, and its chemical identity, non-specifically bound active proteins are readily lost from the surface under the constant shear stress, and may re-expose the thrombogenic biomaterial surface and/or generate unwanted biological activity elsewhere in the body.

A more attractive approach that overcomes many of these disadvantages uses specific (covalent) binding of the protein to the graft surface, a technique that maintains the biologic activity of the bound protein. Polyester arterial graft surfaces are largely inert, and have few available functional groups for reaction to link proteins. In normal textile applications, polyester is occasionally given a treatment in aqueous alkaline solutions. This results in surface saponification of the polyester providing a more hydrophilic surface and (if prolonged) a reduced diameter: hence its being referred to as “denier reduction” (30). The hydrophilicity results from the formation of carboxylic acid groups at the fiber surface and these groups are available for reaction. Using chemical compounds that crosslink two biologic agents, proteins with specific functions have been immobilized onto the graft surface.

The effect of denier reduction on medical polyester materials has been studied (31). Polyester materials were exposed to a range of alkali concentrations for 30 minutes at 100°C. No significant strength or weight loss was found for concentrations of sodium hydroxide of up to 1.0% (Figure 1). Excessive alkali treatment results in the production and removal of short chain fragments and gives no additional benefit in terms of carboxylic acid content. The treated materials were exposed first to the crosslinker 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and subsequently to ¹²⁵I labelled serum albumin (BSA), which is found in abundant quantities in blood. Nonspecific binding of BSA (i.e. without the use of EDC) was significantly greater with the hydrolyzed materials reflecting the physical modification of the surface and the increased formation of secondary bonding (Figure 2). More importantly, the use of EDC provided significantly greater covalent binding of BSA to the hydrolyzed materials. The best combination of protein binding and maintenance of physical properties, the 0.5% hydrolyzed material, was chosen for further study. This hydrolyzed material was exposed to a range of BSA concentrations after EDC treatment, and an optimum protein binding of 330ng/mg polyester was demonstrated using a 14.6μM albumin solution.

We used this approach to covalently immobilize the potent, specific antithrombin agent recombinant hirudin (rHir) to alkali-modified polyester and characterize the *in vitro* thrombin inhibition by this novel biomaterial surface (32). rHir, a 6,965 Dalton recombinant protein synthesized from the leech protein hirudin, is the most potent specific inhibitor of thrombin (33), inhibiting the enzymatic, chemotactic, and mitogenic properties detailed above (34,35,36). This leech anticoagulant holds several advantages over heparin: 1) rHir inhibits thrombin directly whereas surface bound heparin requires the transport of antithrombin III prior to inhibiting thrombin, 2) heparin enhances platelet aggregation (37), 3) rHir inhibits both the proteolytic and fibrin binding sites of thrombin (38,39) and 4) heparin activity is regulated by platelet heparitinases (40). BSA was again selected as the “basecoat” protein, and was reacted with the heterobifunctional crosslinker sulfo-succinimidy-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC). These BSA-SMCC complexes were then covalently linked to sodium hydroxide-hydrolyzed Polyester (HD) segments via EDC. rHir was initially reacted with Traut’s reagent in order to create sulfhydryl groups, and was then covalently bound to these HD-BSA-SMCC surfaces (HD-BSA-SMCC-S-rHir). Using a 1:50 molar ratio of sulfo-SMCC resulted

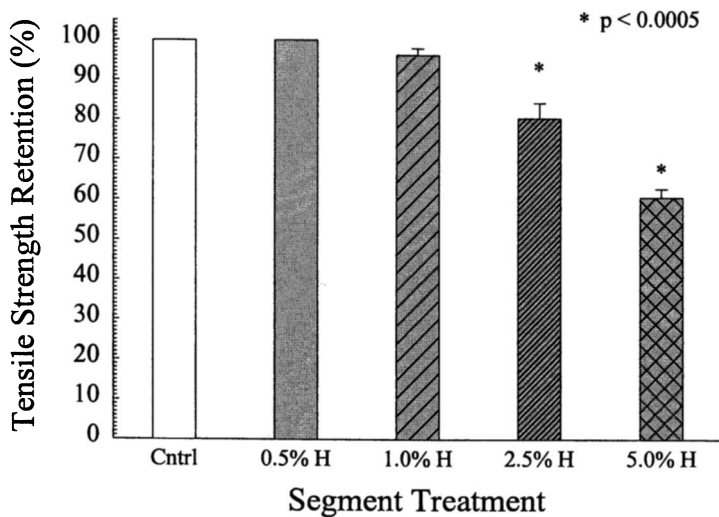


Figure 1. Alkali treatment of polyester: retention of tensile strength with alkali concentration. (Reproduced with permission from reference 31)

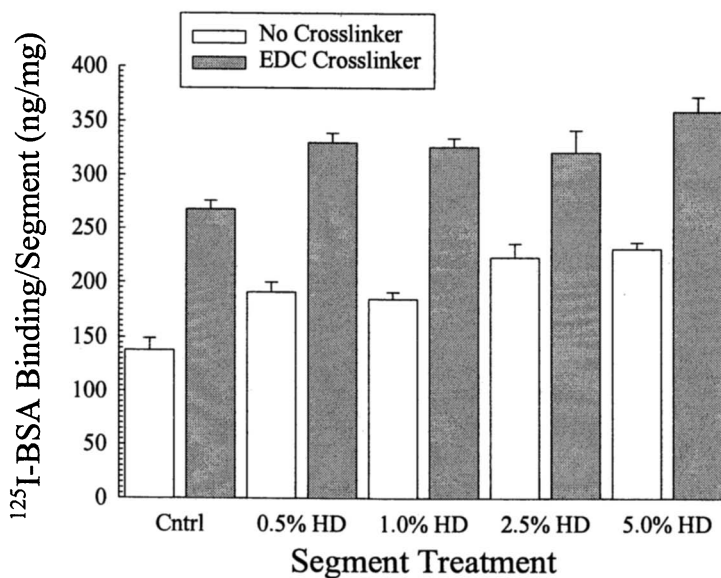


Figure 2. ¹²⁵I-BSA binding to control and hydrolyzed Dacron segments in the presence and absence of EDC crosslinker (Reproduced with permission from reference 31)

in a 22-fold greater covalent binding of rHir (111 ng/mg Polyester) as compared to control segments

These segments also possessed the greatest thrombin inhibition of the segments when evaluated using a chromogenic substrate assay for thrombin. Further characterization of the HD-BSA-SMCC-S-rHir segments demonstrated that maximum thrombin inhibition was 20.43 NIHU, 14.6 times greater inhibition than control segments (1.4 NIHU). Thrombin inhibition results were confirmed by ^{125}I -thrombin binding experiments that demonstrated that the 1:50 HD-BSA-SMCC-S-rHir segments had significantly greater specific thrombin adhesion as compared to control segments. Non-specific ^{125}I -thrombin binding to and release from the 1:50 HD-BSA-SMCC-S-rHir segments was also significantly less than the control segments. Thus, these results demonstrate that rHir can be covalently bound to a clinically utilized biomaterial while still maintaining its ability to bind and inhibit thrombin.

Knitted polyester grafts (6mm inner diameter) with covalently bound rHir were subjected to simulated physiologic flow conditions to determine the stability of the antithrombin coating (41) (Figure 3). Both constant flow of shear rate 300 s^{-1} , and pulsatile flow of maximum shear rate 780 s^{-1} were used for a seven-day period. The loss was moderate in both cases (approximately 50% of the rHir and 20% of the albumin). The ability of these modified surfaces to remove thrombin from the perfusion stream was comparable with that found in the parallel static experiments: 3 NIHU of thrombin/cm² was bound by the rHir grafts, compared to 0.6 NIHU of thrombin/cm² for albumin-coated grafts. Similarly, the total amount of thrombin inactivated over the seven days was 125 NIHU for surface bound rHir versus 10 NIHU for surface bound albumin.

Based on the encouraging results obtained in these simulated flow studies, knitted polyester patches were subjected to the same modification with rHir (42). Segments with covalently bound rHir and control (BSA-treated) polyester patches were implanted in the thoracic aortas of canines and exposed to non-heparinized aortic blood flow for two hours. The materials were explanted and the loss of rHir was found to be 20%. The remaining antithrombin activity was assessed: explanted patches were able to inhibit 7 NIHU of thrombin. Gross and microscopic examination of the explanted patches showed that the controls had a thick surface acellular layer (pseudointima) composed of fibrin rich thrombus, while the rHir modified patches had no gross thrombus, and a thin pseudointima of platelets and plasma proteins (Figure 4).

Modification of Graft Surfaces for Cell Growth

The biocompatibility of a vascular graft (or other implantable device) depends on the nature of the biomaterial surface. Host cells generally do not incorporate themselves into unmodified grafts: such incorporation requires several factors including cell-specific adhesion and basement layer of protein. For vascular grafts,

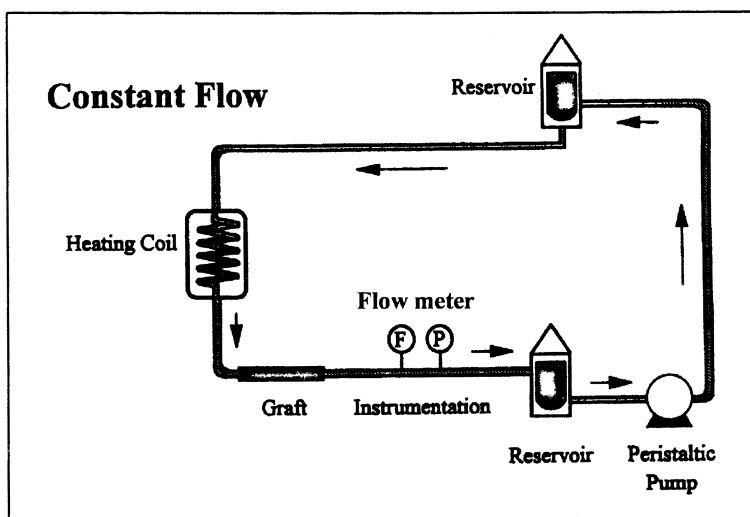


Figure 3. Apparatus for exposing graft segments to flow conditions (Reproduced with permission from reference 41)

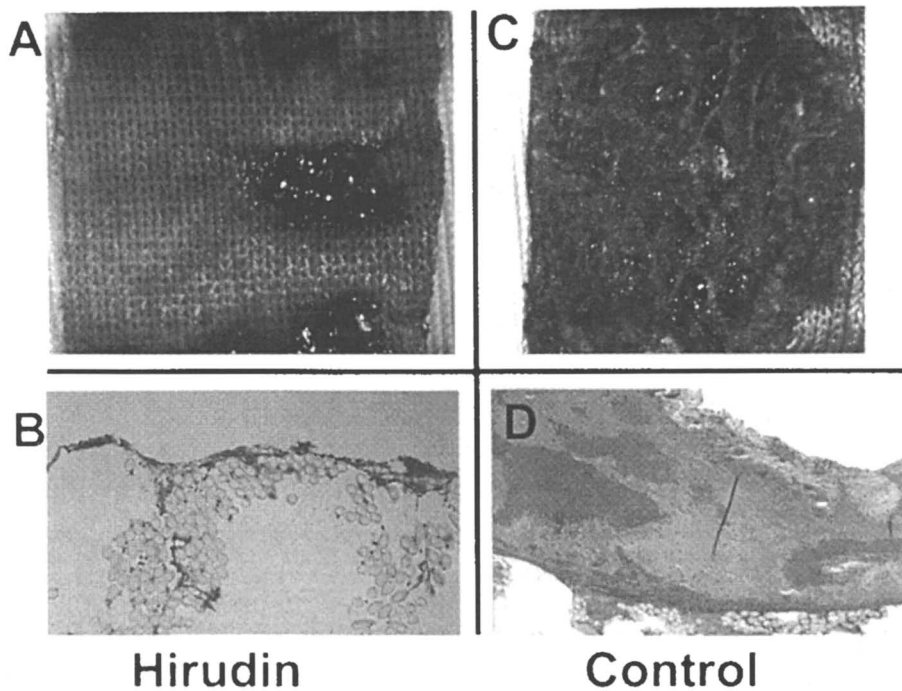


Figure 4. Macroscopic evaluation of the ^{125}I -rHir patches post-explantation (A), the patches had virtually no gross thrombus formation on the luminal flow surface. Control BSA patches had dense carpet of thrombus that lined the entire flow surface (C). Microscopic assessment of these patches confirmed these results. The ^{125}I -rHir surfaces had primarily a thin layer of platelets sealing the pores of the patches with minimal fibrin formation (B). Control BSA surfaces had dense, well-formed thrombus composed of fibrin and platelets (D). (Reproduced with permission from reference 42)

an ideal surface should promote vascular endothelial cell adhesion, growth on the luminal surface and ultimately direct incorporation of host tissue throughout the material. Such incorporation would greatly increase the healing characteristics, and thus the effectiveness or patency (maintenance of blood flow) of the graft. Successful adhesion of vascular cells would also go a long way to answer the two other problems discussed, that of blood clotting and infection, since the body's own cells provide a natural level of resistance.

A considerable volume of work has been conducted with this in mind. Cell adhesion to prosthetic grafts using endothelial cell seeding techniques has been extensively employed (43,44,45). Adhesive proteins such as fibronectin, fibrinogen, vitronectin and collagen have served well in graft seeding protocols (46,47). The cell attachment properties of these matrices can also be duplicated by short peptide sequences such as RGD (Arg-Gly-Asp) (48). These adhesive proteins, however, have several drawbacks. Bacterial pathogens recognize and bind to these sequences (49,50), as do non-endothelial cell lines (45,51). Patients requiring a seeded vascular graft have few donor endothelial cells, therefore cells must be grown in culture (52). Endothelial cell loss to shear forces remains a significant obstacle (53).

Polyester surface modification has also been employed to change host response to the foreign body, serving as an approach for improving endothelial cell adherence. After seeding, endothelial cells have been shown to attach and grow on a variety of proteins coated onto vascular graft materials (47,54). Bioactive oligopeptides (55,56) and cell growth factors (57) have been immobilized onto various polymers and shown to affect cell adherence and growth. Additional studies have described the incorporation of growth factors into a degradable protein mesh, resulting in the formation of capillaries into the graft wall (58). These incorporation methods, however, have limitations. Growth factor is rapidly released from the matrix, and matrix degradation re-exposes the thrombogenic surface, thus endothelialization is not uniform. The release of non-endothelial specific growth factor is not confined to the biomaterial matrix, thereby exposing the "normal" distal artery to the growth factor.

A number of proteins are available as possible mediators for vascular cell incorporation. A lectin was linked directly to the surface of hydrolyzed polyester using the techniques described above (59). Lectins are proteins that bind oligosaccharides, and UEA I (from *Ulex Europaeus I*,) is highly specific for glycoconjugates on the human vascular endothelial cell surface. A covalent linkage between COOH on a polyester surface, and the UEAI was established via the crosslinker EDC to provide an oligosaccharide-mediated endothelial cell attachment to the polyester. Experiments with radiolabelled UEA I showed that up to $1.35\mu\text{g}/\text{cm}^2$ could be bound. Subsequent exposure of this modified surface to a range of cell lines showed a 100-fold increase of attachment of five lines of endothelial cell compared to the untreated polyester. In contrast, no monocytic or mesenchymal cells adhered to the modified surface.

More recently, this work examined the binding of human vascular endothelial cell growth factor (VEGF), a more endothelial cell specific protein, to encourage

endothelial cell incorporation in polyester (60). The first step was to determine if chemical modification affected the mitogenic properties of VEGF. ^{125}I radiolabelled VEGF was modified to contain sulfhydryl groups using Traut's reagent. (^{125}I -VEGF-SH). Concurrently, canine serum albumin (CSA) was reacted with the crosslinker sulfo-SMCC. The two were reacted together to form the complex CSA-SMCC-S- ^{125}I -VEGF, which was purified via gel filtration (Figure 5). Fractions were gamma counted and the amount ^{125}I -VEGF/fraction was determined. Using these fractions, the mitogenic activity of VEGF was determined using a mitogen-activated protein (MAP) kinase assay that measures stimulation of endothelial cell activity via interaction of VEGF with the surface receptor (61), resulting in phosphorylation of the proteins ERK1 and ERK2. Unstimulated endothelial cells (EC) did not activate MAP-kinase, serving as the negative controls (Figure 6). EC's stimulated with increasing concentrations of native VEGF showed concentration-dependent stimulation of MAP-Kinase (positive control). VEGF-complex fractions 16 through 18.5 all activated MAP-Kinase as demonstrated by the phosphorylated forms ERK1 and ERK2 (molecular weights p44 and p42). Unbound fractions of VEGF (VEGF-SH), represented by fractions 22-23.5, also activated MAP-kinase in a similar fashion to native and BSA-VEGF complex.

^{125}I -VEGF was then bound to polyester segments utilizing the same technology described for rHir immobilization. Collagen was utilized as the basecoat in order to provide a surface to which endothelial cells can adhere. Hydrolyzed polyester knitted grafts (6mm internal diameter) were incubated with EDC followed by incubation with sulfo-SMCC modified ^{125}I -collagen, resulting in 359 ± 29 ng collagen bound/mg Polyester. These grafts were then reacted with Traut's modified ^{131}I -VEGF overnight, thereby covalently binding ^{131}I -VEGF (93 ± 5 ng/mg Polyester) to the surface.

This graft surface was then exposed to an *in vitro* constant flow model in order simultaneously to assess the structural stability of surface bound ^{125}I -collagen and ^{131}I -VEGF⁴¹. A gravity-fed flow of 400ml/min, monitored with an electromagnetic flowmeter, was directed through grafts connected in series. Perfusate for the system was 550 ml of a 5% albumin/PBS solution containing 400mg Ofloxacin to prevent infection. Polyester grafts had 79 ± 2 % of the ^{125}I -collagen and 79 ± 1 % of the ^{131}I VEGF remaining covalently bound to the surface after exposure to arterial flow for 7 days (Figure 7). These amounts were comparable to the static controls that had 81 ± 2 % of the ^{125}I -collagen and 82 ± 1 % of the ^{131}I -VEGF remaining, thus protein release was not flow induced. In both conditions, protein release gradually occurred over the seven-day evaluation period.

Creation of an Infection-Resistant Graft Surface

Any invasion of the skin carries with it the risk of infection. This applies to simple surface wounds, some 4-6% of which become infected. The use of antibiotics in their treatment has recently been reviewed (62). Surgical procedures use a similar range of textile biomaterials for wound closure and healing, and may also involve

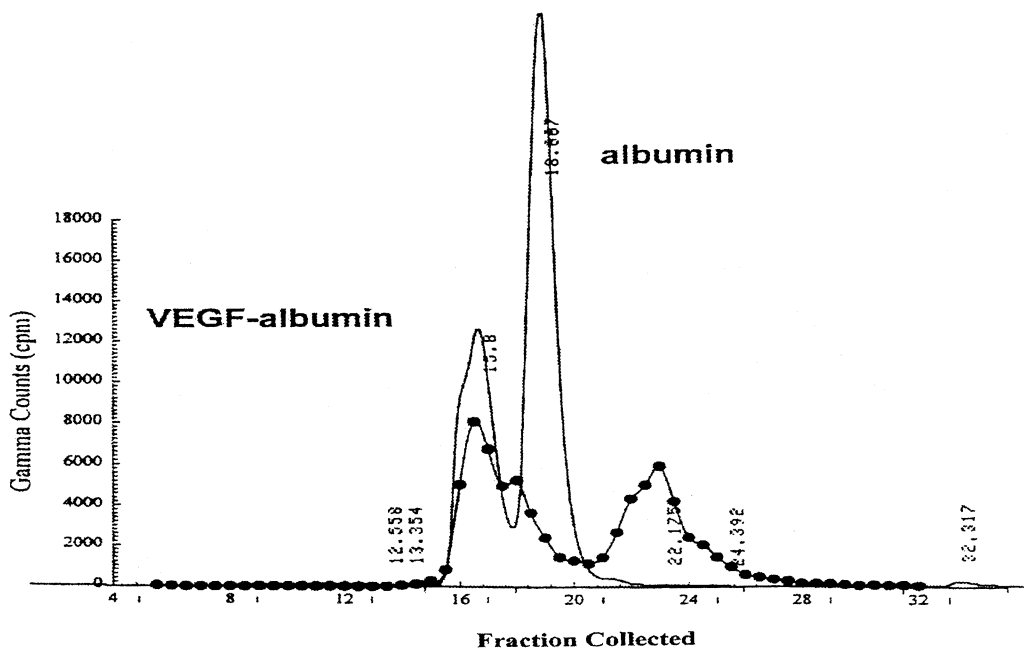


Figure 5. Profile of CSA-SMCC-S- ^{125}I -VEGF conjugate purification using a Superdex 75 HR10/30 gel filtration column.

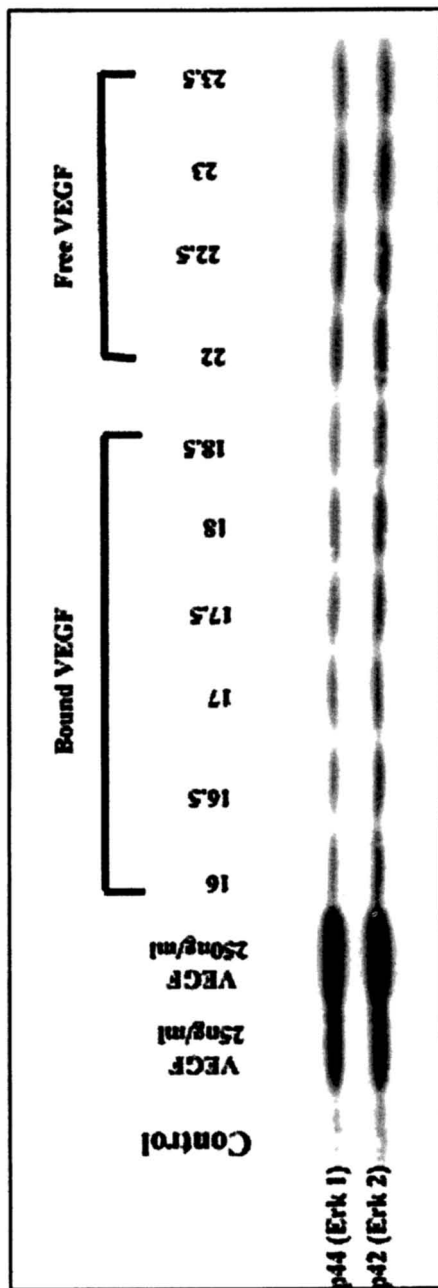


Figure 6. Determination of mitogenic activity using a MAP-kinase assay.
(Reproduced with permission from reference 60)

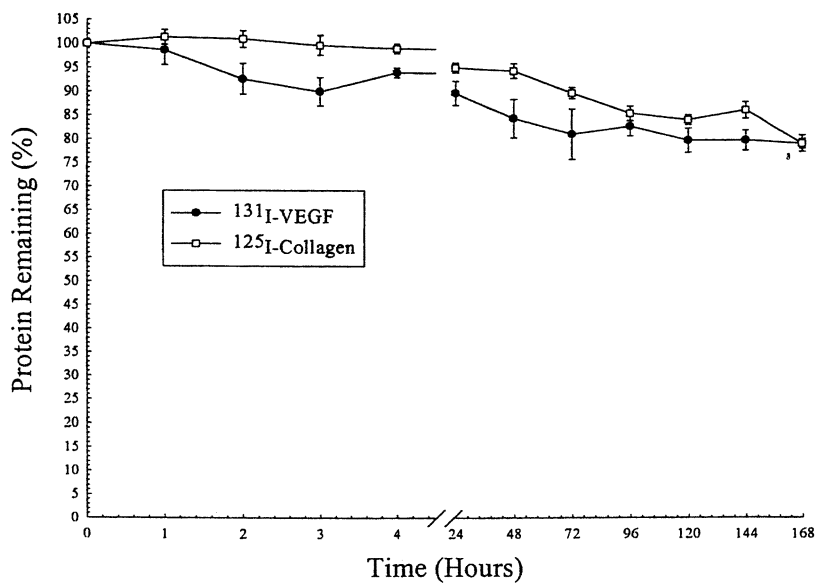


Figure 7. Retention of ^{125}I -collagen and ^{131}I -VEGF under flow conditions

implantable devices (catheters, vascular grafts, heart valves). Infection of these materials is of major concern despite recent advances in sterile procedures used in the clinical setting, and was the subject of a FDA/EPA/CDC/AAMI joint conference (63). The delivery of antibiotics to wounds in general has been the subject of study, and within the larger field of slow-release drug delivery systems, implantable biodegradable materials have been used (64).

Infection in prosthetic vascular grafts is of particular concern (65). Infection of these arterial substitutes is a source of significant clinical morbidity and mortality (66,67,68,69,70,71). Prosthetic graft infections occur in 2-6% of all clean cases performed (66,67,68,69,72,73,74,75,76,77) with morbidity and mortality related to the anatomic position of the graft (68). The economic impact of the problem can be estimated. In 1989 some \$150M was spent on the implantation of synthetic arterial grafts (71). A 2% infection rate represents \$3M annually. The cost of treating the infections, plus the mortality that occurs in some 25% of infected cases must be added. When figures are extended to cover infection in other biotextiles, and are adjusted for inflation, the magnitude of the problem can only be estimated, but is extremely significant.

Inoculation of the biomaterial presumably occurs at the time of implantation or as a result of transient bacteremia in the immediate post-operative period (67,70,78). Perioperative parental antibiotics, while having a defined role in wound infection prophylaxis often fail to permeate the avascular spaces immediately around prosthetic grafts and the carbohydrate-rich bacterial biofilm once pathogens have adhered (79,80 81,82) *Staphylococcus aureus* (*S. aureus*) is responsible for 65-100% of acute infections (67,78). These infections are typically quick to develop and generate an intense response by the body's defense mechanisms. An ever increasing problem, which has been documented both in animal models (83) and in humans (66,69,75,84) is the susceptibility of vascular prostheses to late infection. *Staphylococcus epidermidis* (*S. epidermidis*) recently emerged as the leading isolate from infected vascular conduits (20-60%) with infection appearing late after implantation. These cases are clearly not affected by low level antibiotic transiently present at the time of operation, which may in fact lead to the development of resistant organisms.

The treatment of biomaterials with antibiotics began in the mid-1970's, particularly in the area of prosthetic grafts for which infection is a significant problem. The mode of action of most antibiotics requires that they be unbound to act effectively as bacteriocides or bacteriostats, so permanent binding or incorporation of antibiotics to/within the material structure is ineffective. Success requires the slow sustained release of antibiotic over time. Thus far none of the approaches tried has provided a clinically available conduit in which consistent, steady release of antibiotic occurs.

Current clinical practice involves dipping the conduit in an antibiotic solution immediately prior to implantation (85). The level of antibiotic in the surrounding tissue has been shown to increase graft resistance against a low concentration inoculum of bacteria. However, there is rapid elution of the antibiotic from the graft into the blood stream and surrounding tissue, within minutes of establishing flow within the graft, and there is no prolonged effect against a high level of inoculum.

This approach has the merit of simplicity, and is better than no treatment, but given the common delay in onset of infection is of limited value.

Somewhat more sophisticated approaches than the simple co-application of antibiotics have involved the modification of the textiles, typically involving the use of some binding material (78,81,85,86,87,88,89,90,91,92,93,94,95,96,97,98). One approach, which has been the subject of several investigations, is the ionic binding of antibiotics by surfactants (90). Cationic surfactants (e.g. tridodecylmethyl ammonium chloride) are sorbed at the anionic surface potential of a polymeric material, and in turn anionic antibiotics can be weakly bound to this surface (87,88,91). In these cases, the antibiotic still elutes from the graft over a time period in which less than 50% of the antibiotic is present after 48 hours and less than 5% is present after three weeks (89). While this antibiotic coverage may be adequate for small, localized contaminations, large contaminations are not addressed. The methods also involve the introduction of additional materials into the body. Other researchers have attempted a rifampin/collagen release system with some success in a bacteremic challenge dog model (78). Gentamycin has been applied to polyester using a fibrin sealant (98). Release in these systems, however, may be quite varied depending on the rate of collagen or fibrin matrix degradation. The clotting propensity of the biomaterial may also be adversely affected by the sealant.

Noticeably all of the above work avoids the examination of any direct fiber/antibiotic interaction. Dyes for textiles have substantivity, and will "exhaust" from a bath preferentially into a fiber, attracted by physical forces of attraction. We have attempted to use similar direct interactions in order to provide continued infection resistance without exogenous binders.

Initial efforts in this regard examined the use of commercially available dyes as anchors for antibiotic molecules, and even the determination of antibiotic activity of some dyes (99). This approach was unrewarding. In contrast, the direct use of antibiotics was examined. Fluoroquinolone antibiotics are particularly suitable in such applications. They are stable to dry heat and to hot aqueous media; they have an appropriate molecular size, and (in the absence of any method for predicting physical interactions) a somewhat dye-like structure. The most common commercial quinolones currently available are ciprofloxacin (cipro) and ofloxacin (oflox) (Figure 8). The interaction of cipro with fibrous polyester materials has been examined. The direct application of cipro to polyester was unsuccessful: under aqueous conditions at 130°C (the normal conditions for dye application on polyester) cipro was stable, but no exhaustion was observed. Attempts to apply the cipro as its silver salt (given the known antibiotic properties of silver ions), and with its carboxylic acid group in the form of an ethyl ester were likewise unsuccessful.

On polyester, successful application of antibiotic was achieved using an alternative textile dyeing procedure referred to as "pad-heat" (100). In this method, a fibrous polyester material was dipped in a solution of antibiotic (cipro or oflox) and then dried. The materials were then subjected to the action of dry heat at 210°C for 2 minutes. Quinolone associated fluorescence in the cross-sections of polyester fibers confirmed antibiotic presence within the fibers. By measuring the absorbance of applied and released Cipro from polyester, the amount of Cipro bound to fiber was

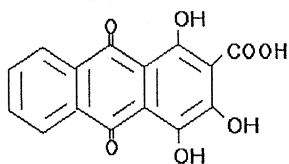
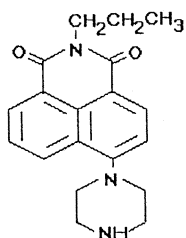
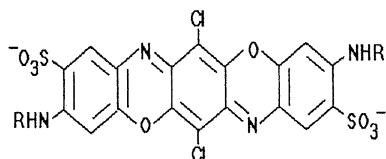
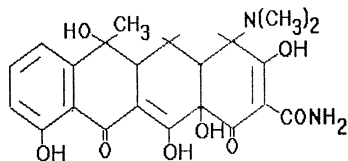
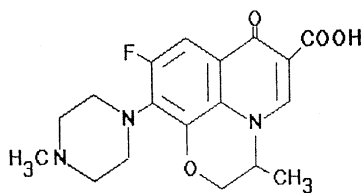
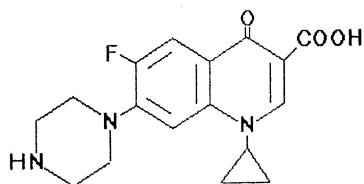
DYES**A****B****C****ANTIBIOTICS**

Figure 8. Structural comparisons of dyes and antibiotics

calculated to be $33 \pm 3.0 \mu\text{g}/\text{cm}^2$ ($n=12$). The release of antibiotic *in vitro* was examined. All pad/heat grafts were washed for 10 minutes in phosphate-buffered saline solution (PBS) prior to testing to remove any loosely bound antibiotic. To assess release pharmacokinetics, treated polyester segments were placed into 5 ml of PBS at 37°C. These segments were incubated in a 37°C water bath and sampled at 10 minutes, 24, 48, 72, and 96 hours. Fresh PBS (5 ml) was replaced after each sampling. The concentration of antibiotic in the wash was determined spectrophotometrically and the percent of bound cipro released was determined. Control samples for the following studies were polyester segments dipped into an equal concentration of antibiotic solution (a common practice in the operating room), then allowed to air dry. The graph of the release from the cipro treated specimens is given in Figure 9. This important control is not included in similar previously published studies.

Fiber samples from the washing model were also tested for antibiotic release in a zone of inhibition assay. One cm^2 pieces of polyester were washed for various times (with buffer changes as outlined previously), sterilized, and then plated on agar streaked with *S. aureus* (ATCC 29213). The zone of inhibition was measured after an overnight incubation. The control sample lost antimicrobial activity within 48 hours, while the pad/heat treated polyester continued to demonstrate anti-staphylococcal activity past 336 hours (Figure 10).

A complete battery of standard microbiologic assays (101) has been completed for the *S. aureus* and cipro used in these studies. Results revealed a minimum inhibitory concentration (MIC) of 2.3 μM ($n=6$), and a minimum bactericidal concentration (MBC) of 6.5 μM ($n=6$) for cipro, and demonstrated that antimicrobial activity is heat stable at 135°C for 1 hour. In a 2 ml volume of broth, 1 cm^2 treated fabric samples demonstrated activity against a sizable inoculum, inhibiting the growth of 10^7 organisms and killing 10^6 .

This infection resistant graft material was tested in an *in vivo* model (102). One cm^2 polyester segments (plain, cipro dipped, or pad/heat treated) were implanted in the dorsal subcutaneous tissue of rabbits and directly contaminated with 10^6 *S. aureus*. After one week, the samples were sterilely harvested. Wounds were blindly graded on a scale from 1 (no evidence of infection, good tissue incorporation) to 4 (suppurative infection extending outside of the graft pocket, no gross tissue incorporation) (Figure 11). Plain polyester was easily infected in this model (mean grade 3.1 ± 0.6 , 92% culture positive). Notably, however, a significant ($p<0.05$) wound grade difference between the dipped (2.3 ± 1.0) and pad/heat (1.4 ± 0.6) samples was demonstrated. Determination of adherent bacteria present on the implanted polyester pieces by sonication and culture studies again revealed a significant difference between the dipped (56% culture positive) and pad/heat (12% culture) groups ($p<0.025$). Histologic studies confirmed good tissue incorporation of the pad/heat samples (Figure 12).

This textile application method holds several key advantages over the antibiotics bound in other studies. The antibiotic attaches to the fibers without molecular modification, thus retaining full antimicrobial activity. No cross linking agents are

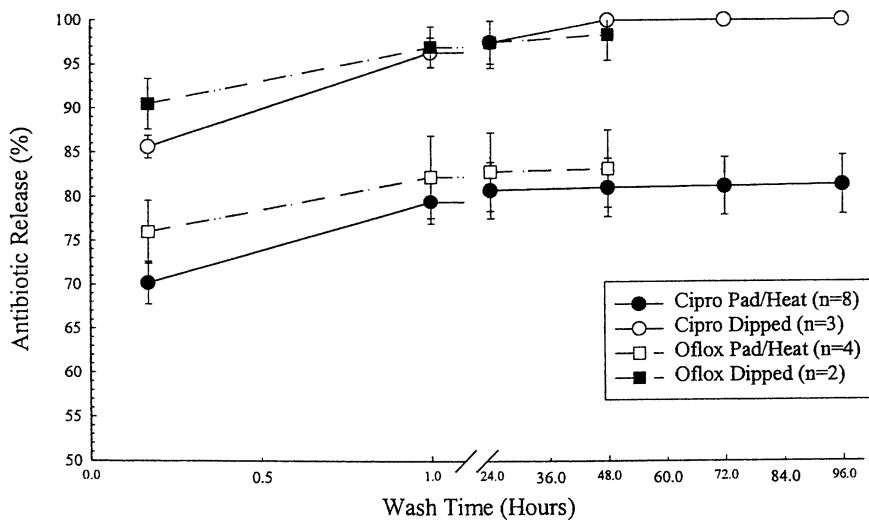


Figure 9. Loss of antibiotics from polyester specimens: pad-heat treated vs. dipped (Reproduced with permission from reference 100)

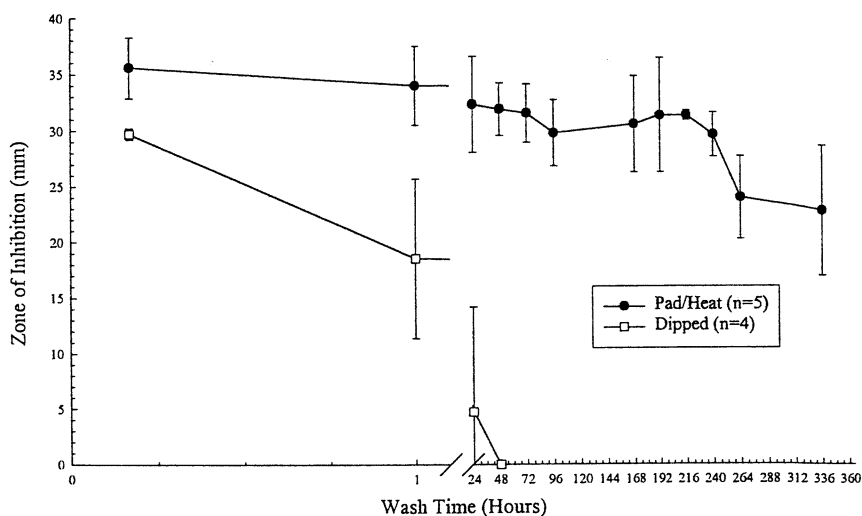


Figure 10. Zone of inhibition of polyester specimens after extended washing (Reproduced with permission from reference 100)

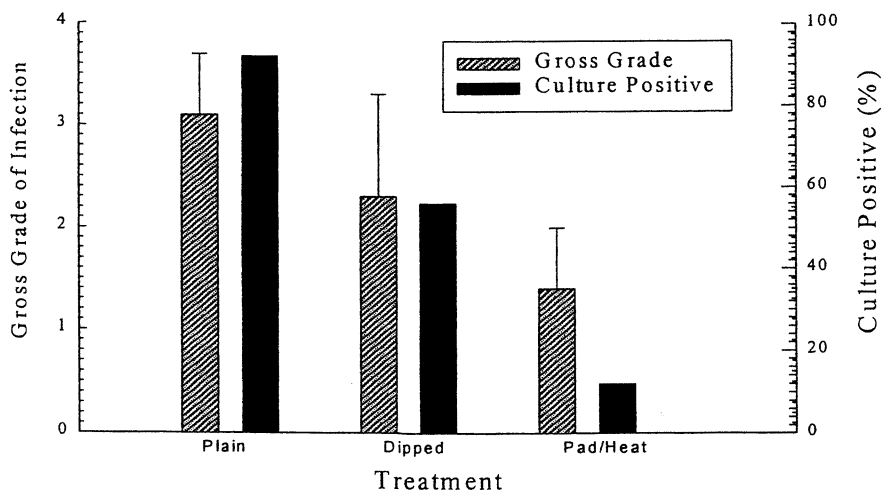


Figure 11. Wound grading, and culture of treated polyester segments retrieved from wounds of figure 12. (Reproduced with permission from reference 102)

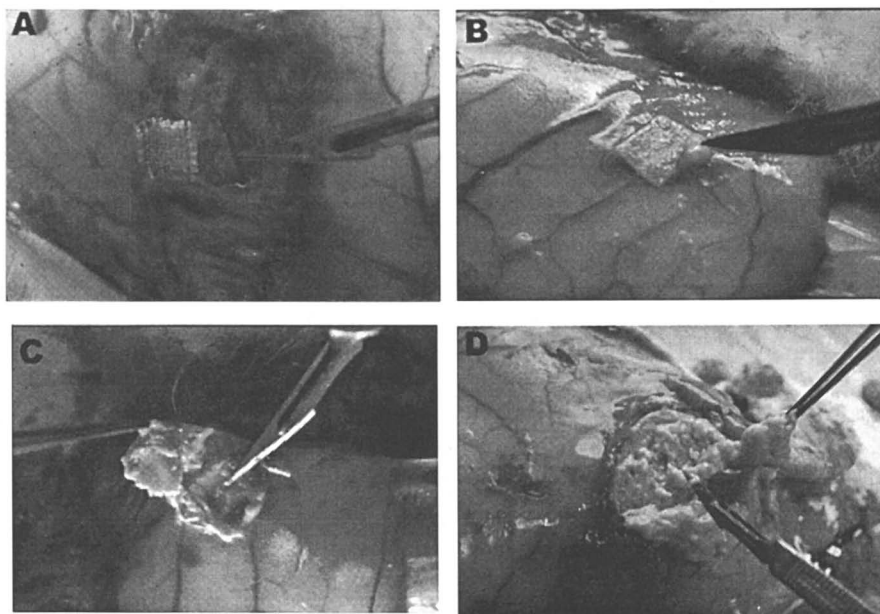


Figure 12. Macroscopic assessment of Dacron explants after exposure to bacteria. Pad/heat segments predominantly showed good tissue incorporation with no evidence of infection (A). Dipped segments showed signs of infection with some tissue incorporation (B/C). Untreated segments had suppurative infection extending outside of the graft pocket, with no gross tissue incorporation (D) (Reproduced with permission from reference 102)

needed, avoiding concerns over drug carrier toxicity, biocompatibility, and mutagenicity. Antibiotic leaching is controlled and sustained.

Application of Surface Modification Technology to Polyurethane

While polyurethanes are not currently used in arterial grafts, the good tissue and blood compatibility of polyurethanes makes them an important family of biomaterials. They are frequently used for implantable devices, including heart valves, artificial organs, blood filters, catheters, wound dressings, pacemaker leads, and prosthetic grafts. They are segmented polymers, formed from diisocyanates and polyols. Structural variation leads to materials ranging from rigid plastics to flexible foams. The most common method for manufacturing segmented polyurethanes is a two-step reaction. In the first step, an excess of diisocyanate is reacted with a polymeric diol, typically a polyester-, polyether-, or polyalkyl-diol, to produce an isocyanate-terminated prepolymer. In the second step, this prepolymer is allowed to react with a diol, called the chain extender. Early biomedical polyurethanes were polyether-based polymers. Although they had excellent stability *in vitro*, they showed surface degradation *in vivo* resulting from several degradative reactions (103). The development of polyurethanes using polycarbonate-based diols overcame these problems (104,105) and they are widely used today. A typical material is formed from poly(1,6-hexoyl-co-1,2-ethyl-carbonate)diol and 4,4'-diphenylmethane diisocyanate (MDI), with 1,4 butanediol as the chain extender. This polyurethane demonstrated not only improved compatibility with blood but also maintained the biodegradability of the basic polycarbonate polyurethane.

Having demonstrated the promise of a thrombin resistant polyester graft surface on polyester, additional studies have been performed in order to synthesize polyurethane with carboxylic acid sites (cPU) present on the polymer backbone to match those functional groups present on the hydrolyzed polyester (106). Carboxylic acid groups were incorporated into the polymer by using the chain extender 2,2-bis(hydroxymethyl)propionic acid in place of 1,4 butanediol (107). BSA was reacted with sulfo-SMCC, and this complex was linked to the carboxylic acid groups via EDC. ^{125}I -rHir was modified with Traut's reagent, and then reacted with the sulfo-SMCC remnant of the BSA attached to the polyurethane. Using these procedures, 190 ng ^{125}I -rHir/cm² cPU was covalently bound, some 20-fold greater than found for the control specimens with non-specifically bound ^{125}I -rHir. The modified specimens were also able to inhibit ^{131}I -thrombin (2.6 NIHU) to a much greater extent than controls (0.70 NIHU). The binding of ^{131}I -thrombin was similarly greater on the treated specimens. Thus, rHir can be linked to a polyurethane surface and retain its biologic activity.

As mentioned earlier, thrombus formation is a greater problem in small diameter grafts, and maintaining patency of these grafts is a significant challenge. The general procedure for modifying the surface of carboxylic acid-containing polyurethane with rHir described above was applied to a 4mm internal diameter graft of the material

(108). Determination of accessible carboxylic acid groups was determined using the cationic dye Methylene Blue (Figure 13). Once again, the binding of ^{125}I -rHir was successful, and some 150 fold greater than for the non-specifically bound analog, with correspondingly greater ^{131}I -thrombin inhibition and binding (Figure 14). In vivo implantation studies are currently in progress. The encouragement of endothelial cell incorporation by binding proteins such as VEGF to the carboxylic acid moieties, paralleling the work on polyester described earlier, is in its preliminary stages.

The application of cipro to cPU materials has been examined (109). The same experimental polyurethanes described above for protein binding studies were tested, i.e. polycarbonate based polyurethanes with ("Polyurethane A") and without ("Polyurethane B") carboxylic acid moieties present. Initial work showed that the cipro was preferentially absorbed from an aqueous solution by the polyurethane, i.e. that dyeing took place. A range of dyeing conditions (pH, temperature, concentration of cipro, liquor ratio, and dyeing time) was examined in order to obtain maximum uptake. The optimum conditions for the uptake of ciprofloxacin were determined to be at a liquor ratio of 20:1, a pH of 8.6, and a temperature of 55°C. An equilibrium uptake was established at a time of 3.5 hours. These optimum conditions were used to apply a range of cipro concentrations to polyurethane A and derive the sorption isotherm shown in Figure 15. The plot suggests a Langmuir distribution, and this is supported by the linearity ($R^2 = 0.923$) of the reciprocal plot of Figure 16. (A log/log plot of the same data had $R^2 = 0.76$) The saturation value, and partition coefficient K were derived from the intercept and slope of the reciprocal plot. The standard affinity was calculated based on the K value (110). The saturation value (0.45g/kg) corresponded closely with the known concentration of carboxylic acid groups in cPU (polyurethane A), indicating again that the carboxylic acid groups are the "sites" for dyeing. It is postulated that the mode of interaction between cipro and these carboxylic acid groups is hydrogen bonding between acid groups. The lack of uptake by the corresponding polyurethane lacking carboxylic acid groups is further evidence for this. Using the value for the distribution coefficient, K , obtained above, and making a number of assumptions (for example, that the interaction is nonionic, and that activities are equal to concentrations) a value for the standard affinity of cipro for this polyurethane of 4.69kJ/mol was obtained. This is a value that is of the same order of magnitude as, but lower than, the usual range of quoted values for standard affinities for a wide range of dye fiber systems, and corresponds to the comparatively low exhaustion obtained here. The value of such a calculation, if it can be suitably refined, is to correlate the attraction between antibiotic and fiber with rate of release and thus the activity/time of subsequent anti-microbial activity, and make this a predictable item. The anti-microbial activity of Polyurethane A treated in this way was determined using the same protocol as the cipro-treated polyester discussed previously. cPU "dyed" under our optimum conditions with 2% owf cipro showed a sustained zone of inhibition (25mm or better) after washing times of 96 hours, the maximum tested Figure 17. In contrast, butane diol extended polyurethane (polyurethane B) exposed to the same dyeing conditions lost all activity after 15

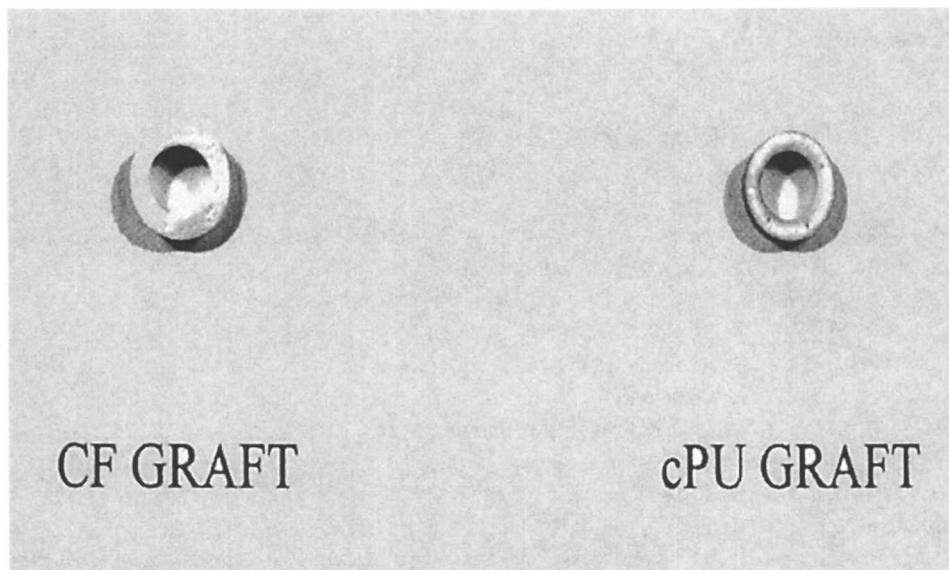


Figure 13. Cross-section of control and carboxylated polyurethane (cPU) coated Chronoflex grafts dyed with methylene blue.

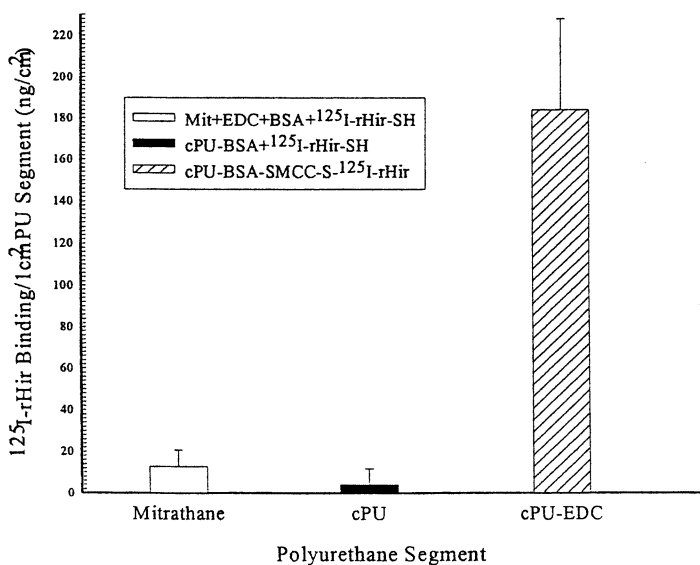


Figure 14. ^{125}I -rHir binding to polyurethane materials (Reproduced with permission from reference 106)

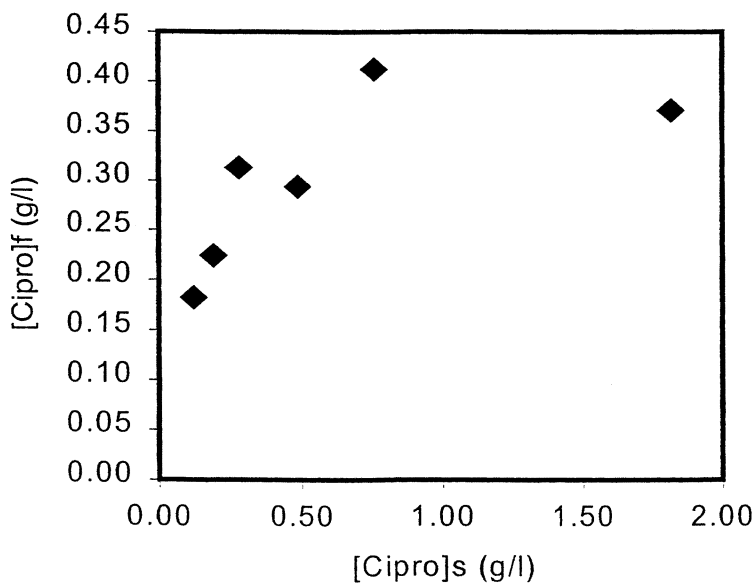


Figure 15. Equilibrium isotherm of cipro applied to COOH-modified polyurethane (Reproduced with permission from reference 109)

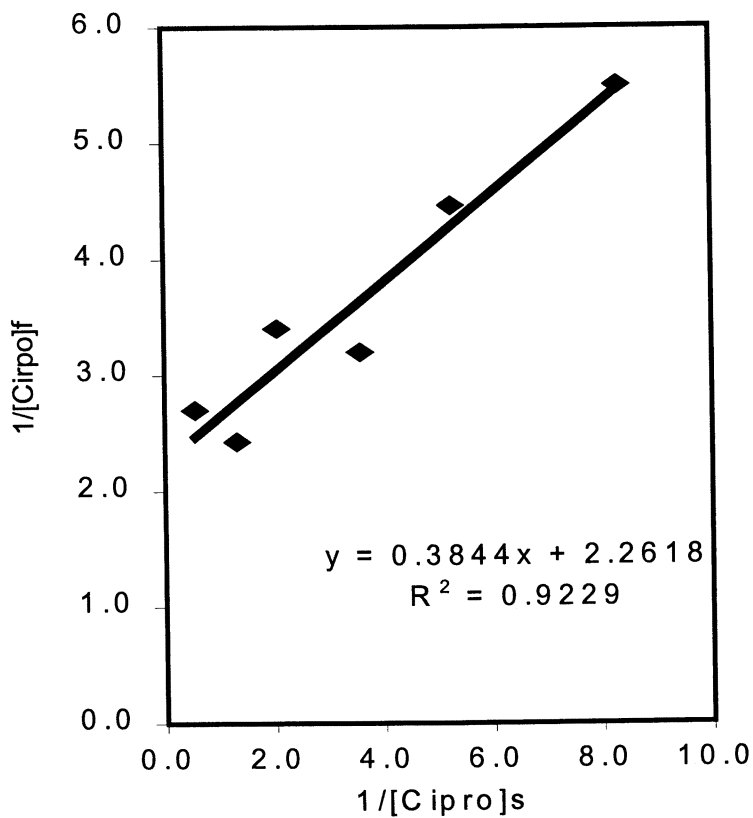


Figure 16. Reciprocal plot of data of figure 15.
(Reproduced with permission from reference 109)

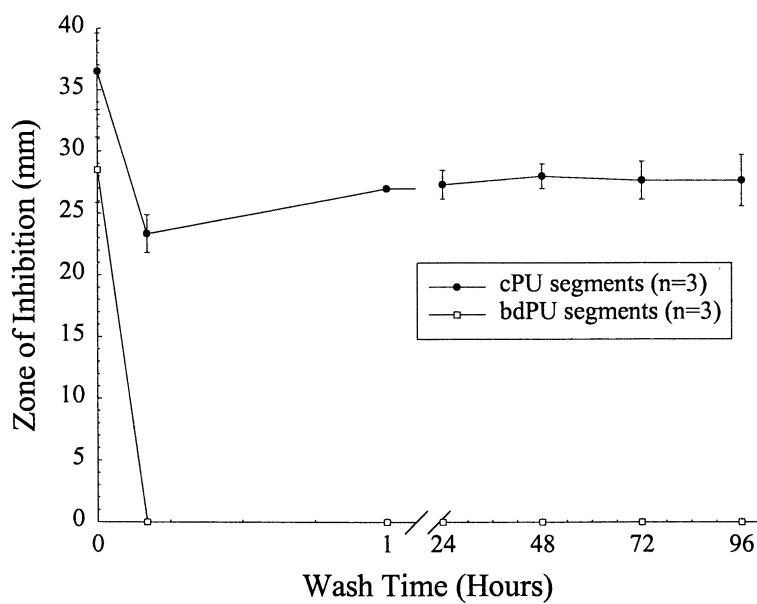


Figure 17. Zone of inhibition of polyurethanes “dyed” with antibiotic

minutes washing. The ready release of cipro, albeit over a long period of time, corresponds to the low standard affinity calculated above.

Conclusions

Antibiotics can be non-substantively incorporated into the polymer surface using a dyeing technology, and subsequently released over an extended period of time. This provides sustained infection resistance to these materials. Carboxylic acid groups developed on the surface of implantable polyester biomaterials using established textile processing techniques have proven to be useful for covalently binding proteins such as rHir and VEGF. These immobilized proteins maintain biologic activity as demonstrated in several *in vitro* studies. The same functional groups incorporated within a medically useful polyurethane can also provide protein binding sites, and have been shown to generate substantivity of an antibiotic for the material. Ultimately, the usefulness of this technology will be dictated by subsequent *in vivo* studies.

Clearly there is great scope for extending this work and future experiments will focus on evaluating various polyurethane formulations (e.g. varying the carboxylic acid content, incorporation of alternative functional groups), and testing different antibiotics. The polyurethanes can be used as materials in their own right, or as coatings to pre-existing devices (e.g. catheters, vascular grafts) to create a desired functional group on a relatively non-reactive surface. The basic methodology can also be extended to the broader family of biomaterials, and might thus develop new applications for these materials.

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Chapter 10

Biologically Active Biodegradable Biomaterials

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The chemical incorporation of biologically active compounds into synthetic biodegradable biomaterials was successfully synthesized by using nitric oxide derivatives (e.g., 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl) as the biochemical agents and synthetic aliphatic polyesters like polyglycolide as the biodegradable biomaterials. The resulting new biomaterial was characterized and its *in vitro* hydrolytic degradation property was studied to examine the release profiles of the chemically incorporated nitric oxide derivative from polyglycolide. The biological activity of this new class of biodegradable biomaterial was tested by examining its ability to retard the proliferation of human smooth muscle cells *in vitro*. It was found that this new class of biologically active biodegradable biomaterial has indeed one of the well-known biological functions of nitric oxide: retardation of the proliferation of smooth muscle cells. The potential biomedical applications of this new biomaterial include the treatment of hyperplasia in the cardiovascular system, promoting wound healing in healing-impaired patients, and those nitric oxide-related diseases.

Introduction

The interests in biodegradable polymeric biomaterials for biomedical engineering use have increased dramatically during the past decade. This is because this class of biomaterials has two major advantages that non-biodegradable biomaterials do not have. First, they don't elicit permanent chronic foreign-body reaction due to the fact that they would be gradually absorbed by the human body and do not permanently retain traces of their residue in the implantation sites. Second, some of them have recently been found to be able to regenerate tissues, so-called tissue engineering, through the interaction of their biodegradation with immunologic cells like macrophages. Hence, surgical

implants made from biodegradable biomaterials could be used as a temporary scaffold for tissue regeneration. This approach toward the reconstruction of injured, diseased or aged tissues is one of the most promising fields in the future of medicine.

Although the earliest and most commercially significant biodegradable polymeric biomaterials are originated from linear aliphatic polyesters like polyglycolide and polylactide from poly((-hydroxyacetic acids), recent introduction of several new synthetic and natural biodegradable polymeric biomaterials extends the domain beyond this family of simple polyesters. These new commercially significant biodegradable polymeric biomaterials include poly(orthoesters), polyanhydrides, polysaccharides, poly(ester-amides), tyrosine-based polyarylates or polyiminocarbonates or polycarbonates, poly(D,L-lactide-urethane), poly((-hydroxybutyrate), poly((-caprolactone), poly[bis(carboxylatophenoxy) phosphazene], poly(amino acids), pseudo-poly(amino acids) and copolymers derived from amino acids and non-amino acids.

The earliest and most successful and frequent biomedical application of biodegradable polymeric biomaterials have been in wound closure (1). All biodegradable wound closure biomaterials are based upon glycolide and lactide families. For example, polyglycolide (Dexon from American Cyanamid), poly(glycolide-L-lactide) random copolymer with 90 to 10 molar ratio (Vicryl from Ethicon), poly(ester-ether) (PDS from Ethicon), poly(glycolide-trimethylene carbonate) random block copolymer (Maxon from American Cyanamid), and poly(glycolide-(-caprolactone) copolymer (Monocryl from Ethicon). Some of the above materials like Vicryl have been commercially used as surgical meshes for hernia and body-wall repair. Besides wound closure application, biodegradable polymeric biomaterials are commercially satisfactory as drug control/release devices. Some well-known examples in this application are polyanhydrides and poly(ortho-ester). Biodegradable polymeric biomaterials, particularly totally resorbable composites have also been experimentally used in the field of orthopedics, mainly as components for internal bone fracture fixation like PDS pins. However, their wide acceptance in other parts of orthopedic implants may be limited due to their inherent mechanical properties and their biodegradation rate. Biodegradable polymeric biomaterials have been used experimentally as vascular grafts, vascular stents, vascular coupler for vessel anastomosis, nerve growth conduits, augmentation of defected bone, ligament/tendon prostheses, intramedullary plug during total hip replacement, anastomosis ring for intestinal surgery, and stents in ureteroureterostomies for accurate suture placement. The details of the biomedical applications of biodegradable polymeric biomaterials and their chemical, physical, mechanical biological and biodegradation properties could be found in other recent reviews (1-7).

There is one common characteristic among all these biodegradable biomaterials: they don't "actively" participate in the process of wound healing, tissue regeneration & engineering. In other words, these biomaterials are not "alive" and can't remodel or/and release cytokines upon stimulation like normal tissues. These biomaterials, however, elicit inflammatory and foreign body reactions and play a "passive" role in wound

healing. It would be ideal if these synthetic biomaterials could be engineered so that they could become “alive” after implantation and hence actively participate in the biological functions with the surrounding tissues, such as the ability to modulate inflammatory reactions, to facilitate wound healing or to mediate host defense system to combat diseases. In this chapter, we would describe the new biodegradable biomaterials having nitric oxide (NO) function.

Biodegradable Biomaterials Having Nitric Oxide Function

Nitric oxide (NO•) is a very small but highly reactive and unstable free radical biomolecule with expanding known biological functions. This small biomolecule and its biological functions have recently become one of the most studied and intriguing subjects as recently reviewed by several investigators (8-18). NO• is extremely labile and short-lived (about 6 to 10 seconds).

NO• and its radical derivatives have been known to play a very important role in a host of expanding biological functions, such as inflammation, neurotransmission, blood clotting, blood pressure, cardiovascular disorders, rheumatic and autoimmune diseases, antitumor activity with a high therapeutic index, antimicrobial property, sensitization or protection of cells and tissues against irradiation, oxidative stress, respiratory distress syndrome, and cytoprotective property in reperfusion injury, to name a few (8-30). NO• acts both as an essential regulatory agent to normal physiological activities and as cytotoxic species in diseases and their treatments. Nathan et al. reported that nitric oxide is a potent antiviral compound against two disfiguring poxvirus and herpes simplex virus type-1, which causes cold sores in humans (12). Levi et al. also found that nitric oxide could protect the human heart against low oxygen supply, a condition known as myocardial ischemia, by widening blood vessels so that more oxygen-rich blood reaches the heart (14). Elliott et al. reported that a new NO• -releasing nonsteroidal anti-inflammatory drug has the benefit of accelerating gastric ulcer healing (31,32). It is important to know, however, that excessive introduction of NO• into the body may have adverse effects like microvascular leakage, tissue damage in cystic fibrosis, septic shock, B-cell destruction, and possible mutagenic risk, to name a few (18,19,27,33-35).

NO• and NO•-derived radicals are not normal biological messengers whose trafficking depend on specific transporters or channels. Instead, nitric oxide radicals released by cells like macrophage and endothelial cells would diffuse randomly in all directions from the site of release. Because of this unusual property, the only way to control the biological functions of nitric oxide is to control its site of synthesis. This suggests that the only way to deliver the desirable biological functions of nitric oxide is through nature. Existing science and technology are not able to modulate the release of nitric oxide according to our wish for a variety of therapeutic purposes.

Synthesis & Characterization of Synthetic Biodegradable Biomaterials Having NO Function.

We recently used a patented chemical method to incorporate nitric oxide derivatives into a series of synthetic biodegradable biomaterials (36,37). Upon the hydrolytic degradation of the host biomaterials, nitric oxide derivatives could be released to the surrounding buffer media and the rate of release could be controlled by the nature of the biodegradable biomaterials. The amount of the nitric oxide derivatives that can be incorporated into biodegradable biomaterials would depend on the molecular weight of the biomaterials. Figure 1 illustrates the chemical scheme of this patented method of incorporating 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (TAM) as the source of nitroxyl radicals nitric oxide into synthetic biodegradable polyesters like polyglycolide or polylactide.

Due to the free radical characteristic of Tempamine nitroxyl radicals, the radical incorporated polyglycolide (PGA) must exhibit an EPR spectrum that has the characteristic of nitroxide. Figure 2 is such an EPR spectrum of TAM-PGA.

This EPR spectrum shows a considerable broadening of linewidth when comparing with an EPR spectrum of free TAM nitric oxide. An EPR characterization of nitroxyl radicals is based on the measurement of the signal intensity of an EPR spectrum. This measurement can provide fundamental information of free radicals, such as linewidth that bears a relationship to the tumbling motion of free radicals, g -value which largely depends on the immediate environments of the free radicals, and hyperfine splitting constants which describe the classical multiplicity of EPR spectrum due to the interaction of the unpaired electron spins with nuclear spins.

The considerable broadening of the EPR spectrum of the TAM-PGA biomaterial (Figure 2.A) when compared with free TAM nitroxyl radical (Figure 2.B) is attributed to the viscous macromolecular environment surrounding TAM nitroxyl radicals that were chemically bonded to PGA chain ends. These nitroxyl radicals can't move as freely as free nitric oxide due to the restricted PGA chain segmental motion. This relationship between free radical motion and the characteristic of its immediate environment would provide a useful means to study the release pattern of nitroxyl radicals that were chemically bonded to biodegradable substrates.

Because TAM nitroxyl radicals were incorporated only into the carboxylic chain ends of PGA macromolecules, their physical, thermal and mechanical properties are insignificantly different from the parent PGA macromolecules, such as similar melting temperature and heat of fusion. This lack of change in fundamental properties between TAM-PGA and parent PGA biomaterials should be beneficial because the similar processing conditions that have been used to fabricate PGA for a variety of clinical applications could also be used to fabricate the new TAM-PGA. In addition, the knowledge of the well-known biodegradation properties of PGA could be applied to estimate the release pattern of TAM nitroxyl radicals from PGA upon its biodegradation. The main difference between the parent and TAM-PGA, however, is their degradation products and their subsequent biological properties.

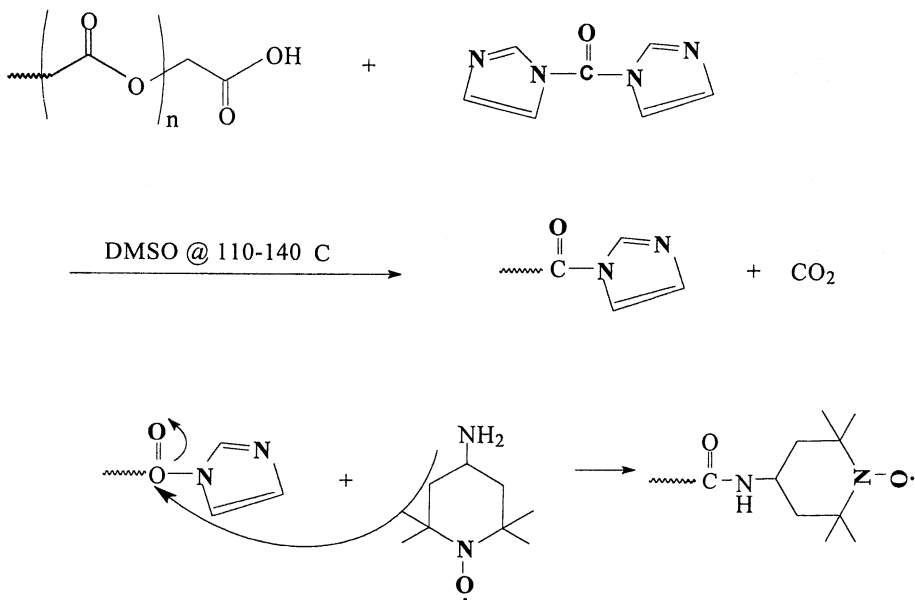


Figure 1. Chemical scheme for incorporating 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (TAM) into the carboxyl chain ends of linear aliphatic polyesters like polyglycolide.

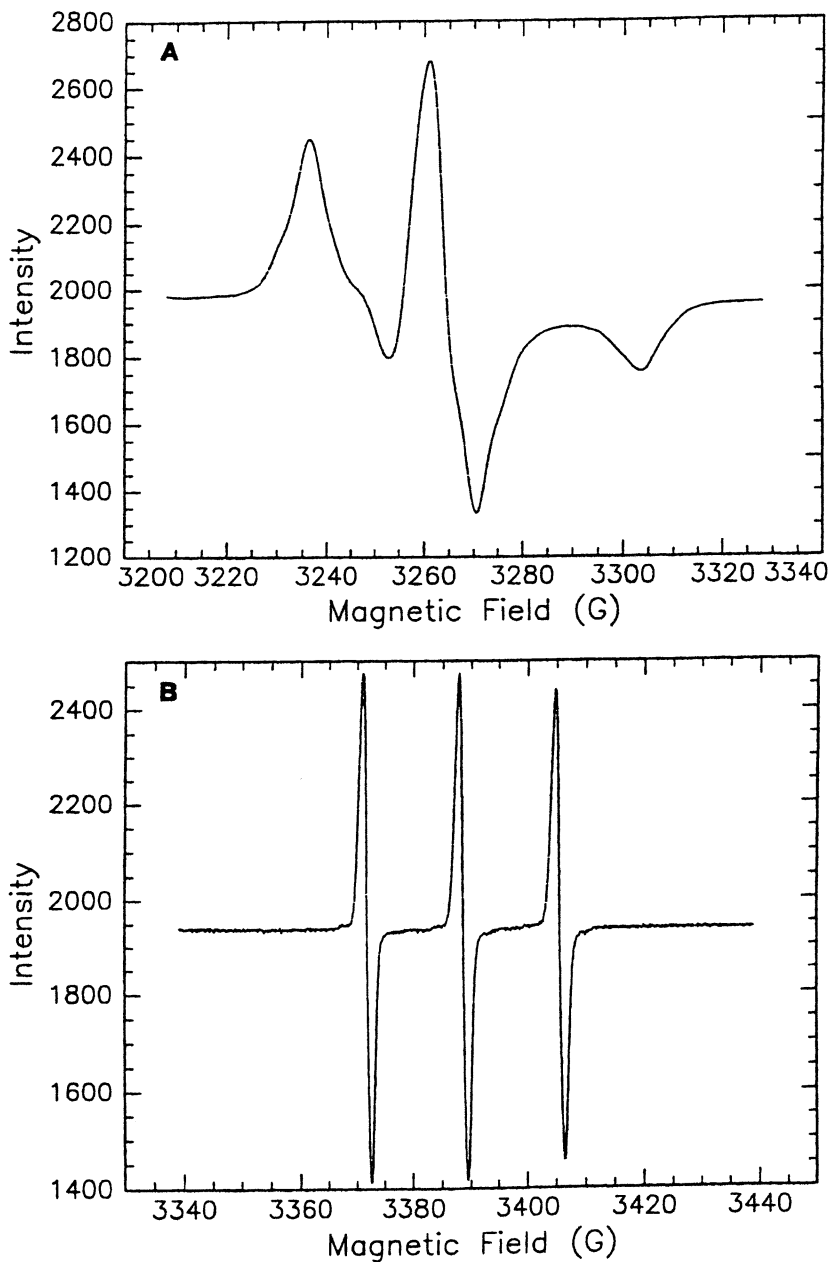


Figure 2. Electron paramagnetic resonance (EPR) spectra of 4-amino-2,2,6,6-tetramethylpiperidine-1-oxy (TAM) radical in free form (b) and in conjugation with polyglycolide (a).

***In Vitro* Hydrolytic Degradation of Synthetic Biodegradable Biomaterials Having NO Function.**

Since the expected biological functions of the TAM-PGA must come from the nitroxyl radicals that would be released into the surrounding environment upon hydrolytic degradation of PGA, the amount and rate of release of TAM nitroxyl radicals should have a direct impact on the applicability of the newly synthesized TAM-PGA biomaterials to medicine. Figure 3 illustrates such an *in vitro* release pattern of TAM radicals from PGA. The release of Tempamine nitroxyl radicals upon PGA hydrolysis followed a double exponential behavior in which significant amounts of nitroxyl radicals were released before 20 days followed by a gradual release thereafter.

It was well known that the nitroxyl free radicals could participate in one-electron reduction reaction in an acidic medium to yield relatively stable diamagnetic products, hydroxypiperidines structure (NOH) and *oxopiperidinium* cations (NO⁺). Figure 4 shows such one-electron reduction and oxidation reaction of free nitric oxide derivatives.

Because the conversion of TAM nitroxyl radical (i.e., chemical reduction of nitroxyl radical) to hydroxypiperine structure in an acidic environment would remove the free radical characteristic of TAM, EPR could not detect the presence of hydroxypiperine and the spin numbers of TAM would continue to decrease with time. The extent of this conversion and hence the number of spins of TAM would depend on the strength of acidity of the medium. Figure 5 illustrates such an effect of pH on the peak intensity of EPR spectra of TAM nitroxyl radicals in glycolic acid media. As the pH of the media decreased from 7.44 to 4.0 (EPR spectrum b) and 3.0 (EPR spectrum d), the EPR spectra peak intensities were reduced accordingly. This would suggest that more TAM nitroxyl radicals would be reduced to hydroxypiperine at a lower pH and fewer TAM nitroxyl radicals would remain in an acidic medium and exhibit weaker EPR signal.

In addition, the nitroxyl group could also be easily polarizable by acids toward its ionic resonance form resulting in an increased electron-nuclear coupling constant (hyperfine splitting constant) since the spin density of the nitroxyl bond has been influenced by the proton activity in the acidic media.

The acidifying of the degradation media can be demonstrated by the decrease in pH as shown in Figure 6. The result indicated a similar double exponential reduction ($y(x) = 3.83 e^{-0.39x} + 3.57 e^{-0.0018x}$) in pH as the # of spins vs time in Figure 3. The pH of the medium was reduced from the initial 7.4 at 0 day to 3.5 during the first 14 days of hydrolysis and there was very little reduction in pH thereafter. A comparison between Figures 3 and 6 showed remarkable similarity between the release pattern of TAM nitroxyl radicals and the profile of the reduction in pH of the degradation media.

The observed *in vitro* release pattern of TAM nitroxyl radicals from the TAM-PGA biomaterial and the close similarity between the TAM release pattern and the media pH reduction profiles suggested that pH must play a major role. Based on the well-known hydrolytic degradation mechanism of PGA biomaterial, we postulated the hydrolytic degradation mechanism of TAM-PGA biomaterial as illustrated in Figure 7. TAM-PGA

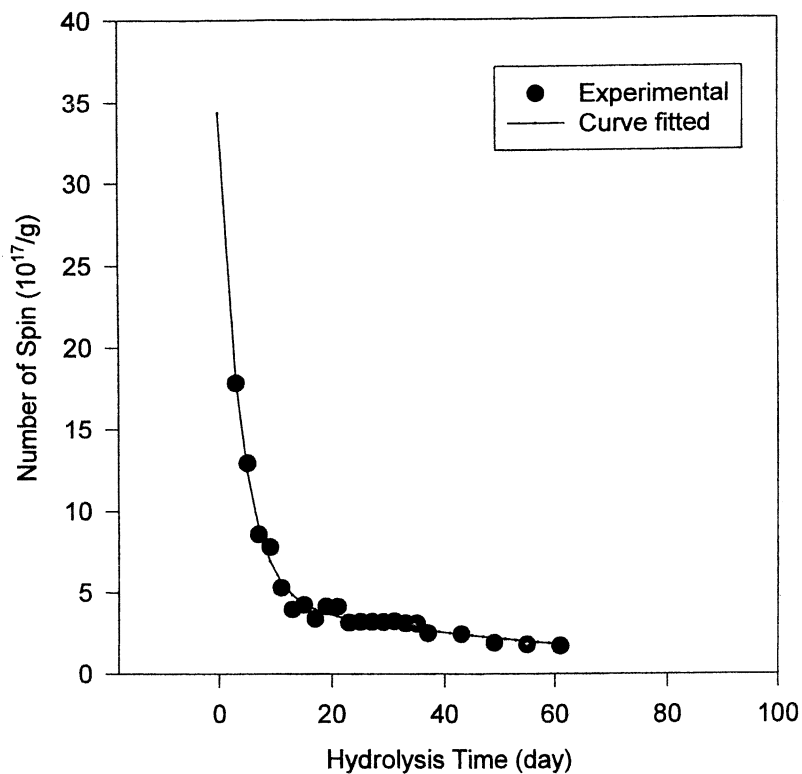


Figure 3. The kinetics of *in vitro* release of 4-amino-2,2,6,6-tetramethylpiperidine-1-oxy (TAM) nitroxyl radicals from TAM-Polyglycolide biomaterials in buffer media of original pH 7.44 at 37°C.

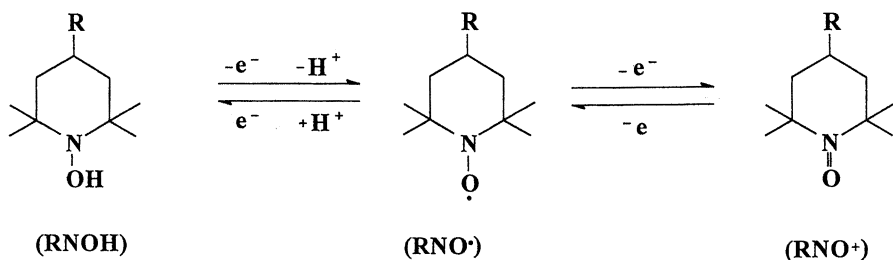


Figure 4. The reversible one-electron reduction and oxidation reactions of nitroxyl free radicals.

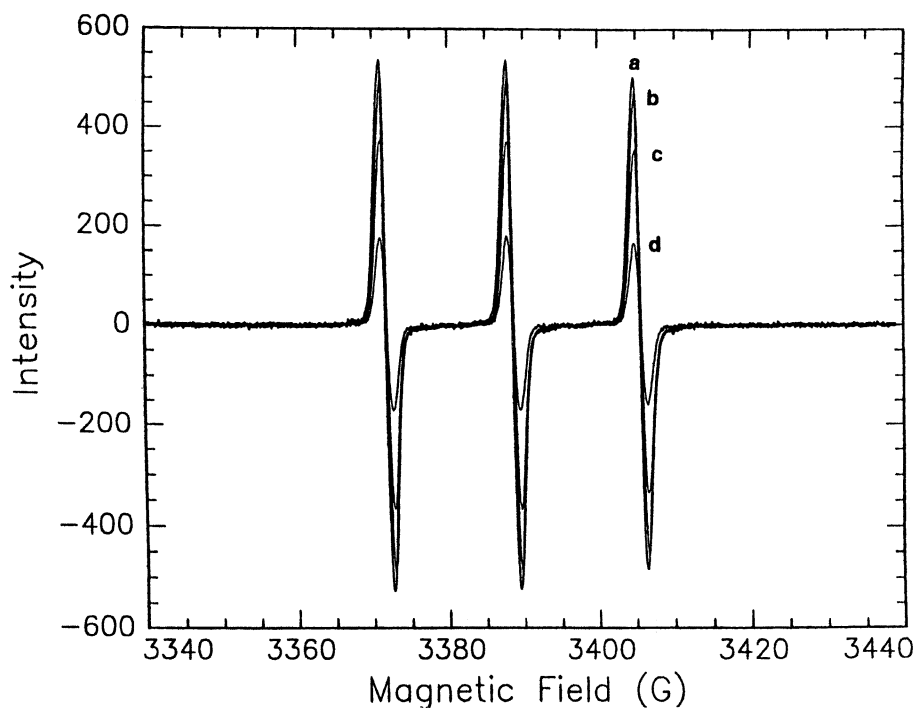


Figure 5. The effect of pH of the media on the EPR spectra intensity of 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (TAM) nitroxyl radical at 10(g/ml). The media was glycolic acid. (a). pH 7.44; (b) pH 4.0; (c) pH 3.5; (d) pH 3.0.

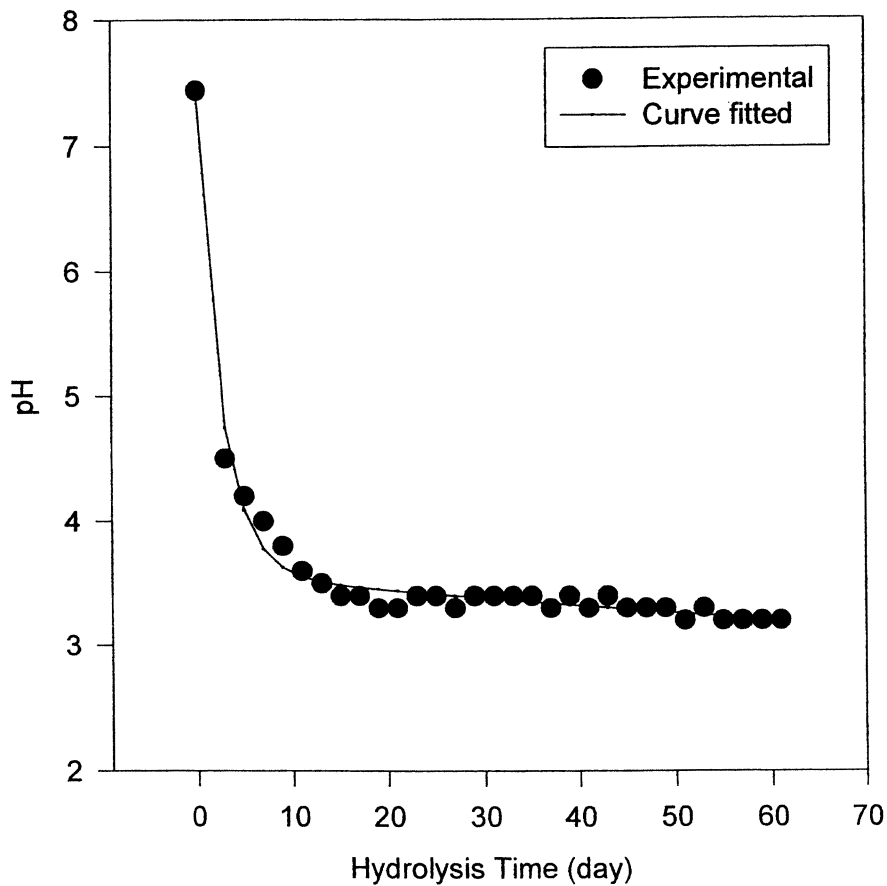
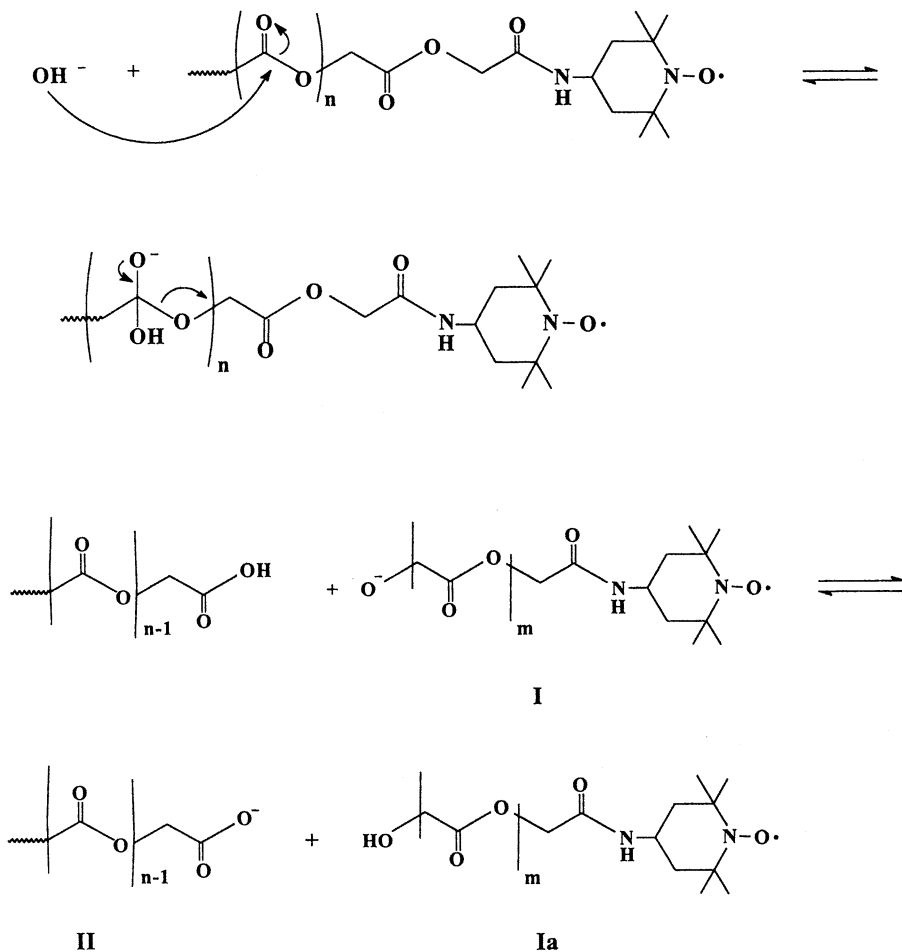


Figure 6. The change in pH of the buffer medium used for the *in vitro* release study of TAM-Polyglycolide at 37°C.

a

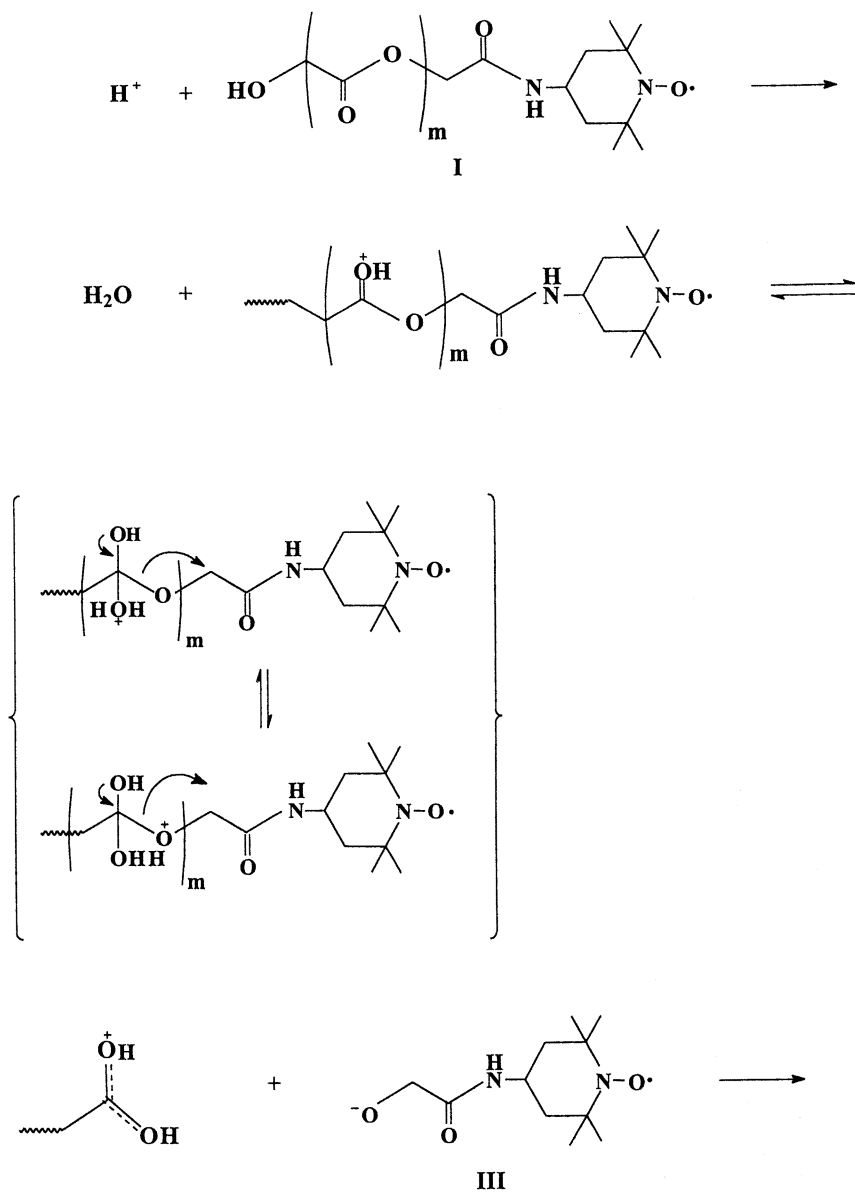


a). Alkaline hydrolysis of the ester linkages in polyglycolide backbone during the early stage of immersion.

Figure 7. The hydrolytic release mechanism of 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (TAM) radicals from TAM-Polyglycolide biomaterials via alkaline and acid hydrolytic degradation. (a) Initial alkaline hydrolytic degradation of the ester linkages in polyglycolide backbone during the early stage of immersion. (b) Acidic hydrolytic degradation of the ester linkages in polyglycolide backbone during the middle stage of immersion. (c). Acidic hydrolytic degradation of the amide linkage to free 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (TAM) radical from the polyglycolide substrate during the late stage of immersion.

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b

Figure 7. *Continued.*

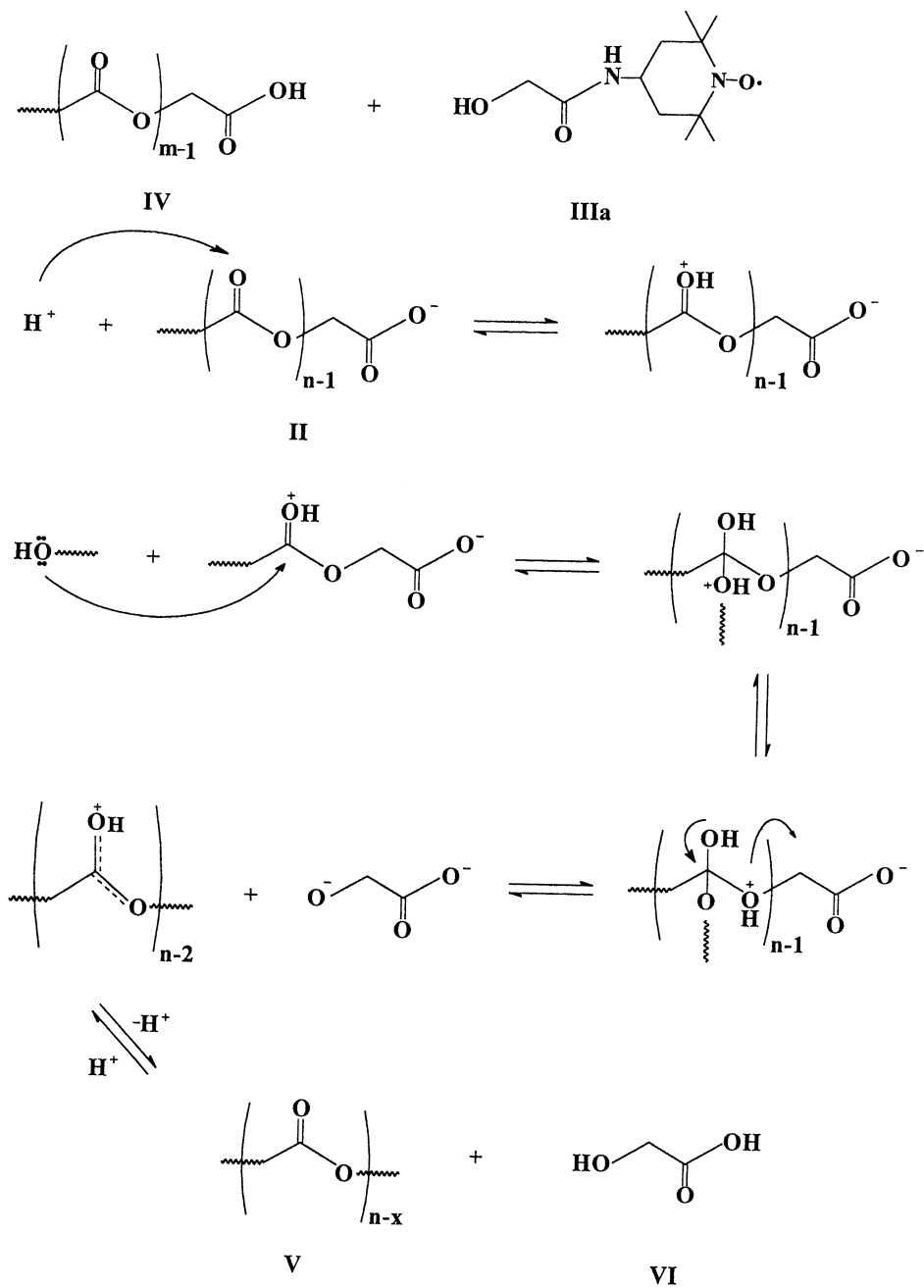
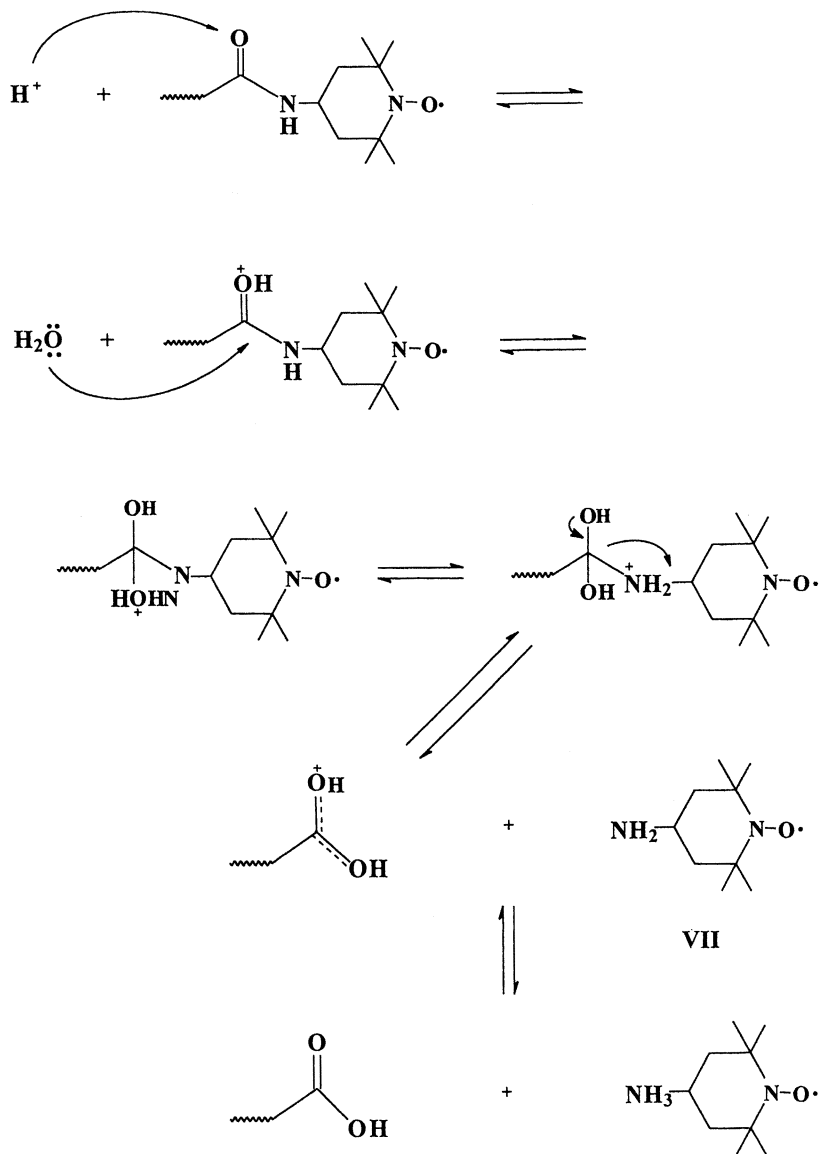


Figure 7. Continued.

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c

Figure 7. *Continued.*

biomaterials experienced both alkaline and acid catalyzed hydrolytic degradation as evident in the change of pH of the media (Figure 3).

Alkaline hydrolysis occurred during the very early stage (within a few days) because of the initial pH of the buffer (7.4). The possible products of this early stage alkaline hydrolysis of TAM-PGA were expected to be carboxylic fragments of PGA segments (II in Figure 7a) and the TAM-PGA fragments (Ia in Scheme 3.4a). As hydrolysis time proceeded further, the PGA fragments would eventually be degraded into glycolic acid or/and its cyclic dimers (glycolide) as the pH of the media became more acidic. The length of the PGA segment of the TAM-PGA fragments (Ia) would also be reduced via ester hydrolysis in both alkaline and acidic media till all the ester linkages were scissioned (III or/and IIIa in Figure 7b). Because the amide linkages where the TAM nitroxyl radicals were attached to PGA were far more hydrolytic resistant than ester linkages, we didn't expect the formation of free TAM nitroxyl radicals via the scission of the amide linkage (VII in Figure 7c) until the late stage of hydrolysis.

Based on the proposed hydrolytic degradation mechanism of TAM-PGA biomaterials shown in Figure 7, one should expect only one type of TAM nitroxyl radicals (I or Ia in Figure 7a) in the very early stage of hydrolysis; as hydrolysis proceeds further, a mixture of TAM nitroxyl radicals with different PGA chain length, i.e., different m in I or Ia, would be the predominant products in the degradation media. At the late stage of hydrolysis, all PGA segments of the TAM-PGA fragments were hydrolyzed and eventually the amide linkages where the TAM radicals were attached would be hydrolyzed to free TAM radicals (VII in Figure 7c).

These different types of TAM radicals due to different PGA chain lengths attached might be evident in the EPR spectra shown in Figure 8 which was a series of EPR spectra from 3 different stages of hydrolysis time.

At 3 days of hydrolysis, its EPR spectrum had a unique character on its first peak, i.e., a spectral feature with split peaks, that was characteristically different from the typical 3 EPR peaks of a homogeneous nitroxyl radical in solution. This spectral feature could assume that there were a mixture of TAM-PGA nitroxyl radicals with various PGA chain lengths (heterogeneity). In other words, there might be at least two different segmental lengths with different tumbling rates of TAM nitroxyl radicals, i.e., two different $A_{||}$ tensor and g factors. The largest EPR spectral feature of the first peak was observed at 23 days hydrolysis. This EPR spectral feature persisted for a long period up to 40 days of hydrolysis. At the late stage of hydrolysis (55 days), however, this spectral feature of TAM radicals disappeared and the typical 3 EPR spectrum of nitroxyl radicals reappeared. This suggested that a more homogeneous type of nitroxyl radicals like VII or IIIa shown in Figure 7 was present in the media. Due to the lack of restriction imposed by the PGA long chain segments, the tumbling motion of free TAM nitroxyl radicals in aqueous solution was easier than their polymeric counterparts and was reflected in the typical nitroxyl radical hyperfine spectrum.

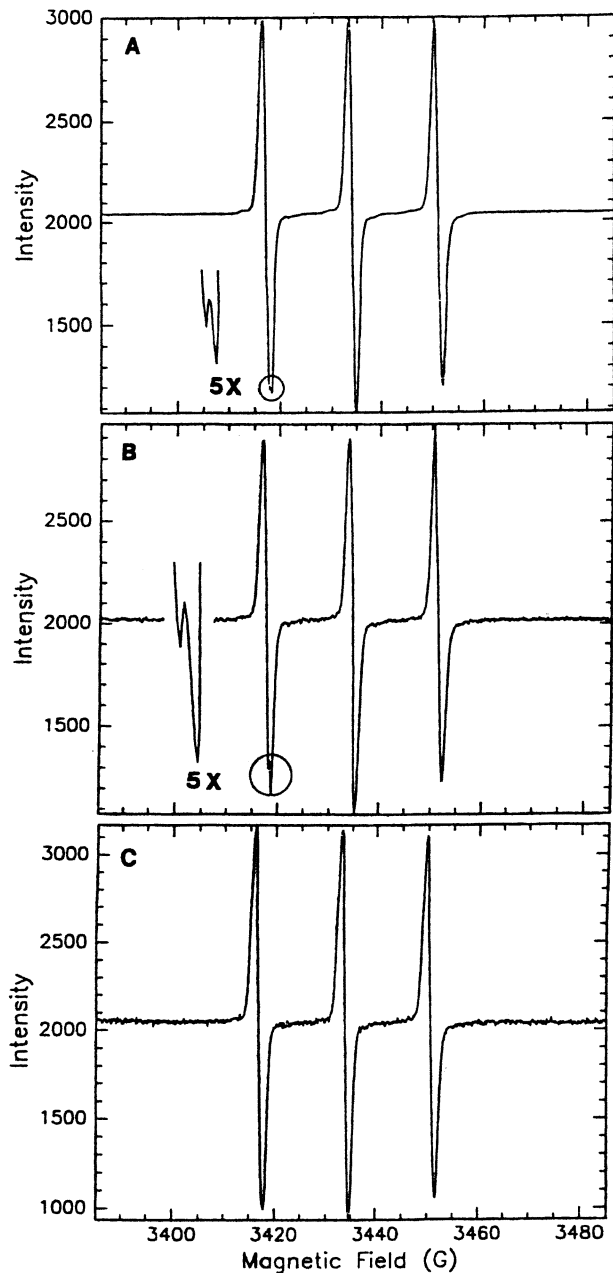


Figure 8. Electron paramagnetic resonance (EPR) spectra of the degradation products from the *in vitro* hydrolytic degradation of TAM-polyglycolide in buffer media over a period of 55 days at 37°C. (A). 3 days; (B) 23 days; (C) 55 days. Note the presence of split peaks in (A) and (B) that was not there in (C).

***In Vitro* Biological Activity of Synthetic Biodegradable Biomaterials on Human Smooth Muscle Cells.**

The biological activity of TAM-PGA can best be illustrated by the level of retardation of smooth muscle cells (SMC) in cell culture. This is because NO has been theorized to be able to retard SMC proliferation in humans. As shown in Figure 9, TAM-PGA showed profound retardation of the proliferation of SMC *in vitro*. There was virtually no change in the number of live SMCs in the culture medium having TAM-PGA over the entire period of cell culture, while the number of live SMCs in the culture medium control had been more than double (134% increase) during the same culture period. This level of SMC retardation of TAM-PGA biomaterial was found to be similar with free TAM nitroxyl radicals at 1 (g/ml.) Figure 9 also shows that any higher concentrations of TAM nitroxyl radicals in the culture media (> 1 (g/ml)) would appear toxic to SMC, as evident in the reduction in SMC population from the initial number (0 day), particularly at 100 (g/ml).

The preliminary *in vitro* SMC culture data suggest that the newly synthesized TAM-PGA biomaterials have the same biological function as free nitric oxide in terms of the retardation of SMC proliferation. Although the intermediate and final degradation products from the hydrolysis of TAM-PGA biomaterials (I/Ia, III/IIIa and VII) are chemically different from pure nitric oxide they exhibited the same retardation of SMC proliferation as free TAM nitroxyl radicals. Thus, it appears that both the free TAM and the TAM-PGA nitroxyl radicals would have the same biological function as pure nitric oxide. The long PGA chain segments where the TAM nitroxyl radicals were attached appeared not to interfere with the biological functions of the nitric oxide portion of the TAM-PGA molecules. Since the level of retardation of SMC proliferation by TAM-PGA biomaterials at a concentration of 1 mg/ml was found to be similar to the pure TAM nitroxyl radicals at 1 $\mu\text{g/ml}$ over the entire culture period, it appears that the amounts of TAM that were incorporated into PGA chain ends based on the stipulated chemical reaction conditions were adequate for this particular purpose.

Potential Biomedical Applications.

Some examples of the potential use of this new generation of biologically active biodegradable polymers are the treatment of intimal hyperplasia after balloon angioplastic procedures, delivery of anticancer drugs, wound closure materials with improved healing and antimicrobial capability, and synthetic vascular grafts that would not clot. In the anticancer drug area, the nitroxyl radical incorporated biodegradable biomaterials could be used as the vehicles to precisely deliver the antitumor property of nitroxyl radicals to tumor sites via the biodegradation release of the incorporated nitroxyl radicals. The nitroxyl radical incorporated biomaterials could also be used to improve the efficacy of radiation therapy in cancer because nitroxyl radicals are known to be able to considerably sensitize tumor cells toward radiation. The benefit would be a lower side effect of radiation therapy because a lower dosage of radiation could be used without compromising the therapeutic effect. In addition to the therapeutic effect and

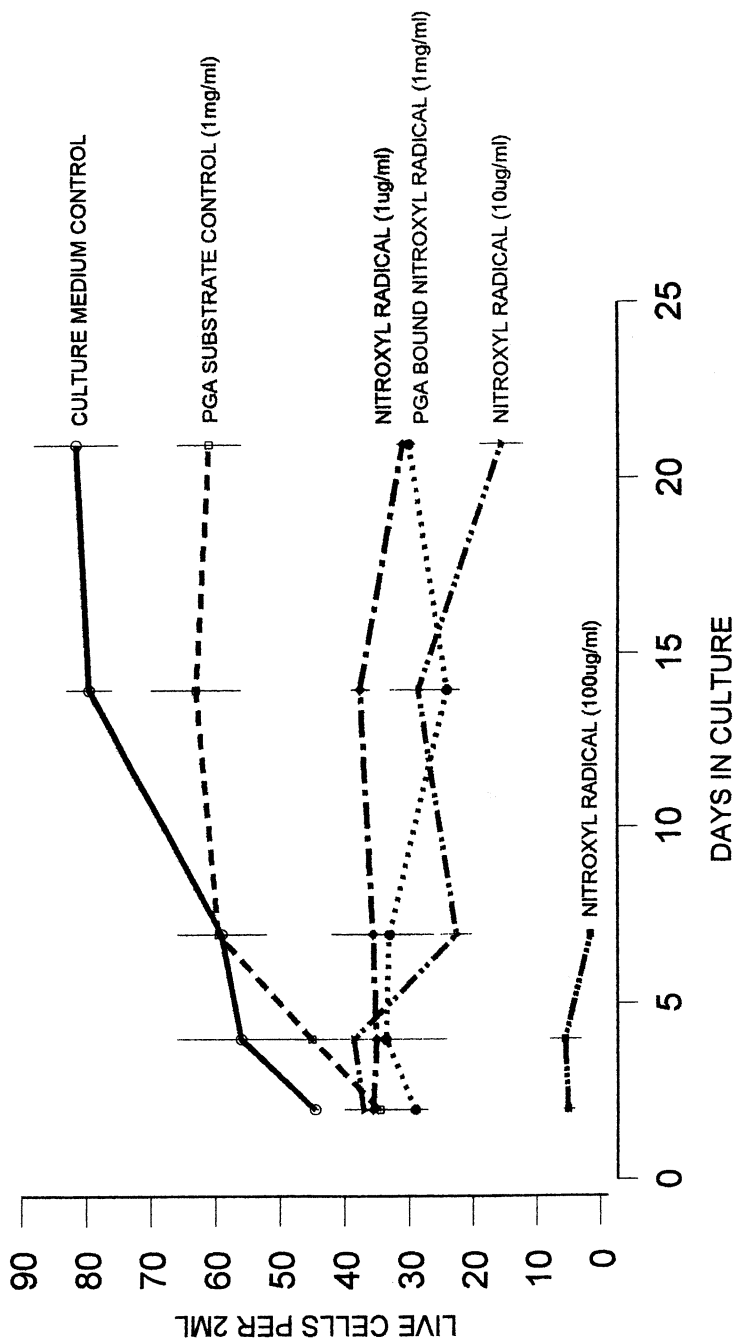


Figure 9. The effect of TAM-Polyglycolide on the proliferation of human smooth muscle. The cell density at 0 day was $5.0 \times 10^4/2$ ml.

reconstruction of injured or diseased tissues, the nitroxyl radical incorporated biomaterials may also be used as a useful tool for a fundamental study of a host of biochemical reactions involving free radicals and superoxide anions because these nitroxyl radical labelled biomaterials could react with any reactive free radicals and neutralize them. These modified biomaterials could also be used to mimic the functions of superoxide dismutase (SOD), a naturally occurring enzyme to neutralize superoxide anions and other reactive radicals. Thus, the modified biomaterials may be used to control local inflammatory reaction induced by wounds or/and surgical implants.

Acknowledgement

The author wishes to thank his former graduate student, Dr. Keun-Ho Lee, for his thesis that the bulk of this chapter was based upon. The *in vitro* smooth cell proliferation was conducted by Prof. Fred Quimby and his research staff, Suzanne Klaessig, Dept. of Pathology, College of Veterinary Medicine, Cornell University.

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Chapter 11

Antimicrobial Polymers and Fibers: Retrospective and Prospective

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Approaches for imparting antimicrobial activity to polymeric materials have evolved from ancient practices to eclectic, modern strategies. The mechanisms by which microbes attach to polymeric surfaces and acquire resistance to antimicrobial agents are critically reviewed. Representative microorganisms (primarily bacteria, fungi, algae and viruses) are listed that are deleterious from a health or medical perspective and/or cause unwanted damage in materials. A variety of chemical approaches have been effective and include micrencapsulation, polymerization, covalent bond formation and simple insolubilization. Effectiveness of these agents (primarily biocides but also selective antibiotics) against various types of microorganisms and the modes by which they prevent microbial contamination and growth is discussed. Newer synthetic and naturally occurring agents are noted as well as strategies that prevent microbial attachment are discussed. The latter include modification of polymer surfaces, chemicals that disorient biochemical signals to microbes, and other useful concepts and approaches. The interest in this area is relevant to an array of applications where biological activity on fiber and other polymeric surfaces is of concern as biological discoveries reveal that many pathologies are caused by microbial infection.

A historical perspective of antimicrobial polymers and fibers (excluding the last decade) has been published [1]. The first scientific documentation in this area is generally agreed to be the use of phenol (the first antiseptic) by Lister for bandages in surgery [2]. Other recent reviews of general interest include two excellent books on the history of antimicrobial agents [3] and disinfection, sterilization and preservation [4]. However, neither monograph devotes much attention to modified antimicrobial materials.

The scope of this review briefly focuses on important historical concepts and achievements with these materials, but also elaborates on advances and trends in the past decade in seven areas: (a) representative pathogenic microorganisms and those that cause damage to materials; (b) mechanisms by which microbes attach to the surface of fibrous and polymeric materials and their persistence, i.e., the amount of time they are viable on the surface; (c) classification of antimicrobial agents with regard to their target sites on the microorganisms, and their degree and scope of efficacy; (d) modes of action of durably affixed antimicrobial agents on fibrous and other polymeric surfaces and a chemical functional group classification of such agents; (e) mechanisms by which microbes acquire or exhibit resistance to antimicrobial and/or antibiotic agents; (f) new synthetic and naturally-occurring antimicrobial agents and (g) new strategies for overcoming the microbial resistance of these agents in materials.

Terms and Definitions

The definitions in this area have evolved over time and a brief summary of these (original and current definitions) are given in a historical review on antimicrobial fibers and polymers [1]. A selected and concise list of definitions and terms is given in Table I and adapted from those listed by Block [5]. Effectiveness of antimicrobial agents and antibiotics range from sterilization (complete destruction of all forms of life) to static and -cidal activity to the minimum requirement of a sanitizer (reduction of bacterial contaminants to safe levels as judged by public health laws). Although only bactericide and bacteriostat are defined, the corresponding suffixes -cidal and -static equally apply to the effectiveness of other antimicrobial agents such as fungistat, sporicide and virucide. These terms and definitions will be frequently used throughout the review and are important in assessing the potency and scope of agents that affect microbes.

Representative Microorganisms

Polymeric substrates, in addition to other materials, can act as fomites for microbial pathogens and can also be biologically degraded by many species of fungi and selected species of bacteria. Disease transmission is a very important consideration for intracorporeal or implantable devices in the human body (e.g., vascular grafts and sutures) and for extracorporeal devices such as catheters and hollow fibers for dialyzers. Depolymerization, discoloration and accompanying loss in mechanical and other functional properties of polymeric substrates is caused primarily by fungi, algae and some bacteria. Cellulosic fibers (e.g., cotton and rayon) are particularly susceptible to such microbial damage. However, most fibrous materials and many polymers have no inherent resistance to disease transmission unless they are chemically or physically modified for this purpose.

Table 2 shows a list of microorganisms and selected species in those classes that are pathogenic and/or cause polymer degradation. This concise compilation reflects microorganisms of continuing and current interest that are known to cause health problems and recurring epidemics. Many commonly occurring gram-positive and gram-

Table 1. Terms and Definitions Relevant to Antimicrobial Materials

| <i>Term</i> | <i>Concise Current Definition</i> |
|----------------------------|---|
| antimicrobial agent | Any agent that kills or suppresses growth of microorganisms. |
| antibiotic | Organic chemical substance produced by microbes and synthetically that has capacity in dilute solution to destroy or inhibit growth of bacteria and other microorganisms. |
| sterilization | Act or process that destroys all forms of life, especially microorganisms. |
| biocide | Substance that kills all living organisms, pathogenic and nonpathogenic. <i>De facto</i> , a sterilizing agent. |
| germicide | Substance that destroys primarily pathogenic microorganisms. |
| bactericide | Agent that kills bacteria. |
| bacteriostat | Agent that prevents growth of bacteria, but does not necessarily kill them or their spores. |
| disinfectant | Agent that frees from infection, usually chemical but could be physical like UV light. Kills pathogenic microorganisms but not necessarily spores. Refers to substances applied to inanimate objects. |
| fomite | Contaminated inanimate object that serves in the transmission of disease. |
| antiseptic | Substance that prevents growth of microbes by static or -cidal activity. Term usually reserved for topical application to living tissue. |
| sanitizer | Agent that reduces number of bacterial contaminants to safe levels as judged by public health laws. Usually applied only to inanimate objects. |

^a Majority of definitions adapted from ref. [5]

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Table 2. Microorganisms Pathogenic/Parasitic on and Deleterious to Polymeric Substrates

| <i>Species</i> | <i>Effect on user and/or substrate</i> |
|-----------------------------------|--|
| Gram-positive bacteria | |
| <i>Staphylococcus aureus</i> | Pyogenic and nosocomial infections |
| <i>Staphylococcus epidermidis</i> | Skin and systemic infections |
| <i>Mycobacterium tuberculosis</i> | Tuberculosis |
| Gram-negative bacteria | |
| <i>Escherichia coli</i> | Nosocomial infections |
| <i>Klebsiella pneumoniae</i> | Pneumonia, other infections |
| <i>Pseudomonas aeruginosa</i> | Infection of wounds, burns |
| Fungi | |
| <i>Candida albicans</i> | Diaper rash, digestive and urogenital tract infections |
| <i>Trichophyton rubrum</i> | Athletes' foot |
| <i>Aspergillus fumigatus</i> | Allergic and invasive aspergillosis |
| <i>Chaetomium globosum</i> | Rot and mildew of materials |
| Viruses | |
| <i>HIV</i> | AIDS, cancer |
| <i>Hepatitis</i> | Inflammation of liver, jaundice |
| <i>Hanta</i> | Respiratory failure, 50% fatality rate |
| Protozoa | |
| <i>Trichomonas vaginalis</i> | Acute vaginitis or urethritis |
| Prions | Spongiform encephalopathies (e.g., mad-cow disease) |

negative bacteria have been and continue to be implicated in a variety of infections and highly pathogenic diseases. *Staphylococcus aureus* is responsible for many nosocomial (hospital-acquired) and non-nosocomial infections. It is usually one of the most frequently evaluated microorganisms for its resistance to biocides and antibiotic. New resistant *staphylococcal* strains, especially methacillin-resistant, are highly contagious and aseptic conditions are required when this type of infection is present. The recurrence of tuberculosis in HIV-infected and other immuno-compromised persons requires similar concern in hospitals and other confined environments. *Pseudomonas aeruginosa* is a particularly opportunistic gram-negative pathogen that frequently occurs in wounds and burns, but also is commonly acquired in confined environments such as hospitals, prisons and nursing homes. Although *Staphylococcus epidermidis* is parasitic rather than pathogenic, this bacterium, along with *Staphylococcus aureus*, has been implicated in various types of nosocomial infections with medical devices such as sutures and catheters. Certain strains of the gram-negative bacterium *E. coli* may lead to pathologies such as hemorrhage (with the rare Enterohaemorrhagic strain called EHEC), septic shock, non-healing wounds and infections of the skin and of respiratory and urogenital tracts. *Klebsiella pneumoniae* can readily cause pneumonia and other infections.

A wide variety of fungi are pathogenic and/or caused damage and discoloration to fibrous and polymeric substrates. *Candida albicans* has been isolated in cases of diaper rash and a variety of digestive and urogenital tract infections. *Trichophyton rubrum* and other *Trichophyton* species are known to cause athletes' foot and other skin disorders. *Aspergillus fumigatus* has been determined to be the cause of allergic reactions and asthma as well as more dangerous infections of the lungs (called invasive aspergillosis). *Aspergillus niger* and *Chaetomium globosum* were recognized many decades ago as two of the most opportunistic fungi in the degradation of cellululosic fibers.

Viruses and other less well known microorganisms have received much attention because of their ability to be transmitted by fomites and producing deadly and disabling diseases such as HIV, various forms of hepatitis and more recently hanta virus. Protozoa such as *Trichomonas vaginalis* are known to cause acute vaginitis and urethritis. Prions (small proteinaceous particles which resist inactivation by procedures that modify nucleic acids) have caused much concern in the United Kingdom because of their ability to cause spongiform encephalopathies (most publicized was "mad-cow" disease that is technically know as BSE or bovine spongiform encephalopathy) and their modes of transmission are not well characterized nor understood.

Attachment and Viability of Microorganisms on Surfaces of Materials

Fixation or attachment of microbes to polymers and fibrous surfaces and their viability (or persistence) after attachment to the surface have recently received increased attention. Their attachment and persistence are well-recognized in the mechanisms for microbial growth and transmission of disease. Exploratory studies in the early 1920's

determined that infiltration by fungal hyphae led to depolymerization and rot/mildew of fibrous cellulosic substrates (such as rayon and cotton). These observations were followed by extensive work by the U.S. Army Quartermaster Corps to prevent this phenomenon by applying mildew-resistant agents to tents and other materials exposed to weathering in primarily hot and humid climates [6]. Facile and rapid test methods for early detection of microbial attack on polymeric materials have been devised that rely on chemical changes caused by enzymes released by microorganisms such as production of ergosterol by specific fungi and of ammonia by urealytic bacteria [7]. The fixation of microorganisms to all types of surfaces (including polymeric substrates) is critically discussed and reviewed in a comprehensive monograph that focuses on the physical chemistry of surfaces [8]. An illustration of this attachment is shown in the four step sequence in Figure 1 [9]. Initial approach of microorganisms to a surface may occur by the processes of diffusion, convection or active movement (latter usually referred to as Brownian motion). Initial steps in the adhesion of microbes have been described by colloid chemical theories such as the DLVO theory in which the Gibbs energy is a function of the distance between the microbe and the surface and the overall interaction is the sum of electronic and Van der Waals interactions between the microbes and a solid surface. Separation distance and ionic strength are the two most important parameters that will predict initial adhesion of the microbe to a surface [9]. This adhesion occurs when a primary irreversible energy minimum is achieved between the microbes and the surface. Attachment of the microbes to the surface is then achieved by formation of a polymer or fibrils to the surface. Subsequent colonization of the microorganisms rapidly leads to the formation of a biofilm or microcolonies on the material's surface. Although viruses adsorb rather than adhere to surfaces, their attraction to surfaces may be described by the same chemical colloidal theories used to describe attraction of bacteria and fungi to surfaces.

Costerton and his colleagues [10] have reviewed the information on bacterial biofilms including his recognition of this phenomenon in the late 1970's. A partial, but extensive, list of human infections involving biofilms is given. These include a variety of gram-positive (such as *staphylococci* and *streptococci* species) and gram-negative bacteria (such as *E. coli* and *P. aeruginosa*) that have been isolated from musculoskeletal infections, necrotizing fasciitis ("flesh-eating bacteria) and acquired in hospitals and other confined environments (with sutures, catheters, vascular grafts, prostheses and orthopedic devices). Bacterial biofilms are groups of bacterial colonies organized within an extensive mucopolysaccharide exopolymer called the glycocalyx. They are structured in such a manner that they contain channels in which nutrients can circulate and in which cells in different regions of the biofilm can exhibit different patterns of gene expression. It is currently thought that biofilms are analogous to tissues of higher organisms and their sessile (permanently attached to a substrate) bacterial cells are much less susceptible to antibiotics and biocides than nonattached planktonic cells [10]. The biofilm resistance to antimicrobial agents is complex and will be discussed in some detail later in the section on microbial resistance.

The persistence or viability of representative bacteria, fungi and viruses on fibrous

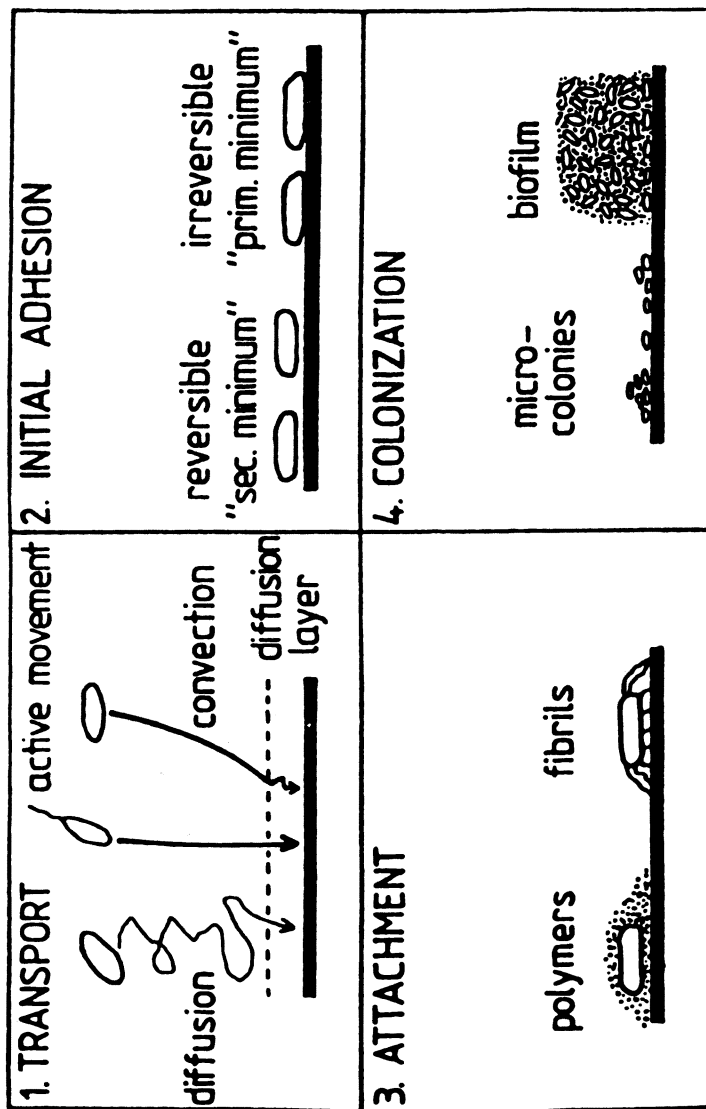


Figure 1. Schematic representation of the sequencing steps in the colonization of surfaces by microorganisms

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and polymeric substrates has been critically reviewed by the author [11,12]. Unfortunately, there have been very few studies in this area since 1975 when it was demonstrated that most fibrous polymers were conducive to microbial growth and viability (with bacteria, fungi and viruses) and thus none were inherently microbiostatic nor microbicidal. Aerosol contamination of fibrous surfaces at low relative humidity was optimum for persistence of bacteria and pathogenic viruses while solution contamination under high humidity conditions resulted in lower persistence of the microorganisms. Viability is somewhat related to difficulty in decontamination and it is well known that most forms of hepatitis are highly persistent and not removed by germicides that readily remove other viruses and most bacteria and fungi from surfaces. Sterilization and the accompanying act of disinfection are discussed in the next section.

Effectiveness of Biocides and Antibiotics Against Microorganisms

There are several ways of classifying biocides and antibiotics. The classification in this section is based on (a) the extent to which the microbes are killed and destroyed, (b) the scope or breadth of microbial action which the agents exhibit and (c) the one or more microbial functions that are disabled or attacked by the agent to exhibit static or -cidal activity.

Sterilization of materials is the most hygienic and dramatic interaction between antimicrobial agents and the host microbes. Most sterilization methods may be classified as either physical (heat, filtration, ultraviolet and ionizing radiation) or chemical (gaseous and liquid agents) and have been comprehensively and critically reviewed [12].

Pressurized steam is the oldest and most frequently used method. However, "cold" sterilization must be employed if plastics, films, elastomers or fabrics are damaged by heat, moisture or that combination. High energy radiation has become more frequently used to sterilize polymeric materials such as bandages, plastic syringes, catheters, gloves and sutures.. However, cold sterilization may have to be considered as an alternative technique if the ionizing radiation causes unacceptable loss in mechanical and related properties. Cold sterilization methods normally employ gaseous ethylene oxide and to a lesser extent propylene oxide, formaldehyde and CO₂. The carcinogenic nature of ethylene oxide and implications that formaldehyde may cause nasal tumors has led to strict standards in their use (particularly ethylene oxide). Glutaraldehyde (in liquid form) is being increasingly used as an alternative broad-spectrum cold chemosterilant because of its less hazardous nature, rapid inactivation of all forms of microbes and its lack of deleterious effects on most materials. Sterilization by filtration is employed primarily to remove pathogenic and other unwanted microorganisms in liquid and/or air in the manufacture of diverse products such as antibiotics, vaccines, cosmetics, and industrial gases. Filters used are a variety of polymeric membranes, films and fibrous materials.

Microorganisms may be classified with regard to their difficulty in being removed

from decontaminated surfaces by disinfectants or antiseptics. A useful disinfection scale for most representative microorganisms is shown in Table 3. Class A is the most susceptible to and Class F the least susceptible to prototype biocides [13]. It is well documented that spore-forming microorganisms are generally harder to remove than non-spore forming microorganisms. The table also shows a broad response to inactivation of viruses that range from quick inactivation of the HIV virus to very difficult inactivation of hepatitis A on environmental surfaces. This is exemplified with only 3 of 20 common disinfectants (hypochlorite, glutaraldehyde and a quaternary ammonium salt containing sizeable amounts of HCl) able to inactivate surfaces contaminated with hepatitis A [14]. Protective clothing has been recommended for exposure to prions that can contaminate surfaces and transmit a variety of lethal and insidious spongiform encephalopathies [15]. Furthermore, it has been documented that these proteins are even more difficult to inactivate than most hepatitis viruses. The prions have been shown to be resistant to agents such as glutaraldehyde, oxidizing agents, iodine, strong acid, proteinases, ultraviolet light, ionizing radiation at usable doses; only partial inactivation occurs on heating them at temperatures as high as 360°C. The only chemical agents that are currently known to be completely effective against prions are concentrated solutions of strong alkali [15,16].

Conversely, antimicrobial agents and antibiotic may be classified with regard to their ability to inhibit microbial growth (static) or kill microbes (-cidal) and the breadth of their effectiveness against many types of microorganisms. If a biocide or antibiotic is effective against many classes of microorganisms, it is known as a "broad-spectrum" antimicrobial agent. Russell and Russell [16] have classified biocides used over the past 50 years with respect to their breadth of antimicrobial activity.

Surfactants, most antibiotics, formaldehyde-releasers and biguanides, bisphenols and silver compounds are only effective against non-spore forming gram-positive (G+) and gram-negative (G-) bacteria. Alcohols, imidazole derivatives, chlorhexidine, isothiazolones, phenolic antioxidants and quaternary ammonium salts are antifungal (F) in addition to be effective against non-spore forming bacteria (G+ and G-). Alkylating agents (such as ethylene oxide), glutaraldehyde and succinaldehyde-based products, chlorine-release agents, iodophors and peroxygen agents have the broadest activity of all and are also effective against spore-forming microbes (S), viruses (V) and in some cases even protozoa (P) in addition to being effective against bacteria and fungi.

Another way of classifying antimicrobial agents and antibiotics is by the single or multiple modes of action that they exhibit to inhibit or kill microorganisms. These modes have been critically reviewed in two book chapters [16,17]. Cellular targets of antimicrobial action (primarily bacteria and fungi because viruses and prions do not have metabolic functions) include the cell wall, outer membrane, inner or cytoplasmic membrane, interference with protein synthesis, binding of nucleic acids, oxidation of -SH groups, reaction with amino groups, promotion of leakage of low molecular-weight intracellular constituents such as potassium salts and amino acids and disruption of viable electron transport and proton motive forces (pmf). The mechanistic categories are shown in Figure 2. It is important to note that some biocides and sterilants (particularly

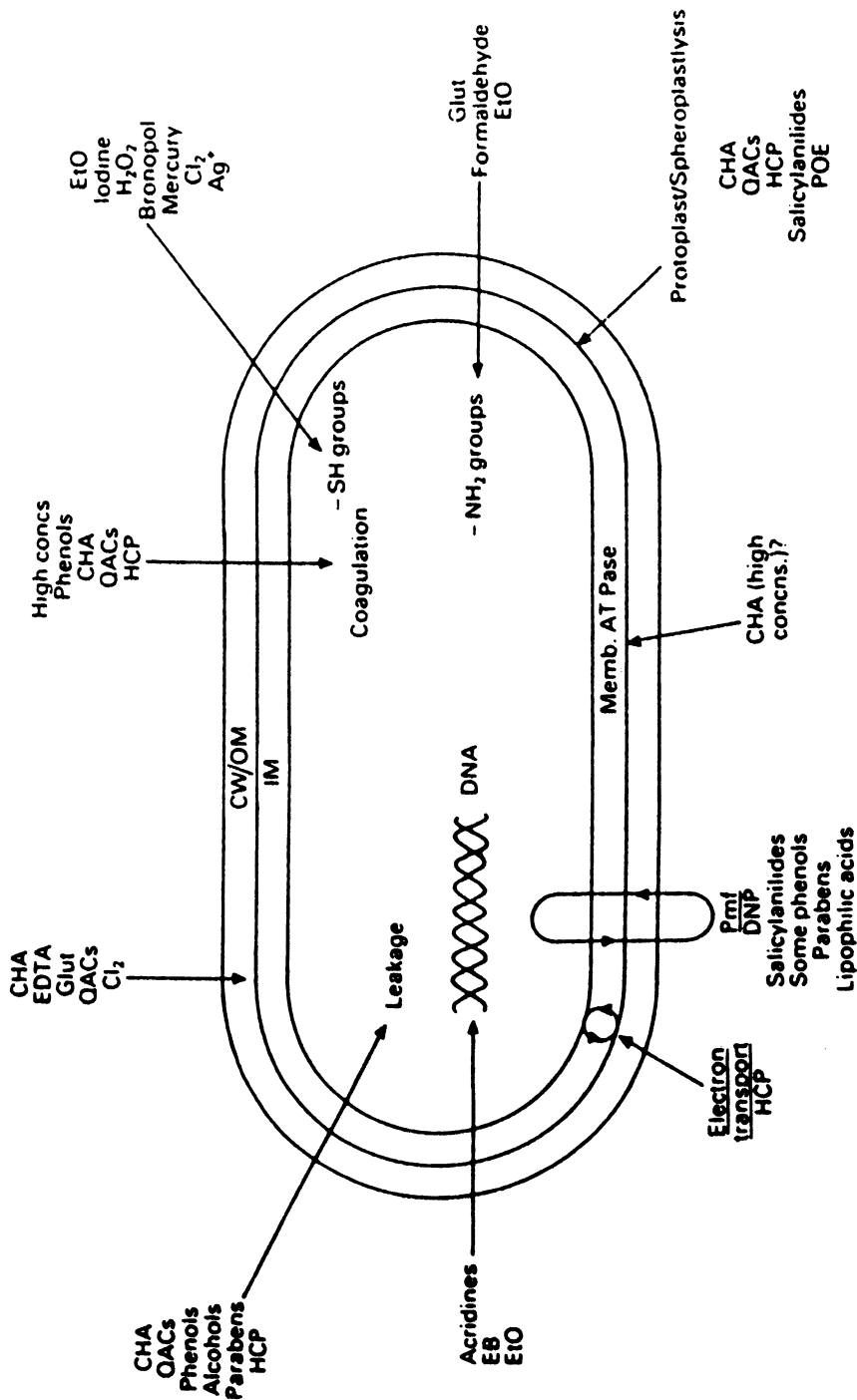


Figure 2. Target sites and effects of biocides. Many biocides have more than one target site and effects may be concentration dependent. Abbreviations: EDTA, ethylenediamine tetraacetic acid; Glut, glutaraldehyde; CHA, chlorhexidine diacetate or gluconate; QACs, quaternary ammonium compounds; HCP, hexachlorophene; Cl₂, chlorine-releasing agents such as hypochlorite; POE; phenoxyethanol; DNP; dinitrophenol; EB, ethidium bromide; EO, ethylene oxide. CW, cell wall (Gram-positive); OM, outer membrane (Gram-negative); IM, inner or cytoplasmic membrane; pfm, proton motive force

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Table 3. Approximate Disinfection Scale for All Organisms in Order of Increasing Resistance (Response to Commercial Disinfectants)

| <i>Microbial Susceptibility Group^a</i> | <i>Microorganisms (Dried on Carriers)</i> |
|---|--|
| A | Retroviruses (AIDS), ortho and paramyxoviruses, herpes viruses, vaccinia, corona, other enveloped viruses, gram-negative rods and some filamentous fungi; some gram-positive cocci |
| B | <i>Staphylococcus aureus</i> , some diphasic and filamentous fungi, yeasts and algae, some gram-negative rods, hepatitis B (?) |
| C | Adenoviruses |
| D | <i>Mycobacterium tuberculosis</i> (BCG strain) ^b , rotaviruses, reoviruses, some mold ascospores |
| E | Picornaviruses (polio, rhino) Parvoviruses (SS DNA), Hepatitis A |
| F | Bacterial endospores (<i>Bacillus</i> , <i>Clostridium</i>); viroids |
| G | Prions (chronic infectious neuropathic agents; slow viruses) |

^aExceptions will be found to exist among and between the various susceptibility groups listed in Table 2, but the broad outline of comparative susceptibility has become a basic principle of disinfectant biology.

^bUnfortunately, little information is available on human strains, such as H37Ra, H37Rv, the various scotochromogens, drug resistant forms, *Mycobacterium avium* intracellulare, and species of *M. fortuitum* and *M. chelonii* as well as pathogenic actinomycetes.

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ethylene oxide and glutaraldehyde) have multiple modes of action or target sites that tend to make them much more effective than agents that only have a single mode of action on microbial functions and metabolism.

Long-Term Effectiveness of Biocides and Antibiotics on Polymeric Substrates

Antimicrobial agents containing a variety of functional groups and bioactive cations and anions have been applied to polymeric and fibrous substrates. However, when the requirements for the applications require durable or long-term effectiveness of microbistatic or microbiocidal action, only a few active agents are suitable because they diffuse out the material too rapidly. Applications that require long-term biocidal or biostatic activity are diverse and include items used outdoors that are durable to weathering (combined effect of mildew/UV degradation), intracorporeal devices such as catheters and sutures, protective clothing worn to stop transmission of pathogenic organisms and various apparel and non-apparel items used in hospitals and other confined environments in which the material of interest is durable or semi-durable rather than disposable.

A historical review chronologically documents successful treatments for fabric preservation, application of antibacterial agents to fibrous materials and the associated concepts for achieving long term effectiveness of these treatments [1]. The deleterious effects of fungi and some bacteria on cellulosic fibers were elucidated in the early 1900's, but the first modern day patent for protecting cotton fabric from weathering (by mineral dyeing with lead chromate) was issued in 1884. A subsequent period of intense activity was characterized by the discovery that cellulose acetate was rot-resistant (1920) and by the successful formulation of a variety of durable anti-mildew agents on cotton fabrics (1940-1970) using such agents as chlorinated phenols and metal complexes of 8-hydroxyquinoline in conjunction with waterproofing and/or compounds that protected against ultraviolet light. The advent of many synthetic fibers that were inherently resistant to attack by mildew and rot-producing fungi (especially polypropylene) led to less effort in this area after 1970. In contrast, the concept of antibacterial fabrics was first mentioned in 1941 and the first patent to claim bacteriostatic fabrics [containing bis(halophenols)] durable to multiple laundering did not issue until 1951. A landmark paper by Gagliardi in 1962 described the principles and strategies for antibacterial fiber finishes [18]. In this paper, the methods of applying antimicrobial agents for durability to fabrics were delineated and included homo- and copolymerization, resin treatment, covalent bond formation and the regeneration principle. The latter was formulation of the concept that an inactive chemical species attached to the fiber could be continually regenerated by physical or chemical agents to become biostatic or biocidal. This concept was successfully reduced to practice in only the last few years and will be discussed in detail in the section on new concepts and agents for antimicrobial polymers and materials. The mechanism of controlled release was also explained in this paper and

serves as the basis of most effective antimicrobial agents. Controlled release is the slow and usually steady diffusion of the agent off the fiber in the presence of sufficient moisture to insure that the agent can be maintained on the fibrous or polymeric surface for extended periods of use, yet be effective in providing antimicrobial action. In the early 1970's, two other modes of antimicrobial action for materials were patented and developed. The first development involved application of an inherently biostatic coating based on a silicon polymer with a pendant quaternary ammonium group to a variety of surfaces (Figure 3). To date, this is the only antimicrobial approach that claims to be effective as a biostatic surface rather than the commonly employed controlled release methodologies [19]. An improvement in the antimicrobial effectiveness was made by having two long chain pendant groups in the quaternary ammonium moiety rather than only one disclosed in the earlier patent [20]. The second approach involves microencapsulation in sandwiched structures from reservoirs (Figure 4) that release bactericides in treated mattresses and other materials [21].

In addition to chemical agents and antibiotics, the advent of the blood-borne pathogens (e.g., HIV, hepatitis and hanta viruses and the slow virus prion agents) have necessitated the development of materials that are physical barriers to liquids rather than possess biocidal activity. Materials suitable for this type of protection have been critically reviewed, and include treatment of substrates with water-repellent agents (waxes and fluorocarbons) or microporous coatings that allow transmission of water vapor but not liquids due to unique pore structures [22]. The problem has been to produce gowns, gloves and related items that are thermally comfortable and breathable yet retain a good margin of safety in not allowing penetration (called strikethrough) of pathogenic viruses in liquid media.

Microbial Resistance to Biocides and Antibiotics

The development of strains of bacteria resistant to antibiotics (e.g., *Staphylococcus aureus*) has received much attention in the popular press and the public is generally aware of this phenomenon and its attendant health risk. However, the mechanisms by which microbes exhibit this resistance are even more complex and varied than the modes by which biocides and antibiotics suppress microbial growth or destroy them. As noted in the excellent review on biocides, it has only been in the past 10 to 20 years that we have acquired some understanding on how bacteria and other microorganisms exhibit resistance to antimicrobial agents [16].

Before considering the mechanisms by which microorganisms exhibit resistance, it is well documented that different microorganisms have different responses or susceptibility to biocides. This is commonly known as the MIC or minimum inhibitory concentration that will affect the microbe. For example, hexachlorophene has a MIC of only 0.05 ug/ml with *S. aureus*, 12.5 ug/ml with *E. coli*, but a value of 250 ug/ml with *P. aeruginosa*; conversely, the antimicrobial agent bronopol (2-bromo-2-nitro-1,3-propanediol) has a much narrower MIC range for these three bacteria (31.5 ug/ml for *E. coli* and *P. aeruginosa* and 62.5 ug/ml for *S. aureus*) [16].

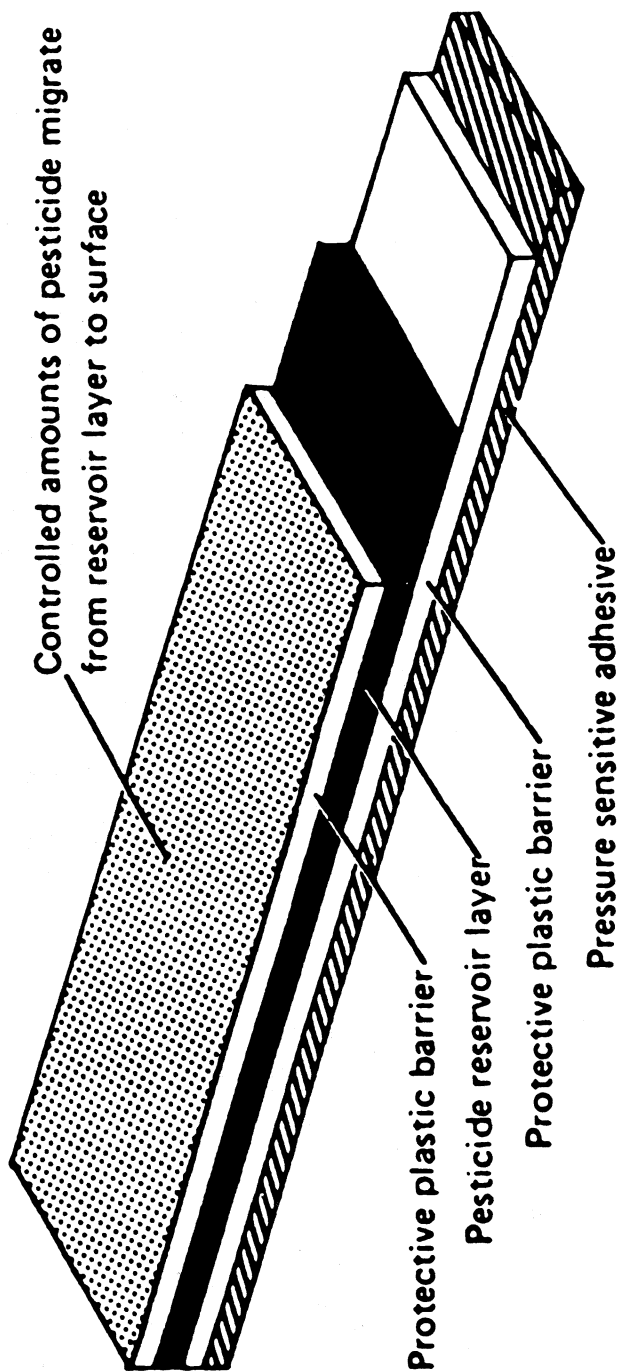


Figure 4. Microencapsulation technique for controlled release of pesticides from a polymeric surface

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Mechanisms of bacterial resistance to antibiotics have received the most attention. However, in the last decade, it has been determined that bacterial resistance to both biocides and antibiotics may occur by one or two general modes: intrinsic (natural or innate) or acquired. Much less information is available on the mechanisms of fungal, viral and protozoal resistance. Intrinsic resistance is known to be greater in mycobacteria (causes diseases such as tuberculosis and leprosy) and bacterial spores (such as *Bacillus subtilis*) due to the waxy material in the cell wall of the former and the coat and cortex in the latter. These structural features impede attack of the biocide, but the specific manner in which they exhibit this resistance is not well understood and is a fertile area for further study. Gram-negative bacteria are usually more resistant to biocides and antibiotics because of the structure and composition of their outer membrane [16]. In addition to these structural aspects, the ability of bacteria to form biofilms as a physical barrier to attack by biocides and antibiotics is another intrinsic form of microbial behavior. At least three hypotheses have been proposed (with varying degrees of experimental verification) to explain why bacterial biofilms are effective in resisting antimicrobial attack: (1) failure of the antimicrobial agent to penetrate the full depth of the biofilm and reach the metabolic and functional components of the bacteria; (2) existence of slow or no growth cells in the biofilm with limited nutrient requirements and thus reduced susceptibility to chemical agents; and (3) some cells adopt a distinct and protected biofilm phenotype, i.e., a biologically programmed response to growth on a surface [10, 23].

Recent studies on biofilms have been concerned with modeling of a biofilm with computers to describe the activity and interaction of antimicrobial agents on the biofilm [24] and investigation of the interactions between bacterial films using attenuated total reflection Fourier transform infrared spectroscopy (FTIR) [25]. Processes incorporated into the computer model were bulk flow in and out of a reactor to simulate the dynamics of a chemostat, transport of dissolved species within the biofilm, external mass transfer, depletion of biocide, growth and death of microorganisms, transport of cells within biofilm, and detachment of biofilm and disinfection. The model is considered useful because it includes previous experimental observations such as rapid disinfection followed by biofilm regrowth, slower detachment than disinfection and reduced susceptibility of microorganisms in biofilms. The FTIR study is believed to be useful in analysis of both bacterial and fungal biofilms.

This analytical data may provide information on transport of the antimicrobial agent to bacteria embedded in the biofilm and interactions between the agent and the biofilm components.

Acquired microbial resistance has been extensively investigated with various antibiotics, but similar studies with biocides are fewer and more recent. It has been confirmed that many biocides can also be rendered ineffective or less effective by acquired microbial resistance. This acquired resistance to both biocides and antibiotics may be genetic and/or biochemical [16, 26]. Chromosomal gene mutation and acquisition of plasmids and transposons by the microbes are the genetic modes that have been observed. As shown in Table I, plasmid-mediated biocide resistance can occur by

**Table 4. Possible Mechanisms of Plasmid-mediated Biocide Resistance
Adapted from [16].**

| <i>Mechanism</i> | <i>Biocide(s)</i> | <i>Comments</i> |
|---------------------------------|--|--|
| Inactivation | Mercury compounds Chlorohexidine Formaldehyde | Hydrolases and reductases May be chromosomally rather than plasma-mediated Formaldehyde dehydrogenase |
| Decreased uptake | Silver nitrate Chlorohexidine QAC's^a | Inactivation unlikely Not proven Not proven |
| Cell surface alterations | Formaldehyde | Outer membrane proteins appear to be involved |
| Efflux | Acridines, QAC's Crystal Violet Chlorohexidine | MRSA^b strains |

^a QAC's = quaternary ammonium salts. ^b MRSA = methicillin-resistant strains of *S. aureus*.

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Cambridge University Press.

biocide inactivation, its decreased uptake, cell surface alterations and efflux (genetic alteration of microbial structure to readily eject incoming biocides). In the cases of biocide inactivation, specific enzymes produced by the microbes are usually involved in the defense mechanism [16]. Acquired resistance has also been classified as biochemical: alteration of a target site by the microbe resulting from changes in enzyme composition and protein structure. This leads to modification of envelope structure or efflux behavior modifying biocidal transport and enzyme modification [26]. However, it should be noted that all three of these mechanisms of acquired resistance can also be caused by genetic mutations as well as simple biochemical changes.

New Developments in Synthetic and Naturally-Occurring Antimicrobial Agents

New antimicrobial agents and modified polymeric materials that have appeared in the last decade may be subdivided into four categories: (a) improved or modified chemical compositions based on classes of agents previously known to impart antimicrobial effects; (b) surface modification to minimize or inhibit microbial growth and possibly biofilm formation; (c) modified surfaces that are rendered bioactive by external stimuli such as specific chemical agents or light and (d) incorporation of naturally occurring antimicrobial substances in fibrous or other polymeric substrates. Representative approaches in each category are shown in Table 5.

There are at least four improved or modified antimicrobial agents that merit discussion and that were applied to fibrous substrates and/or exist in polymeric form. The first is an inorganic ion-exchange material (zeolite) that when treated with various transition metal salts (such as silver and copper) and spun into various synthetic fibers, are effective bactericides and fungicides. Since the appearance of the first patent on this topic in the mid-1980's, numerous related patents and publications in this area have followed [27]. The synthesis of new inorganic hydroperoxides from readily available starting materials (magnesium acetate tetrahydrate and hydrogen peroxide) produced agents with antibacterial activity that could be applied to all types of fibers, but were particularly durable to cotton cellulose. These agents (magnesium hydroperoxyacetate and magnesium dihydroperoxide) discovered by the author and his colleague are environmentally benign and are an improvement over earlier inorganic peroxides (based on zinc and zirconium) that would not be as environmentally beneficial [28]. The antimicrobial activity of quaternary ammonium salts is well known. More recently, Kanazawa and co-workers have developed analogous polymeric phosphonium salts and also bound them to cotton fibers as effective antibacterial agents [29]. As previously noted with quaternary ammonium salts, it is necessary to have one of the phosphonium groups as a long chain substituent (C_{12} - C_{18}). Polymeric biguanides have been known for some time as antimicrobial agents. They have been recently employed for odor

Table 5. New Developments in Antimicrobial Polymers and Fibrous Substrates

| <i>Agent or Structural Change Reference</i> | <i>Category</i> | <i>Reference</i> |
|--|---|------------------|
| Zeolites | Inorganic ion-exchange | 27 |
| Magnesium hydroperoxyacetate | New peroxygen agent | 28 |
| Polymeric phosphonium salts | Similar to nitrogen analogs | 29 |
| Polymeric biguanides | Improvement of similar agents | 30 |
| Polyurethane grafts with acrylic acid | Plasma modification of surface | 31 |
| Crosslinked polyols | Physical barrier to microbial growth | 32 |
| Hyandtoin polymers and agents affixed to fibers | Regenerated by OCl^-; "smart material" | 33,34 |
| Substances containing photosensitive groups | Activated by light; "smart material" | 35, 36 |
| Chitosan | Natural antimicrobial polymer blended with fibrous materials | 37,38 |
| Peptides | Naturally occurring antimicrobials | 39,40 |

control in fabrics by reducing the bacteria count in these materials; however, the degree of microbial reduction claimed is not normally sufficient for them to be considered effective biocides [30].

Another strategy is to prevent or minimize biofilm formation or similar microbial processes and thus produce a polymeric surface with good antimicrobial activity. This has been achieved by grafting acrylic acid on polyurethane films by glow discharge or plasma techniques to reduce bacterial adhesion of *S. epidermidis* from 15% (before grafting) to 1% after grafting [31]. A similar material was produced by the author and a colleague when fibrous substrates were coated with crosslinked polyethylene glycol. The modified surface appears to inhibit growth of many bacteria and fungi (such as *B. epidermidis* and *A. fumigatus*) by changing surface energy to prevent microbial attachment [32].

The regeneration principle formulated by Gagliardi in the early 1960's [18] has finally come to fruition in the past few years by development of fibrous materials and polymeric substrates that are chemically activated by bleach [33, 34] and by light [35, 36]. Conceptually, these may also be considered "intelligent materials," i.e. materials that respond to external stimuli, usually in a reversible manner.

The chemical attachment of hydantoin groups to cellulosic fibers, then subsequent activation by bleach has been reviewed by Sun [33]. As shown in Figure 5, conversion of the N-H to a chloramine bond (N-Cl) by bleach or hypochlorite, provides antibacterial activity and can be regenerated as needed. More recent efforts have been the development of N-halamine polymer coatings that can be coated onto fibrous and other polymeric substrates [34]. Photosensitized dyes and polymers (Rose Bengal in fibrous substrates and polymers containing porphyrin and phthalocyanine structures) were effective bactericides [35, 36]. In both investigations, it was believed that singlet oxygen was the primary chemical species responsible for the biocidal activity. It was not mentioned if this effect was readily reversible.

Since bacteria develop natural and acquired defenses to biocides and antibiotics; another approach has been to apply naturally occurring antimicrobial agents to materials or use them in polymeric form.

Chitosan, the deacetylated form of chitin, is a plentiful and naturally occurring aminopolysaccharide obtained from shellfish and other marine species. Most of the research on applying this to fibers and films has been conducted in Japan and to a lesser extent Korea. Micromilled chitosan powder has been blended with rayon fibers, followed by subsequent lamination, to produce a variety of nonwoven fabrics known as Chitopoly. These modified materials were even effective against a methacillin-strain of *S. aureus* [37]. Numerous other publications and patents describe incorporation of chitosan in various forms to produce antimicrobial fibers and polymers. One of the more recent examples is the binding of a quaternary ammonium derivative of chitosan to cotton fabric to produce an antibacterial finish [38]. The other area of current interest is the use of naturally occurring peptides as antimicrobial agents. The use of combinatorial libraries allows one to systematically examine ten to hundreds of millions of peptides for their antimicrobial activity. This was demonstrated with various strains

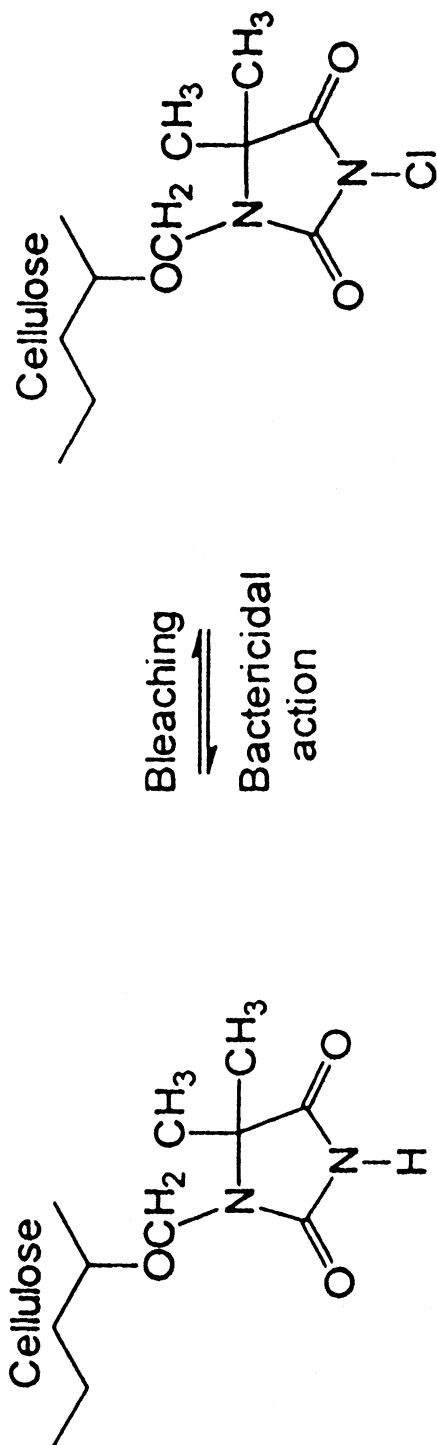


Figure 5. Reversible dehalogenation reaction; active bleaching agent is OCl^-
 (Reproduced with permission from reference 33. Copyright 1994 AATCC.)

of gram-positive and gram negative bacteria [39]. A recent review has critically examined the structure-functional relationships of various antimicrobial peptides and have classified them into five different groups: α -helical and β -sheet secondary structure, cysteine-rich and novel amino acids [40]. High-resolution and solid-state NMR have been invaluable in characterizing antimicrobial peptide structures and peptide-membrane binding mechanisms. Although there is little current activity in binding these antimicrobial peptides to fibers and other polymeric substrates, it is likely that there will be new modified materials as a result of these combinations in future studies.

Challenges, Needs and Opportunities for Development of New Antimicrobial Agents

The aforementioned topics demonstrate both the complexities and opportunities for development of new and effective antimicrobial agents for fibrous and polymeric materials. There are several challenges in their development. These challenges are the production of such materials that are cost-effective, environmentally benign, have selective toxicity to the microbes and not to mammalian and other species, and design of the agent/material to minimize or prevent the microbes from exhibiting their innate or acquired resistance.

The need for such materials can be justified for several reasons. First, it has been mentioned quite frequently in the popular press that several eminent biologists believe that most diseases (including major ones such as cancer, cardiovascular, pulmonary, diabetes, arthritis, etc.) are caused or precipitated in one manner or another by microbial infection. Second, the proliferation of the world's population and the more rapid transmission of diseases by frequent and faster travel, make epidemics more likely and severe. Third, if the earth is trending toward global warming, then the number of pathogenic organisms and their transmission will increase dramatically because most disease-causing microbes grow rapidly in warm, humid environments. Finally, the increasing standard of living in the world will lead to more stringent public health and medical requirements for sterile, decontaminated and antimicrobial materials.

Opportunities to meet the above challenges are numerous. A few are worth mentioning that reflect the current rationale for success in this area. In a recent article in Fortune magazine [41], some of the approaches mentioned were chemicals that disorient biochemical signals to microbes, compounds/polymers that trick the microbes into attaching to fake copies of carbohydrate receptors on cell surfaces, and anti-adhesion compounds that block construction of microbial cell walls. Russell and Russell [16] believe that we need more information on how fungi, protozoa and families or classes of viruses exhibit innate and acquired resistance to biocides and antibiotics. They also note that more agents that are effective in inactivating prions are needed. This author would like to see the development of "intelligent" or "smart" antimicrobial agents that will emit lethal or effective doses of an agent based on changes in microbial growth

that is conducive to the disease or malady that the microbe causes. From another perspective, the antimicrobial agent/material would have “stimulus/response” resistance to microbial growth rather than succumb to the inherent or acquired resistance of the microorganism. It is also appropriate to consider the development of “designer” antimicrobial agents, i.e., agents that are effective in the microclimate or microenvironment in which the material is used or effective against certain microorganisms present in confined environments or on certain parts of the body or effective for specific products and applications. Hopefully, a better understanding on how microbes survive will evolve in conjunction with the development of new and effective antimicrobial agents and materials.

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Chapter 12

Determining Antimicrobial Efficacy and Biocompatibility of Treated Articles Using Standard Test Methods

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Many articles are treated with antimicrobials to kill or inhibit the growth of bacteria and/or mold. Treated articles are subject to a variety of test methods used to confirm antimicrobial activity. Some of these articles come in contact with the human body and therefore must be tested in order to assess their biocompatibility under conditions of intended use. Determining the efficacy and biocompatibility of these treated articles is crucial.

Introduction

Antimicrobials have been around for many years, but never as prevalent in the marketplace as they are today. Just walk into your nearest discount store and you might find many articles that have antimicrobial treatments (e.g., pillows, bed covers, towels, wipes, hand soap, cutting boards, tooth brushes, mop heads, etc.). When you book your next vacation ask the hotel if their sleeping rooms have antimicrobial treated shower curtains, carpet, bed spreads etc. Surprise! treated articles can also be found here. On the other hand, pay attention the next time you find yourself in the hospital or visiting a loved one, you will find a number of treated articles in this environment. (e.g., O.R. drapes, surgical gowns, medical devices, gloves, bed linens, curtains, chair upholstery, carpet etc.)

Antimicrobial agents are added to these articles for several reasons: to reduce the spread of pathogenic organisms, to prevent unsightly appearance of mold and mildew, to reduce device related infections, or to eliminate odors caused by bacteria and mold.

The demand for these antimicrobial treated articles has increased as has the awareness of the governing bodies such as the United States Environmental Protection Agency (EPA) and the Food and Drug Administration (FDA). The EPA has set up an Antimicrobials Division under the Office of Pesticide Programs and are in the process of providing guidelines for the antimicrobial industry. The FDA as always, oversees new or changed devices that enter the market to ensure that they are safe and effective. Currently, the issue of jurisdiction is a confusing one as manufacturers try to meet the requirements of both agencies.

Test Methods

There are a number of test methods that can be used to determine antimicrobial efficacy as well as biocompatibility of treated articles. Standards setting organizations such as the American Society for Testing and Materials (ASTM) and American Association of Textile Chemist and Colorist (AATCC) provide standard test methods that can be used to show antimicrobial efficacy of treated articles. There are also other efficacy test methods that fall outside the purview of these two organizations that are very effective. The ISO Technical Committee 194 has developed the ISO 10993 biocompatibility series which address the biological evaluation of medical devices including standards for those treated with antimicrobials.

Table I. Antimicrobial Test Methods: AATCC, ASTM and Others

| | |
|-----------------------------|--|
| AATCC 30, Part 1, 2, and 3 | 0923 Dow Corning Shake Flask |
| AATCC 100 | Kirby-Bauer Antimicrobial Susceptibility |
| AATCC 147 | New York State 63 |
| AATCC 174, Part 1, 2, and 3 | ASTM E 1428-99 |
| ASTM G-21 | |
| ASTM G-22 | |

Table II. Biocompatibility Test Methods

| |
|----------------------------|
| ISO 10993-5 Cytotoxicity |
| ISO 10993-10 Sensitization |
| ISO 10993-10 Irritation |
| Others |

Test Methods: Efficacy

Each antimicrobial agent has its own unique chemistry and method of delivery in the article treated. Choosing the best method to evaluate the effectiveness of the

antimicrobial treatment is very important. Before deciding which test method should be used, one must consider: are quantitative or qualitative data needed? Where will the product be used and what is its purpose? Will the product be laundered during the life time of usage and will efficacy testing be performed before and after washing? What is the antimicrobial treatment? What claims will the product have? Other than the antimicrobial does the product have topical treatments to prevent absorption of liquids or soiling? The answers to these questions will help indicate the best test methods needed.

Quantitative

Choosing a quantitative (measurement of bactericidal activity of microorganisms) versus qualitative (inhibition of multiplication of microorganism) test limits the number of applicable methods to consider.

For example if a quantitative method is preferred, applicable test options include AATCC Test Method 100, or 174 part 2 which challenge the product with two organisms. Effectiveness is determined after 24 hours of contact. The Dow Corning Shake Flask, which challenges the product with one organism and effectiveness is determined after 1 hour or 24 hours of contact. The New York State 63, used for non absorbent products, which challenges the product with two organisms and effectiveness is determined after 24 hours of contact.

The inoculum of these methods ranges from 1.0×10^4 to 1.0×10^5 cfu/ml. Percent kill is determined after a specified incubation time as shown in table III, IV, and V.

Qualitative

Qualitative methods are quick tests that are sometimes used as a screen to determine if there is any antimicrobial activity prior to performing a quantitative test. If a qualitative method is preferred test options include: AATCC 147, (Figure 1 & 2) and 174 Part 1 (Figure 3) which challenge the product with two microorganisms. Effectiveness is determined by growth or no growth around the product being tested. Kirby-Bauer Antimicrobial Susceptibility Test (Figure 4 & 5), which will show the same type of results as AATCC 147 and 174 Part 1, differing only in the inoculation of the agar and organisms tested; AATCC 30 Part 3, Part 4, and 174 Part 3 (Figure 6 & 7) are tests which challenge products with fungi. Effectiveness is determined by growth or no growth on the products; ASTM G-22 and G-21 which challenge products with bacteria and fungi respectively with effectiveness determined by growth or no growth on the product; and ASTM E1428-99 Pink Stain Test (Figure 8 & 9) which challenge the product with the organism *Streptovorticillum reticulum* with effectiveness determined by growth or no growth around the product.

Environment

The environment (e.g., moist/warm areas, shower, outside/in the body) where a product will be used plays a significant role in determining the most suitable test

Table III. AATCC Test Method 100 or 174 Part 2

| | | |
|-------------------------|---|-----------|
| Test Organism: | <i>Staphylococcus aureus</i> | ATCC 6538 |
| | <i>Klebsiella pneumoniae</i> | ATCC 4352 |
| Sample Size: | 48 mm diameter disk | |
| Number of Layers: | 1 | |
| Neutralizer: | Lethen Broth | |
| Target Inoculum Level: | (1-2) x 10 ⁵ CFU/ml | |
| Inoculum Concentration: | <i>S. aureus</i> = 1.5 x 10 ⁵ CFU/ml | |
| | <i>K. pneumoniae</i> = 1.6 x 10 ⁵ CFU/ml | |

| S.aureus Results (CFU/ml) | | |
|---|-----------------------|-----------------------|
| Zero Contact Time | 24 Hour Contact Time | Percent Reduction (%) |
| Test Sample A - 1.5 x 10 ⁵ | 1.0 x 10 ² | 99.93 |
| Test Sample B - 1.1 x 10 ⁵ | 1.0 x 10 ² | 99.91 |
| Untreated Control - 1.3 x 10 ⁵ | 2.0 x 10 ⁶ | No Reduction |

| K.pneumoniae Results (CFU/ml) | | |
|---|-----------------------|-----------------------|
| Zero Contact Time | 24 Hour Contact Time | Percent Reduction (%) |
| Test Sample A - 1.4 x 10 ⁵ | 1.0 x 10 ² | 99.93 |
| Test Sample B - 1.6 x 10 ⁵ | 1.0 x 10 ² | 99.94 |
| Untreated Control - 1.5 x 10 ⁵ | 2.5 x 10 ⁶ | No Reduction |

Table IV. New York State 63

| | |
|-------------------------|--|
| Sample Size: | 1" square with rounded corners |
| Neutralizer: | Lethen Broth |
| Test Organisms: | <i>Staphylococcus aureus</i> ATCC 6538 <i>Klebsiella pneumoniae</i> ATCC 4352 |
| Target Inoculum: | (1 - 9) X 10 ⁴ CFU/0.2 ml |
| Inoculum Concentration: | <i>S. aureus</i> = 2.5 x 10 ⁴ CFU/0.2 ml <i>K. pneumoniae</i> = 2.1 x 10 ⁴ CFU/0.2 ml |

| Replicate # | 24 Hour Organism Count | | Percent Reduction | |
|-------------|------------------------|-----------------------|-------------------|----------------------|
| | <i>S. aureus</i> | <i>K. pneumoniae</i> | <i>S. aureus</i> | <i>K. pneumoniae</i> |
| 1 | 1.0 x 10 ² | 1.0 x 10 ² | 99.60 | 99.95 |
| 2 | 1.0 x 10 ² | 1.0 x 10 ² | 99.60 | 99.95 |
| 3 | 2.3 x 10 ³ | 3.1 x 10 ³ | 90.80 | 99.85 |
| 4 | 7.0 x 10 ² | 7.0 x 10 ² | 97.20 | 99.67 |
| 5 | 3.2 x 10 ³ | 2.2 x 10 ³ | 87.20 | 99.90 |

Table V. Dow Corning Shake Flask

| | |
|------------------------|--------------------------------|
| Sample Size: | 0.75 ± 0.01 g |
| Test Organism: | <i>Klebsiella pneumoniae</i> |
| Target Inoculum Level: | (1-2) x 10 ⁴ CFU/ml |

| Sample Identification | Organism Count (CFU/ml) | | Percent Reduction |
|-----------------------|-------------------------|-----------------------|-------------------|
| | Zero Time | One Hour | |
| A - Test Sample | 1.5 x 10 ⁴ | 5.8 x 10 ¹ | 99.61 |
| B - Untreated Control | 1.5 x 10 ⁴ | 1.6 x 10 ⁴ | No Reduction |

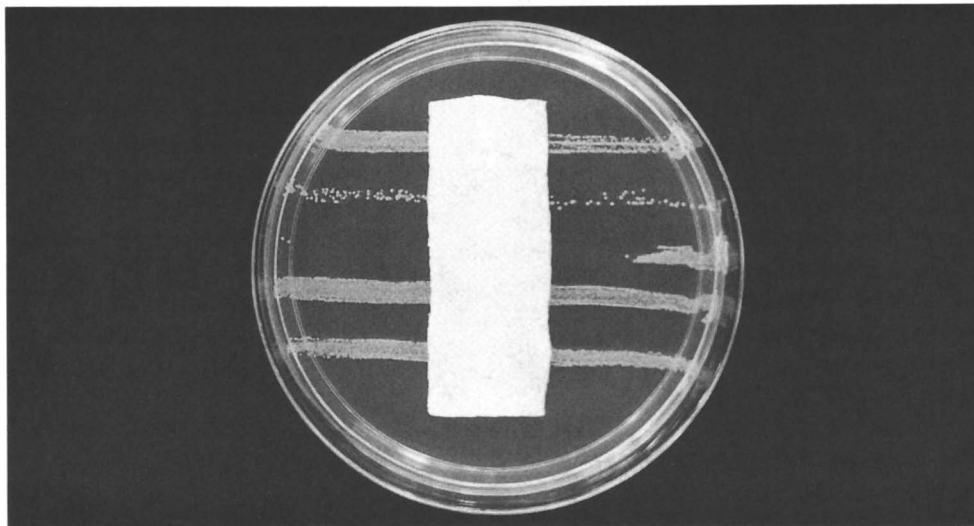


Figure 1. AATCC 147, Untreated, Staphylococcus aureus, showing bacterial growth up to and under a control test article

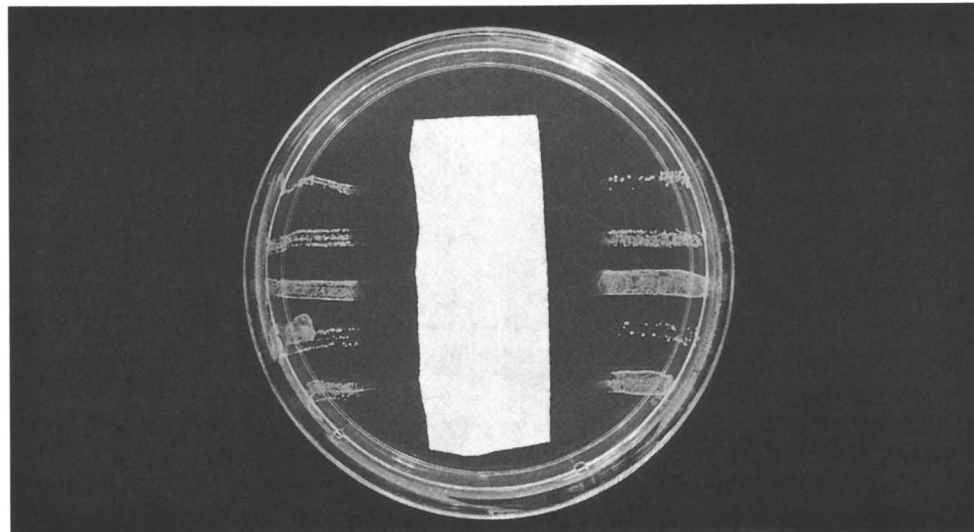


Figure 2. AATCC 147, Antimicrobial Treated, Staphylococcus aureus, showing inhibition of bacterial growth around the test article in the streak line

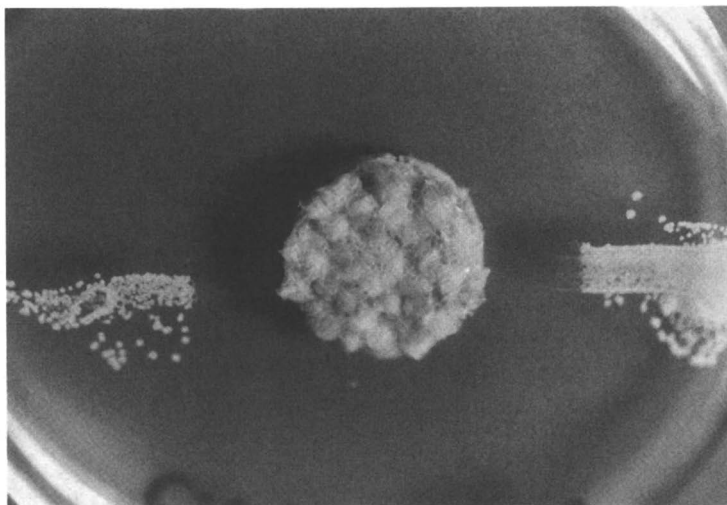


Figure 3. AATCC 174 Part 1, Antimicrobial Treated, Staphylococcus aureus, showing inhibition of growth around the test article in the streak line

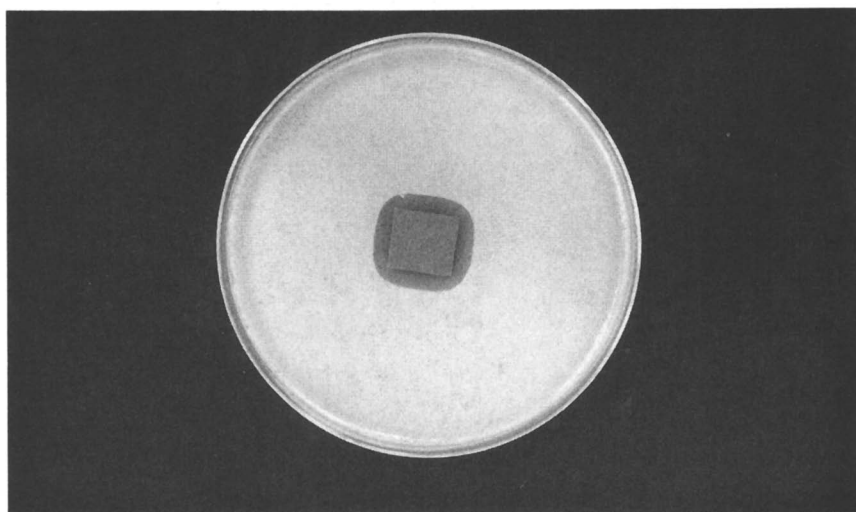


Figure 4. Kirby-Bauer Antimicrobial Susceptibility Test, Klebsiella pneumoniae, showing inhibition of bacterial growth around the test article

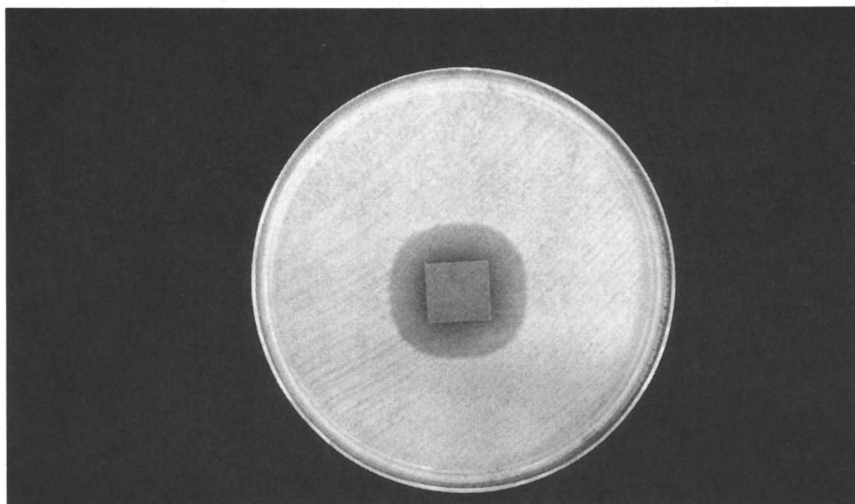


Figure 5. Kirby-Bauer Antimicrobial Susceptibility Test Staphylococcus aureus, showing inhibition of bacterial growth around the test article

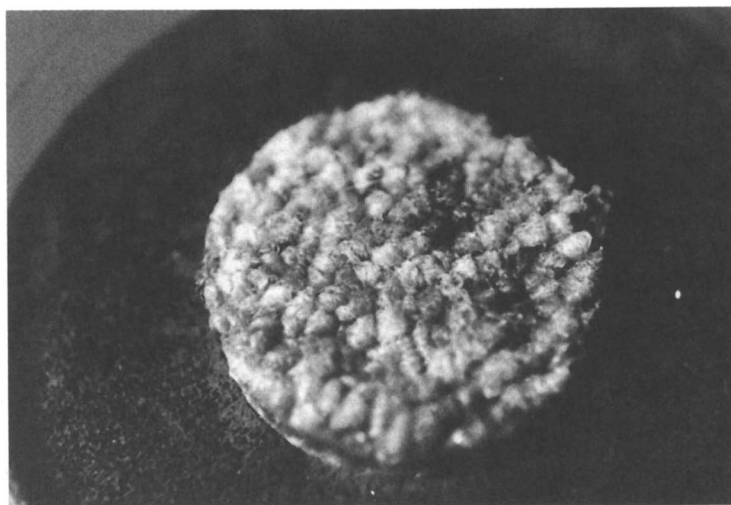


Figure 6. Kirby-Bauer Antimicrobial Susceptibility Test, Staphylococcus aureus, showing inhibition of bacterial growth around the test article

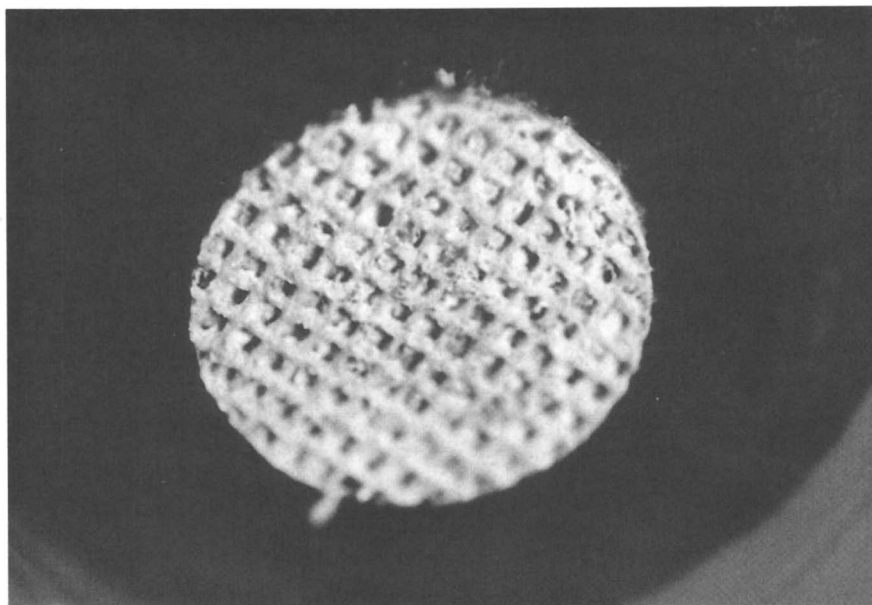


Figure 7. AATCC 174 Part 3, Backing, Aspergillus niger showing growth of fungi on the backing of a carpet sample

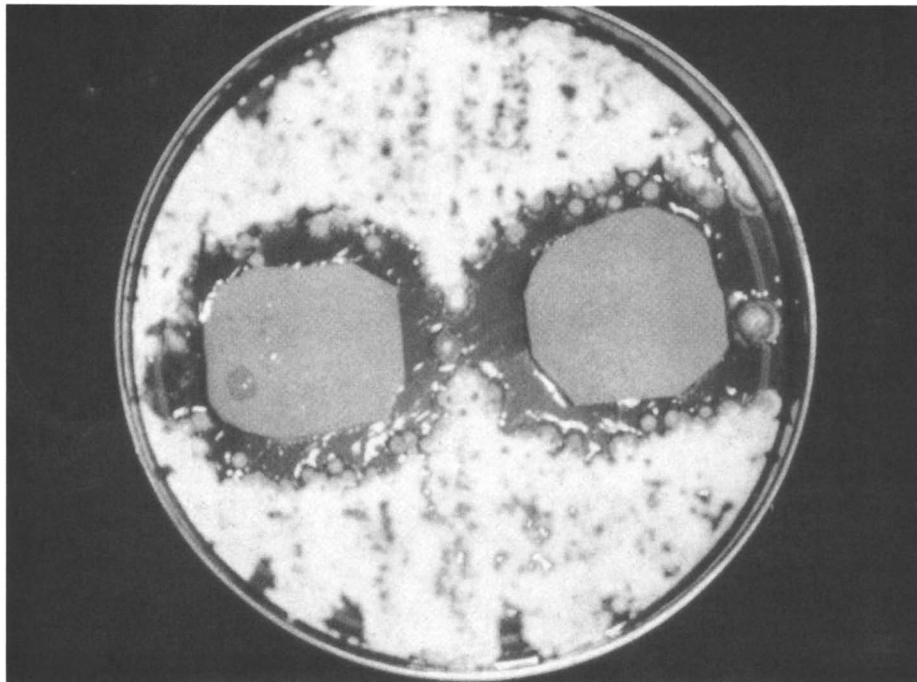


Figure 8. ASTM E1428, Pink Stain Test, Treated, Streptovericillium reticulum, zone of inhibition of fungi around the test article

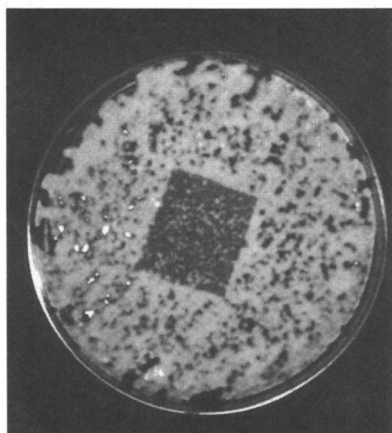


Figure 9. ASTM E1428, Pink Stain Test, Untreated, Streptovericillium reticulum, growth of fungi around and on the test sample

method for the product. Also important are the organisms used to challenge the product. For example a textile used on outdoor furniture is usually exposed to moisture, humidity and cold and/or warm temperatures. The probability of this product being contaminated by mold is very high. A test method like ASTM G-21 that exposes fabric to a mixture of molds with high humidity and moderate temperature would replicate in-use conditions and detect effectiveness of the fungicide treatment under such conditions. Another example is a medical device such as a catheter, with both interior and exterior pathways, which is inserted as a sterile device but has an antimicrobial coating to inhibit the growth of bacteria that may be introduced as it is inserted into the body. Once organisms come in contact with a moist, warm environment infection can be established. A test method such as the Kirby-Bauer Antimicrobial Susceptibility Test that challenges the treated catheter with infectious bacteria, incubated at body temperature (37° C), simulates in-use conditions and demonstrates the efficacy of the antimicrobial treatment.

Laundering

Most textiles are laundered during the lifetime of the product. Accordingly, it is recommended that the product be tested before and after laundering to determine antimicrobial effectiveness. The number of times that a product is laundered and tested should be determined according to how the product will be used. For example if the product is a hospital bed linen then it will probably be laundered every day or every other day. The effectiveness of the antimicrobial should be determined to see how many wash cycles the bed linen can go through and still maintain antimicrobial effectiveness and meet product claims.

Antimicrobial Agent

The antimicrobial agent used on the product is another important factor in designing an appropriate test program. One method is not suitable for all antimicrobial agents. Due to the unique chemistry of a treatment and the mechanics of a given test method, it is important to ask the antimicrobial agent supplier which test method they recommend to qualify the antimicrobial product and to understand the intended use of the product. Relying the supplier recommendation and knowing the intended used of the product can save time and money, especially if the antimicrobial agent is new to the market.

Product Claims

A review of EPA regulations for treated articles should be viewed prior to making any label claims. A copy of these regulations will soon be available through the Antimicrobial Division of EPA. Once it has been determined what product claims will be made, a test program can be designed to prove those claims. For example, if the product literature or packaging claims the antimicrobial product is effective against fungi, *Escherichia coli*, and *Staphylococcus aureus*, then tests performed should challenge the product with these microorganisms to prove antimicrobial

effectiveness. If a product is in the research and development stage and label claims are not available, then knowing where the product will be used is helpful.

Finishes

Finally, modifications to standard test methods must sometimes be made. This is especially true if a product has finishes that prevent water absorption or soiling. These treatments can mask the true efficacy of the antimicrobial product by hindering direct contact between the challenge organisms and the antimicrobial treatment. Modifications might include pre-wetting the sample with a non-ionic wetting solution or sterile deionized water, or laundering prior to testing.

Test Methods: Biocompatibility

A number of important characteristics must be taken into consideration in addition to the efficacy of the products antimicrobial treatment. Biocompatibility is one of those characteristics. According to the International Organization for Standardization (ISO) 10993-1, Biological Testing of Medical and Dental Materials and Devices, all device materials must undergo cytotoxicity, sensitization and irritation testing as a minimum.

It is imperative to determine if the antimicrobial and the device are already in clinical use with safety data on file. If no data is available the antimicrobial and the device must go through a full toxicology work up prior to mating them together. Once this data is available and the two are combined, then only clinically relevant tests must be performed.

Cytotoxicity

Cytotoxicity (ISO 10993-5) is a good first step toward ensuring biocompatibility of a medical device that has an antimicrobial treatment. It is a rapid, standardized, sensitive, and inexpensive means to determine whether a material contains significant quantities of biologically harmful extracts. The high sensitivity of the cytotoxicity test is due to the isolation of the test cells in cultures and the absence of the protective mechanism that assist cells within the body. In standard cytotoxicity test methods, cell monolayers are grown to near confluence in flasks and are exposed to test or control articles directly or indirectly by means of fluid extracts. In the elution test method, which is widely used extracts are obtained by placing the test and control articles in separate flasks of culture media under standard conditions. Each fluid extract obtained is then applied to a cell culture monolayer replacing the media that had nourished the cells. The cells are incubated and examined periodically under the microscope. Cells are observed for visible signs of toxicity. (Figure 10 & 11)

Alternatively, samples of test and control articles can be applied directly to monolayers of cells covered with nutrient medium or to a semi-solid nutrient agar

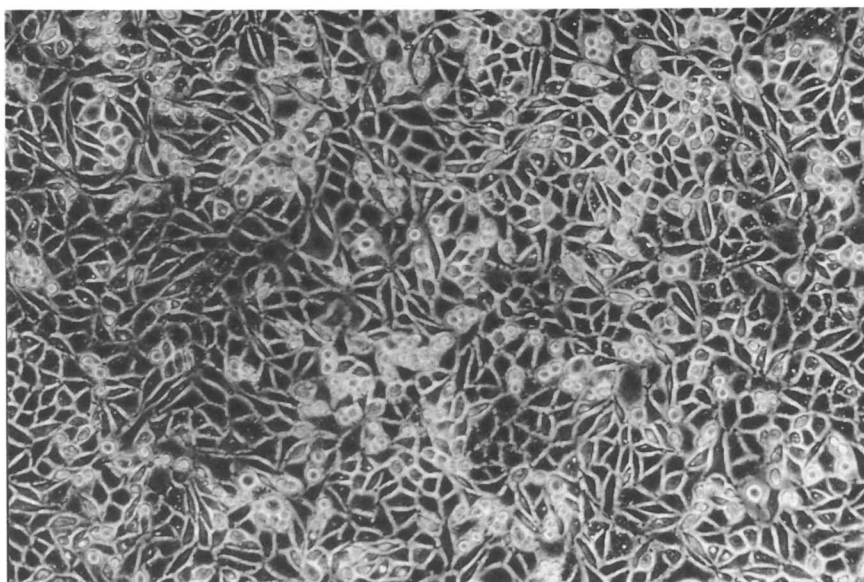


Figure 10. A confluent monolayer (100x magnification) of well defined L929 mouse fibroblast cells exhibiting cell-to-cell contact. This appearance is indicative of a noncytotoxic (negative response in the elution test) method (Reproduced from reference 1. Copyright 1998 Medical Device & Diagnostic Industry.)

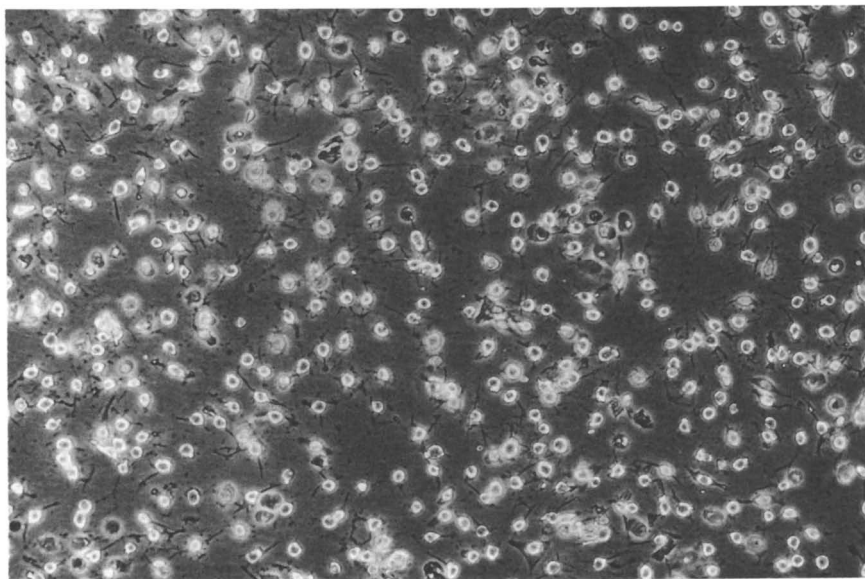


Figure 11. L929 mouse fibroblast cells (100x magnification) that illustrate a positive cytotoxic reaction in the elution test method. The cells are grainy and lack normal cytoplasmic space; the considerable open areas between cells indicate that extensive cell lysis (disintegration) has occurred. (Reproduced from reference 1. Copyright 1998 Medical Device & Diagnostic Industry.)

overlayer that cushions the cells from any physical effects that may be caused by contact with the samples. During incubation, extractables from the samples will migrate into the nutrient medium or through the nutrient agar overlay to the underlying cells. After incubation, the monolayers are evaluated in terms of the presence or absence of a zone of cellular effects beneath and surrounding the sample. A negative result indicates that a material is free of harmful extractables or has an insufficient quantity of them to cause acute effects under exaggerated conditions with isolated cells. However, it is certainly not on its own merit, evidence that the treated article can be considered biocompatible. It is simply a first step. On the other hand, a positive cytotoxicity test result can be taken as an early warning sign that a material contains one or more extractable substances that could be of clinical importance. In such cases, further investigations is required to determine the toxicity of the material.

Sensitization

Sensitization and Irritation ISO 10993-10 are two more test that are required under ISO 10993-1. Sensitization or hypersensitivity reactions usually occur as a result of repeated or prolonged contact with a chemical substance that interacts with the body's immune system. Because most such reactions to biomaterials have been of the dermal cell-mediated type, rather than the humoral or antigen-antibody type, the skin of laboratory animals is used in sensitivity testing. Dermal sensitization reactions are marked by redness and swelling. The guinea pig, a species known to be nearly as responsive to dermal sensitizers as human beings are used in the sensitization test.

Two common test methods that are used include the repeated-patch, or Buehler test and the Magnuson Kligman test method. The Buehler test involves exposing the shaved backs of guinea pigs directly to the test material under occlusive dressings for a minimum of six hours. This procedure is repeated as many as three times a week for three weeks. This part of the test is referred to as the induction phase. Following a two-week rest or recovery period to allow for the development of a delayed response, the animals are challenged by a final exposure to a patch of the biomaterial. The repeated-patch model is used primarily for topical devices such as dermal electrodes, surgical gowns and drapes, in these cases the method of applying these material to the animals simulate clinical use.

In the Magnuson-Kligman test method, fluid extracts of the test materials are prepared in saline or vegetable oil and separate groups of guinea pigs are exposed repeatedly to the two types of extracts. The guinea pigs are first injected with an extract along with an adjuvant to intended to enhance an immune response, then receive a topical application. Following a two week rest or recovery, the animals are covered with a topical patch containing the extract.

The maximization, or Magnuson-Kligman, test method is used for device materials that well contact areas other than skin. The use of saline and oil extracts simulate extraction by bodily fluids. In both methods the area of challenge patch is examined for reactions (redness and swelling) that are not present in negative control animals.

Irritation

The third mandated test according to ISO 10993-1 is Irritation. Irritation is defined as a localized inflammatory response to single repeated, or continuous applications of the test substance without involvement of an immunological mechanism. ISO 10993-10 purposes a four-tier approach to assessing the potential of a material to cause irritation. A device manufacturer should first conduct a review of the literature to determine whether others have reported that the chemical or material under consideration, or structurally related chemicals or materials, can cause irritation. It is essential that the chemical or material of interest already be sufficiently characterized that it can be correlated to those described in the literature. The second step is to use available, validated *in vitro* tests (such as cytotoxicity assays using mammalian cells in culture) to identify, whenever possible, severely irritating materials without using test animals.

When materials have not been ruled out by the first two steps, they should be evaluated using the *in vivo* tests described in the standard. The final step is the use of noninvasive clinical studies in human subjects. This is not presented in the standard as a routine part of an irritation testing program.

Two commonly used *in vivo*, nonclinical tests that are used to evaluate irritation are the intracutaneous and primary skin irritation tests. The intracutaneous reactivity test is aggressive in that it makes use of extracts prepared under exaggerated conditions and places them directly into the skin of test animals, thereby maximizing the chance of finding irritants chemicals if they are present. The primary skin irritation test is less aggressive in that portions of the test material itself are simply placed on the shaved back of albino rabbits. If applicable other irritation tests such as oral, rectal, penile, and vaginal irritation tests are described in annex D of the standard as complements to, not replacements for the primary tests. They are considered relevant for medical devices intended to be applied to those respective mucosal areas of the body.

The three areas of biocompatibility testing, cytotoxicity, sensitization, and irritation mentioned above are by no means all the tests that may be needed when determining the toxicity of a treated medical device. These three test areas are mandated by ISO 10993-1, "Guidance on the Selection of Tests" and its FDA counterpart, blue book memorandum #G95-1, however, other tests listed in this standard may need consideration and are dependent mainly on the device and where it will be used in the human body.

Other Characteristics

Other characteristics that should be considered when applying an antimicrobial treatment to a device are: 1) antimicrobial availability, the amount of the antimicrobial that will be available over the needed period of time; 2) adhesion, if a product is coated with an antimicrobial, it cannot shed or peel. Loss of large particles from the surface of a coated device could distribute the antimicrobial to

non-targeted areas of the body; 3) flexibility, the surface treatment of a device coated with an antimicrobial might add to the thickness of the device and may cause some stiffness, this can be crucial for flexible devices;

4) coverage, the device should be completely covered with the antimicrobial so that when it comes in contact with the body it will be effective in reducing the bacteria it comes in contact with; 5) sterilization; most medical devices are sterilized, therefore, the product must be packaged and sterilized without reducing the effectiveness of the antimicrobial treatment; 6) stability, the antimicrobial treatment must remain active under customary storage and a adequate shelf-life should always be established.

Conclusion

Many different test methods have been discussed for antimicrobial treated products, both in the area of efficacy and biocompatibility. Methods, in both categories, are constantly being reviewed by standards organizations such as ISO, AATCC, and ASTM to determine how they can be improved. When looking at any particular treated product, it is important to consider all areas of testing that might need to take place. Unfortunately, one test will not meet all of the requirements for any given treated product. However, guidance as to what tests should be performed are outlined in the ISO standard and the soon to be published EPA guidelines for treated articles.

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Chapter 13

Antimicrobials for Synthetic Fibers

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Recent work in the field of antimicrobials in textiles demonstrates the durability of the treatment and the different possibilities of incorporating active substances into textiles made of synthetic fibers. Depending on the nature of the fibers, different application techniques and active substances are used.

Introduction - Benefits of an antimicrobial treatment

The consumer nowadays is generally aware of the problems associated with the microbial contamination of textiles. One way to overcome the spread of microorganisms, biodeterioration and infestation is to give the textile an antimicrobial treatment already in the manufacturing stage. There are many reasons for treating a textile antimicrobially. All these different aspects can be summarized in the fact that it achieves better wearing comfort of clothing textiles. This aspect is especially important with textiles made of synthetic fibers. The consumer benefits from the aspect that the development of undesired odor is avoided or at least significantly reduced during activities like sports or when wearing the textile generally. Consumers are demanding functional clothing in the area of casual and sportswear and consequently these market sectors are rapidly growing. The possibilities of applying an antimicrobial treatment are many. Last but not least, by enhancing the wearing comfort, value added is given to the final textile product.

The application of the antimicrobial treatment can be carried out on the finished

woven or nonwoven fabric by using classic textile application processes such as padding or exhaust methods. A highly efficient and durable way of treating man-made fibers is to add the antimicrobial to the spinning mass. The given examples describe both ways of treatment and the performance and durability is discussed.

Typical methods for applying and incorporating an antimicrobial in synthetic fibers and textiles

In the textile industry the same application methods are used for afterfinishing yarns, nonwovens and woven fabrics with antimicrobial-functional formulations as those used for other textile chemicals such as dyes, water and oil repellent finishes as well as sewability improvers and handle-modifying chemicals. For woven fabrics, padding, doctor knife coating and exhaust methods in all their normal technical variants are mainly used. In many cases textile chemicals of the above-mentioned groups are also applied simultaneously from the same aqueous bath together with an antimicrobial finish. In such cases it is absolutely necessary to check the mutual compatibility of the components beforehand. Thus one of the first and most important criteria is to ensure that the textile chemicals to be simultaneously applied are harmonized with one another and that they can be applied at the same pH value, mostly in the weakly acid region. Very often the ionic character given in the technical documentation of the products is used to make the first decision. According to this idea, all the additives used should have either the same ionic character, i.e they all should be cation-active or anion-active, or else all nonionic. Normally nonionic and cation-active additives can be mixed or also nonionic and anion-active additives fit well together. But in general, all new mixtures of different textile additives must be tested in all factors. This is because the rule that chemicals of the same ionic character work well together has a lot of exceptions, one example of which is the following: As tests have shown, it is thoroughly possible that undesired influences can be mutual. Thus, for example, tests showed [7] that with a woven fabric of polyamide simultaneously given an antimicrobial finish with the cation-active Sanitized[®] T 96-04 containing poly(hexamethylenbiguanid)hydrochloride (PHMB) and dyed with the also cation-active Sandocryl Golden Yellow BGRL by the exhaust method at 80 to 98°C and pH 4.5, the dyeing was good but the antimicrobial effect with *Staphylococcus aureus* ATCC 6538 as measured in the agar diffusion test according to SN 195 920 on one and the same specimen exhibited antimicrobial protection values in the scattering range of totally inadequate to good.

It is also interesting in this connection to study the question of how the fastness to washing of the antimicrobial finish behaves when this finish is applied in the dyeing of the polyester woven fabric. Of equal interest is the question of

whether the concentration of the dyes used has an influence on the permanence of the finish. Obviously, the levelness of such finishes is also of interest. The levelness of the dyeing can easily be tested. Less easily tested, however, is the levelness of the antimicrobial finish. In the tests carried out in our own laboratory with the product Sanitized® T 96-20, which contains Triclosan as the active component, no cases of unlevelness were observed. This finding was both with respect to the depth of the dyeing (from pastel to saturated) as well as in the antimicrobial results. Also, no influence on the permanence of the antimicrobial finish could be found [6].

For a successful application it is also necessary that the antimicrobial active substance is soluble in water. The solubility of the antimicrobial formulation should be at least 10 g/l water at 20°C. If the formulation is not water-soluble it must be capable of producing a dispersion in water. This dispersion must be stable during the whole application.

Water-soluble antimicrobial active components

With few exceptions, all substances with an antimicrobial effect as well as those which are used for textile finishing are non-water-soluble. One exception is the cation-active and very thermostable poly-(hexamethylene biguanide) hydrochloride (PHMB), which is available in the form of an aqueous solution, e.g. Sanitized® T 96-04. Unfortunately however, PHMB is unsuitable for finishing most synthetic fibers, for the reason that it exhibits practically no adhesive power with polyamide or polyester fibers.

Another group of excellent water-soluble antimicrobial substances are the quaternary ammonium compounds. They are also strongly cation-active. With these compounds an outstanding antimicrobial effect can be obtained against a great number of microbes. The most noticeable disadvantages of these compounds are their rather low thermostability (decomposition take place at temperatures above around 135°C), and high solubility in water, which virtually rule out a durable finish on textile materials.

Certain other compounds which provide good antimicrobial values and are readily soluble in water exhibit totally inadequate thermostability, e.g. 2-bromine-2-nitropropanediol or the compounds 5-chloro-2-methyl-4-isothiazoline-3-one or 2-methyl-4-isothiazoline-3-one as well as further compounds which serve very well in pot-life preservation, such as all O- and N-formals.

Water-insoluble antimicrobial active compounds

Products that are not or only sparingly soluble in water must be formulated so that they can be used in baths that are sufficiently stable for a successful finishing treatment. As a result, such formulations when applied in water must produce very stable dispersions or emulsions. This normally entails dissolving the active substances in an organic solvent system and adding a surfactant or emulsifier system. These systems have a great influence on the quality level of the antimicrobial finish and are an important part of the know-how of companies that incorporate and market active substances in formulations suitable for industrial application.

When all the microbiologically effective compounds that are well known and correspondingly well documented are systematically tested as to their suitability for afterfinishing textile materials in general and synthetic fibers in particular, the number of products that can be considered suitable is quite modest.

Tests with antimicrobial formulations containing Triclosan:

The following is a more detailed discussion of studies of the afterfinishing treatment of textiles carried out on two different formulations containing the active substance 2,4,4'-trichloro-2'-hydroxydiphenylether, a compound with the common name Triclosan.

Triclosan (CAS No. 3380-34-5) has a melting point of 56 - 58°C. The substance is thermostable up to over 280°C. Triclosan is soluble in most organic solvents such as the simple alcohols, esters and aromates in amounts clearly exceeding 100 g per 100 g solvent. However, only just 0.0010 g [1] dissolve in water at 20°C. Triclosan has an outstanding antimicrobial effect mainly on bacteria. Thus the MIC (minimal inhibition concentration, i.e. the concentration of an active substance which still just shows an effect on test microbes, usually measured in ppm) of Triclosan e.g. with the Gram-positive bacterial strain *Staphylococcus aureus* ATCC 6538 [2], one of the standard test microbes of Swiss Standard SN 195 920, is less than 1 ppm. On the generally much less sensitive Gram-negative bacteria such as *Escherichia coli* ATCC11229, its MIC is still below 10 ppm [3]. This value is excellent, and can also be attained against numerous other bacteria (see [1]).

Triclosan belongs to the class of virtually non-water-soluble active substances, and must be formulated in a manner so that it can be applied in an aqueous finishing step. For this purpose, the active component is built into the formulation in such a way that a stable solution or else a very fine dispersion is formed when it is added to the finishing bath. Ideally, such a formulation should

be so compatible with as many as possible other different textile chemicals, that it can be applied from the same bath together with these. This is normally the last bath before the drying and curing of the textile. The first rough criterion for evaluating the compatibility of different textile chemicals is the ionic character of the products. In any case, a laboratory test is always indispensable.

Moreover, so that the antimicrobial finish of the textile material can be as durable as possible, it must be ensured that the surfactant selected for the formulation does not wash the active component immediately out of the treated textile the first time it is washed. This is because Triclosan is bonded to the substrate in an exclusively physical manner, either by pure adhesion to the surface only or else supplemented by dissolution in the substrate. The structure of the formulation has a considerable influence on the adhesion of the active component to or within the substrate.

With an ideally structured formulation, very good fastness to washing can be achieved using only a small amount of the active component Triclosan, provided the application is also ideal. In such a case, the amount of Triclosan that must be applied in order to achieve a good and durable antimicrobial effect is distinctly lower than the amount required for the same antimicrobial effect when the formulation has a different composition, always presupposing, of course, the identical type of application, test and substrate.

When the formulation is optimized in this way, the amount of Triclosan to be applied for attaining a good and permanent antimicrobial effect can be kept relatively low. This is an enormous ecological advantage, as the amount of Triclosan released to the environment in this case can then be kept appreciably lower, with the same antimicrobial effect. Structuring such formulations represents a considerable portion of technical know-how on the part of the companies which manufacture the formulations.

For applying antimicrobial-effective formulations on woven fabrics, padding or exhaust methods are mostly used. With the padding process, application normally takes place at room temperature or slightly higher. Ideally the pick-up ranges from 50 - 100%. It lies in the very nature of this process that the textile chemicals are applied to the substrate by force. However, with the padding process it should be borne in mind that, depending on the structure of the formulation, substances having very good affinity for the substrate can exhaust from the bath at a greater or lesser rate. This effect then leads to an impoverishment of the bath with respect to at least one component and also to an unlevel application of the chemicals over the length of the substrate. In a number of studies this effect was found to occur mostly with formulations containing cationic surface-active substances.

With the exhaust process the application parameters are usually much wider and the influence of the parameters on the result is much greater than with the

padding method. The main variable parameters with the exhaust method are the liquor ratio, the time, the temperature and the pH value. However, these parameters can be changed during the time of the exhaustion, which in commercial practice is very often done via the temperature. The fact that the exhaustion conditions, particularly the temperature, has a relevant influence on the permanence of the finish in the case of the durable antimicrobial finishing of synthetic fibers with Triclosan among other things, was described as early as 1971 in US Patent 3,788,803 [4]. The conditions that were selected are in the temperature range of 65 to 100°C and ideally between 80 and 95°C. In the German patent application open to public inspection No. EP 0 908 553 [5] a temperature range of even 80 to 135°C is stated for the exhaustion of formulations that can among other things contain Triclosan. These are temperatures that are normally used in the commercial dyeing of polyester woven fabrics.

To accelerate the dyeing of polyester by the exhaust method, especially with temperatures below 100°C, formulations are often used which are usually called carriers. The idea that such formulations, which facilitate the swelling of the fibers, could also improve the absorption of antimicrobial components by the fibers was obvious. Trials were carried out in which the influence of different dyeing accelerants on the durability of the antimicrobial finish was tested. Surprisingly, however, it was found that the influence of such dyeing accelerants on the wash fastness of the antimicrobial finish is negative. This means that on repeated washing, the antimicrobial values of textiles made of polyester break down significantly sooner when the textiles were finished by the exhaust method together with washing accelerants than when no washing accelerants were present in the bath. It can be speculated that dyeing accelerants promote not only the absorption of the antimicrobial components by the fibers, but in the washing process they also favor the washing out of the same components.

It is also interesting in this connection to study the question of how the fastness to washing of the antimicrobial finish behaves when this finish is applied in the dyeing of the polyester woven fabric. Of equal interest is the question of whether the concentration of the dyes used has an influence on the permanence of the finish. Obviously, the levelness of such finishes is also of interest. The levelness of the dyeing can easily be tested. Less easily tested, however, is the levelness of the antimicrobial finish. In the tests carried out in our own laboratory with the product Sanitized® T 96-20, which contains Triclosan as the active component, no cases of unlevelness were observed. This finding was both with respect to the depth of the dyeing (from pastel to saturated) as well as in the antimicrobial results. Also, no influence on the permanence of the antimicrobial finish could be found [6].

The permanence of the antimicrobial finish of two different products, namely Sanitized® T 96-20 from Sanitized AG and Tinosan® AM 100 from Ciba

Specialty Chemicals AG, is compared in various finishes. Both products contain different amounts of Triclosan as the antimicrobially active component. The products were applied by the exhaust method on 100% polyester Dacron 54 spun, with a mass per unit area of 120g/m². All the test specimens were treated at a liquor-to-substrate ratio of 10:1. The pH value in all finishing treatments was adjusted to 4 to 5 with formic acid and in all treatments the dispersant used each time was 1 gram/liter Univadin® DP from Ciba Specialty Chemicals AG and 1 gram/liter ammonium sulfate. For the dyed specimens Foron Yellow SE-6GFL or Foron Blue SE-2R were used. As a matter of course, dyed specimens without antimicrobial finish were also tested for their antibacterial effect. The only dyed specimen exhibit no antimicrobial effect. Exhaustion was started at a temperature of 60°C. This temperature was held for 10 minutes in each case. Afterwards the temperature was raised to 130°C within 30 minutes. The temperature was maintained at 130°C for 20 - 60 minutes, depending on the finish. The specimens were all dried for 5 minutes on the stenter at 100°C. All specimens were washed according to ISO Method 105/C01. The detergent used was ECE Colourfastness Detergent 77 according to ISO 105-C06 or DIN 54017 from Henkel KGaH, D-4000 Düsseldorf. The detergent itself exhibits no antibacterial effect. The specimens were tested by the agar diffusion test with *Staphylococcus aureus* ATCC 6538 according to SN 195 920. The tests described below under 1.5. All tested samples had a good antimicrobial effect against the test germ. The detailed results are shown in tables 1, 2 and 3 at the end of the chapter.

Identical applications with both products, Sanitized® T 96-20 and Tinosan® AM 100 on fabric made of polyamide 6 under the same conditions as on fabric made of polyester gave the same antimicrobial results and the same wash resistance as described in the tables above.

In the following tests dyeing accelerants, i.e. carriers, were used instead of dyes. These carriers were three products from Clariant AG: Dilatin® POE, a nonionic N-alkylphthalimide mixture, Dilatin® NAN, a nonionic/anionic emulsifier-containing hydrocarbon mixture, and Dilatin® EN, a nonionic emulsifier-containing hydrocarbon mixture. As expected, the carriers applied on the same woven fabric of polyester did not exhibit good antimicrobial effects. In these tests exhaustion was also started at 60°C and the temperature was raised to 98°C within 20 minutes and held at 98°C for 20 minutes.

The samples treated with Sanitized® T96-20 and Dilatin® NAN (sample 17) and Dilatin® POE (sample 18) respectively surprisingly showed already a weak growth of the test germ after 20 wash cycles and after 40 wash cycles. A weak growth was found with the sample treated with Dilatin® EN (sample 19). The influence of the carrier was much bigger than expected and also surprisingly in the direction to a significant weaker wash resistance. The detailed results are presented in table 4.

Table 1:

| | |
|---------------------------------|---|
| Antimicrobial product: | Sanitized®T 96-20 |
| Applied amount: | 1% on dry textile |
| Triclosan amount (theoretical): | 500 ppm in treated and unwashed state, on dry textile |

| Specimen | 1 | 2 | 3 | 4 | 5 |
|---|-----|------|-----|------|-----|
| Foron Yellow SE-6GFL [%] | - | 0.04 | 2.2 | - | - |
| Foron Blue SE-2R [%] | - | - | - | 0.03 | 1.7 |
| Exhaust time at 130°C [min] | 20 | 20 | 60 | 20 | 60 |
| Test results: Antibacterial effect in the agar diffusion test according to SN 195 920 with <i>Staphylococcus aureus</i> ATCC 6538 | | | | | |
| Treated and unwashed state | 0 * | 7 | 2 | 2 | 6 |
| 1 washing ISO 105/C01 | 0 | 2 | 0 | 2 | 4 |
| 5 washings ISO 105/C01 | 0 | 0 | 0 | 0 | 0 |
| 10 washings ISO 105/C01 | 0 | 0 | 0 | 0 | 0 |
| 20 washings ISO 105/C01 | 0 | 0 | 0 | 0 | 0 |
| 40 washings ISO 105/C01 | 0 | 0 | 0 | 0 | 0 |
| 60 washings ISO 105/C01 | 0 | 0 | 0 | 0 | 0 |
| 80 washings ISO 105/C01 | 0 | 0 | 0 | 0 | 0 |
| 100 washings ISO 105/C01 | 0 | 0 | 0 | 0 | 0 |

* The test is described in section "Antimicrobial evaluations" for a short interpretation of the given figures: the numbers are zones of inhibition measured in millimetres and mean that 0 and greater is a good antimicrobial effect, "w" stands for weak and in such a case the antimicrobial treatment is borderline, "m" stands for medium growth and "f" for full growth, in both cases the antimicrobial effect of such a sample is insufficient.

Table 2:

| | |
|---------------------------------|--|
| Antimicrobial product: | Sanitized® 96-20 |
| Applied amount: | 10% on dry textile |
| Triclosan amount (theoretical): | 5000 ppm in treated and unwashed state, on dry textile |

| Specimen | 6 | 7 | 8 | 9 | 10 |
|---|----|------|-----|------|-----|
| | | | | | |
| Foron Yellow SE-6GFL [%] | - | 0.04 | 2.2 | - | - |
| Foron Blue SE-2R [%] | - | - | - | 0.03 | 1.7 |
| Exhaust time at 130°C [min] | 20 | 20 | 60 | 20 | 60 |
| | | | | | |
| Test results: Antibacterial effect in the agar diffusion test according to SN 195 920 with <i>Staphylococcus aureus</i> ATCC 6538 | | | | | |
| | | | | | |
| Treated and unwashed state | 9 | 10 | 8 | 10 | 10 |
| 1 washing ISO 105/C01 | 4 | 5 | 4 | 5 | 5 |
| 5 washings ISO 105/C01 | 3 | 2 | 1 | 2 | 1 |
| 10 washings ISO 105/C01 | 1 | 2 | 1 | 0 | 1 |
| 20 washings ISO 105/C01 | 0 | 1 | 0 | 0 | 0 |
| 40 washings ISO 105/C01 | 0 | 0 | 0 | 0 | 0 |
| 60 washings ISO 105/C01 | 0 | 0 | 0 | 0 | 0 |
| 80 washings ISO 105/C01 | 0 | 0 | 0 | 0 | 0 |
| 100 washings ISO 105/C01 | 0 | 0 | 0 | 0 | 0 |

Table 3:

| | |
|---------------------------------|--|
| Antimicrobial product: | Tinosan® AM 100 |
| Applied amount: | 5% on dry textile |
| Triclosan amount (theoretical): | 5000 ppm in treated and unwashed state, on dry textile |

| Specimen | 11 | 12 | 13 | 14 | 15 |
|---|----|------|-----|------|-----|
| | | | | | |
| Foron Yellow SE-6GFL [%] | - | 0.04 | 2.2 | - | - |
| Foron Blue SE-2R [%] | - | - | - | 0.03 | 1.7 |
| Exhaust time at 130°C [min] | 20 | 20 | 60 | 20 | 60 |
| | | | | | |
| Test results: Antibacterial effect in the agar diffusion test according to SN 195 920 with <i>Staphylococcus aureus</i> ATCC 6538 | | | | | |
| | | | | | |
| Treated and unwashed state | 9 | 8 | 6 | 10 | 9 |
| 1 washing ISO 105/C01 | 4 | 5 | 3 | 4 | 3 |
| 5 washings ISO 105/C01 | 2 | 2 | 1 | 2 | 0 |
| 10 washings ISO 105/C01 | 0 | 2 | 1 | 0 | 0 |
| 20 washings ISO 105/C01 | 0 | 0 | 0 | 0 | 0 |
| 40 washings ISO 105/C01 | 0 | 0 | 0 | 0 | 0 |
| 60 washings ISO 105/C01 | 0 | 0 | 0 | 0 | 0 |
| 80 washings ISO 105/C01 | 0 | 0 | 0 | 0 | 0 |
| 100 washings ISO 105/C01 | 0 | 0 | 0 | 0 | 0 |

Table 4:

| | |
|---------------------------------|---|
| Antimicrobial product: | Sanitized®T 96-20 |
| Applied amount: | 1% on the dry textile |
| Triclosan amount (theoretical): | 500 ppm in the treated and unwashed state, on the dry textile |

| Specimen | 16 | 17 | 18 | 19 |
|---|----|----|----|----|
| | | | | |
| Dilatin NAN | - | 2% | - | - |
| Dilatin POE | - | - | 3% | - |
| Dilatin EN | - | - | - | 4% |
| | | | | |
| Test results: Antibacterial effect in the agar diffusion test according to SN 195 920 with <i>Staphylococcus aureus</i> ATCC 6538 | | | | |
| | | | | |
| Treated and unwashed state | 5 | 3 | 1 | 0 |
| 5 washings ISO 105/C01 | 4 | 1 | 0 | 0 |
| 10 washings ISO 105/C01 | 2 | 0 | 0 | 0 |
| 20 washings ISO 105/C01 | 0 | 0 | 0 | 0 |
| 25 washings ISO 105/C01 | 0 | w | w | 0 |
| 30 washings ISO 105/C01 | 0 | w | w | 0 |
| 35 washings ISO 105/C01 | 0 | w | w | 0 |
| 40 washings ISO 105/C01 | 0 | w | w | 0 |
| 50 washings ISO 105/C01 | 0 | w | w | w |
| 60 washings ISO 105/C01 | 0 | w | w | w |
| 80 washings ISO 105/C01 | 0 | - | - | - |
| 100 washings ISO 105/C01 | 0 | - | - | - |

Classic aftertreatment with a covalent bonding mechanism

The following describes the antimicrobial finishing of woven fabrics made of polyamide and polyester with a formulation containing quaternary ammonium compounds having an alkyltrimethoxysilane group, in particular 3-(trimethoxysilyl)-propyl-tetradecyl-dimethyl ammonium chloride in an oligoethylene glycol methyl ether. The trimethoxysilane group is strongly reactive. It can react with protic hydrogen atoms, but nucleophile addition reactions can also be formulated, for example at the carboxyl group of esters and amides. In this manner, quaternary ammonium compounds can be bonded to substrates not only physically but also chemically. As will be described below, this has not only the expected positive effects on the washing permanence of the antimicrobial formulation, but has also been found to have substantial effects on the microbe spectrum, as compared with free quaternary ammonium compounds.

The product used was the test product Sanitized® T 99-19 from Sanitized AG. The product is, as all quaternary ammonium compounds used in practice, soluble in water and it is cation-active.

Products with functional trimethoxysilane groups have long been on the market. These formulations are marketed in methanolic solutions. Representative products of this type are Requat from Sanitized Inc. and Biosil DC 5700 from Dow Corning. An important field of application of these products beside the textile sector is the finishing of glass. Here a readily volatile solvent is obviously suitable. For the antimicrobial finishing of textiles, methanol has the disadvantage of being highly flammable.

As already mentioned, such a formulation can react, at least to some degree, with the textile fibers, forming a covalent bond. The purpose of this chemical bonding between the substrate and the antimicrobial component is a better adhesion of this component, as compared with the same type of molecule, i.e. a quaternary ammonium compound, being attached to the substrate only by physical interactions.

The application of the formulation was carried out at 40°C by the exhaust method. The wetting agent used was 0.2g/l Sandozin® NRW from Clariant AG. The liquor ratio was 20:1 and the exhaustion time was 20 minutes in each case. After the exhaustion phase, the textile test specimens were washed for 5 minutes in water at 30°C and then for 5 minutes in cold water, again at a liquor ratio of 20:1. After centrifugeing, the specimens were dried on the stenter for 2 minutes at 120°C. It was expected that the temperature of the stenter has to be below 140°C otherwise the compounds decompose via the elimination of alkenes, which is the so-called Hofmann degradation. However there are good antimicrobial results found after drying treated fabrics made of polyamide,

polyester and also of cotton up to temperatures of 180°C. Obviously the normally used time of technical textile applications is maximal one minute and it is too short to destroy the product on fabrics.

Woven fabrics, one of polyamide 6, Nylon Spun, with a mass per unit area of 110 g/m² and one of polyester (ECE) S/777 having a mass per unit area of 120 g/m² were each treated as described with 0.5% of the formulation containing 3-(trimethoxysilyl)-propyl-tetradecyl-dimethyl ammonium chloride as the active component. As a matter of course, woven fabrics of cotton and of wool were treated and tested in the same manner, and similarly good results were achieved as with the woven fabrics of synthetic fibers. However, this subject is not of interest here, and will therefore not be further discussed. The finished fabrics, in the treated and unwashed state and after washing, were tested for various microbes in the agar diffusion test. The washing process was carried out according to the Japanese method of Sen-i seithin Eiseikako Kyogikai (S.E.K.). The procedure was as follows: The aqueous bath contains 0.54 g/l ethoxylated nonylphenol with an average of 9.5 EO units (e.g. Imbentin-N/52 from Kolb AG, Switzerland). The specimens are washed while stirring at a liquor ratio of 30:1 for 5 minutes at 40°C, and then the bath is centrifuged out. Afterwards the specimens are rinsed for 2 minutes under cold running tap water and then again centrifuged. This entire rinsing process is then repeated. The specimens are dried at 80°C, ideally on a stenter. The microbes used for testing the antimicrobial effect were *Escherichia coli* ATCC 11229, *Klebsiella pneumoniae* ATCC 4352, *Pseudomonas aeruginosa* ATCC 15442 and *Staphylococcus aureus* ATCC 6538. Testing was carried out according to Standard SN 195 920. Both with the woven fabric of polyamide as well as that of polyester, the antimicrobial results were excellent. With all the above-mentioned test microbes, the polyester fabric gave very good antimicrobial values in the agar diffusion test according to SN 195 920 even after 25 washings at 60°C in a household washing machine, washing with a normal commercial detergent which is antimicrobially non-active. The antimicrobial effect on the treated polyamide fabric which had received ten washings by the above-described method was tested with the above-mentioned bacteria in the Shake Flask Test. This test, which is from Japan, was carried out according to the specifications of S.E.K. [8]. In this test method, 0.75 g of a cut-out piece of the sample is sterilized with ethylene oxide, placed in a phosphate buffer, added to the solution with the test microbes and then incubated while shaking at 37°C in the dark for 24 hours. The bacterial count is taken at the beginning of the test and after 24 hours.

With respect to three of the microbes, namely *Klebsiella pneumoniae* ATCC 4352, *Pseudomonas aeruginosa* ATCC 15442 and *Staphylococcus aureus* ATCC 6538, very good results were achieved. However, against the microbe *Escherichia coli* ATCC 11229, rather poor results were seen. This was all the more surprising, as in the agar diffusion test according to SN 195 920 the same

very good antimicrobial effect was attained against *Escherichia coli* as with the other bacteria. Exactly the same results, by the way, were obtained in the same test with woven cellulosic fabric treated with poly-(hexamethylene biguanide) hydrochloride. Further work is now required in order to clarify when and why the results produced by the Shake Flask Test are so substantially different from the agar diffusion test, and whether the only anomaly is with respect to the *Escherichia coli* bacteria, or whether other microbes give similarly divergent results in the two tests.

In addition, the antimycotic effect on woven fabrics of polyamide and of polyester treated with the silicon-functional quaternary ammonium compound and washed according to the Japanese method was tested according to SN Standard 195 921 against the microfungi *Aspergillus niger* EMPA 18, *Candida albicans* ATCC 10231 and *Trichophyton mentagrophytes* EMPA 334. Excellent results were obtained against the yeast *Candida albicans*, both in the treated and unwashed state and after washing by the method described above, when tested by the agar diffusion test according to SN 195 921, i.e. the test specimen even showed zones of inhibition in the test. With respect to the mold fungus *Aspergillus niger* and the skin fungus *Trichophyton mentagrophytes*, some antimycotic values were still even found on most of the substrates in the treated and unwashed state, which might possibly be explained by the fact that the quaternary ammonium compound was not completely bonded to the substrate. On the washed substrates however, the antimycotic effect was found to have broken down almost completely. This is another very astonishing result. Non-bonded quaternary ammonium compounds of this or a similar type give very good values not only against bacteria but also with microfungi. By the way, very good results can also be obtained against the growth of algae, although this last point was not investigated here, as the question purely relating to the use of the treatment was not of interest. In any case, it seems that, with the exception of the highly sensitive *Candida albicans*, microfungi, in contrast to bacteria, are considerably less sensitive to quaternary ammonium salt bonded to a substrate than to free quaternary ammonium salt. This points to the fact that in the case of microfungi there is another mechanism in the action at least of quaternary ammonium salts bound to a substrate via an alkyl siloxane group. With bacteria it is postulated by Hiroshi Kawai, DOW CORNING ASIA Ltd., that the aliphatic chain penetrates through the cell wall and the cell membrane, thus destroying the bacteria cell.

Another point of great interest was the compatibility with other textile chemicals. The trimethoxysilane group exhibits very high reactivity and it cannot be easily estimated whether the products react with one another in an undesirable way in the bath before or during the application. Compatibility tests were performed using in each case an amount of 0.5% Sanitized® T 99-19, which contains 50%

3-(trimethoxysilyl)-propyl-tetradecy-dimethyl ammonium chloride as the antimicrobial active substance, together with the following textile chemicals, all of which are from Clariant AG:

- 2.0% Sandolube® NV, a sewability improver based on aliphatic hydrocarbons
- 2.0% Sandoperm® MEW, a polysiloxane used as a softener
- 13% Pekoflam® OP, an inorganic flame retardant
- 2.5% Appretan® EM, a polyvinyl acetate for stiffening finishes
- 2.5% Nuva® CSF, a fluorocarbon for water and oil repellent finishing

All applications were carried out by the padding method on polyamide woven fabric. The wetting agent used was Sandozin NRW from Clariant AG, applied in an amount from 0.2 to 0.5g/l bath. In every case, drying was carried out on the stenter. Compatibility was found to exist with all combinations. In all the finishing applications, with the exception of the finishing treatment together with the fluorocarbon, both the technical textile values and the antimicrobial values were good and corresponded to the values that were also attained by the components applied individually, thus permitting the conclusion that the compatibility was very good. In the case of the fluorocarbon, the antimicrobial effect was no longer good with respect to all bacteria. This was in contrast to the excellent oil repellent and water repellent values of the textile. The cause is probably the very hydrophobic surface of the textile. It could be that this prevent a transfer of antimicrobial active molecules to the agar because the surfaces of the two different media, the fabric and the agar are under test conditions not permeable. The interpretation of all type of agar plate tests make statements of test specimen by the indirect measurement of the antimicrobial effect on the agar and not directly on the tested sample. Normally this tests gives reliable and relatively good reproducible results under very strong conditions in a short time.

Tests were also made of the Degree of Whiteness CIE and of Yellowness YI of polyamide woven fabric finished with 0.5 and 1.0% Sanitized® T 99-19, respectively. The test specimens were exposed in the Xenotest 450 for 100 and 200 hours, respectively. Compared with the non-treated control specimen, practically no deviations were found. Even after exposure, the antimicrobial effect remained entire. It can therefore be concluded that the antimicrobial finish with Sanitized® T 99-19 is washfast and lightfast. Table 5 shows the detailed antimicrobial results of agar diffusion tests against different test germs. The test fabric was made of polyamide 6 and was treated with 0.5% Sanitized® T 99-19. The fabric was tested in the treated and unwashed state and up to 12 wash cycles according the method S.E.K. from Japan. It was found that the product effects excellent against all tested bacteria and also against *Candida albicans*, a type of

Table 5:

| | |
|------------------------|---|
| Antimicrobial product: | Sanitized® T 99-19 |
| Applied amount: | 0.5% on dry textile |
| Application: | Padding |
| Substrate: | Polyamide 6, Nylon Spun, 110 g/m ² |
| Pretreatment: | Washing according to S.E.K. |
| Test with bacteria: | Swiss Standard SN 195 920 |
| Test with microfungi: | Swiss Standard SN 195 921 |

| Washings accd. to S.E.K. | 0 | 1 | 3 | 5 | 10 | 12 |
|---|---|---|---|---|----|----|
| <i>Staphylococcus aureus</i> ATCC 6538 | 5 | 3 | 2 | 1 | 0 | 0 |
| <i>Pseudomonas aeruginosa</i> ATCC 15442 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>Escherichia coli</i> ATCC 11229 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>Klebsiella pneumoniae</i> ATCC 4352 | 2 | 0 | 0 | 0 | 0 | 0 |
| <i>Candida albicans</i> ATCC 10231 | 8 | 4 | 4 | 3 | 0 | 0 |
| <i>Aspergillus niger</i> EMPA 18 | m | m | m | m | m | m |
| <i>Trichophyton mentagrophytes</i> EMPA 334 | m | m | m | m | m | m |

yeast, but there was no effect at all against fungi. The identical test was made with a fabric made of polyester Dacron. The detailed test results are presented in table 6. The results against all bacteria were the same as those with the fabric made of polyamide. But against fungi there was in the treated and unwashed state an excellent effect against fungi. The effect against fungi was also still detectable after several wash cycles. The fact that different substrates, treated under identical conditions with exactly the same type and concentration of an antimicrobial product, gives different antimicrobial results against different germs has been found more often. A systematic answer to explain this effect is not known.

The application with 1% Sanitized® T 99-19 and the applications with respectively 0.5 and 1% Sanitized T 99-19 applied by the exhaust method gave practically the same results.

The application with 1% Sanitized® T 99-19 and the applications with respectively 0.5 and 1% Sanitized® T 99-19 applied by the exhaust method gave practically the same results. The specimens were not washed more than 12 times according to the method given. However, up to 25 washings at 60°C were carried out in a household washing machine using a normal commercial washing detergent. Good antimicrobial effects were obtained with respect to all four bacteria and with *Candida albicans*, i.e. there was no growth whatsoever after the washings.

Antimicrobial treatment of synthetic fibers in the spinning mass

Besides the classic method of using an aftertreatment to give the antimicrobial finish, there is also the possibility of incorporating the antimicrobial in the spinning mass during the fiber melt spinning process. This guarantees a homogenous distribution of the active substance in the fiber.

Good results using this technique have been achieved in the sectors of PP, PET and PA fibers. The choice of active substance depends on the actual processing conditions during fiber spinning. For polypropylene (PP) Triclosan-based products are in many cases the products of choice. Based on the physical properties of the masterbatches, different products are offered for this type of application. For reasons of industrial hygiene as well as for continuous and even dosing it is of the utmost importance that there is no blooming out of the active substance from the masterbatch pellets. The processing temperatures in PP fiber production are in the range of 250°C. Here Triclosan-based masterbatches can be used without any restrictions.

Table 6:

| | |
|------------------------|--|
| Antimicrobial product: | Sanitized® T 99-19 |
| Applied amount: | 0.5% on dry textile |
| Application: | Padding |
| Substrate: | Polyester Dacron, 120 g/m ² |
| Pretreatment: | Washing according to S.E.K. |
| Test with bacteria: | Swiss Standard SN 195 920 |
| Test with microfungi: | Swiss Standard SN 195 921 |

| Washings accd. to S.E.K. | 0 | 5 | 10 | 12 |
|---|----|---|----|----|
| <i>Staphylococcus aureus</i> ATCC 6538 | 6 | 0 | 0 | 0 |
| <i>Pseudomonas aeruginosa</i> ATCC 15442 | 0 | 0 | 0 | 0 |
| <i>Escherichia coli</i> ATCC 11229 | 0 | 0 | 0 | 0 |
| <i>Klebsiella pneumoniae</i> ATCC 4352 | 5 | 0 | 0 | 0 |
| <i>Candida albicans</i> ATCC 10231 | 10 | 1 | 0 | 0 |
| <i>Aspergillus niger</i> EMPA 18 | 0 | w | m | m |
| <i>Trichophyton mentagrophytes</i> EMPA 334 | 0 | 0 | w | m |

With synthetic polymeric fibers like polyester (PET) and polyamide (PA) the processing temperatures are about 295°C and 280°C respectively, and only few antimicrobial additives can be used that withstand these temperatures.

Practically no organic-based active ingredients can be used because of their lack of thermostability. The choice in this field of application is silver-based inorganic substances. Synthetic fibers like polyester, polyamide and of course polypropylene can be given an antimicrobial treatment by incorporating silver ions (Ag^+) into the spinning mass during fiber production. The silver ions are typically fixed on an inorganic carrier such as ceramics in Sanitized[®] MB PET 99-56. Masterbatches with the appropriate carriers and the antimicrobial are produced and this material is used in the fiber spinning process.

Inorganic types of antimicrobials are not soluble in the polymer like Triclosan is. Owing to this fact, it is mandatory that the particle size of the additive is small enough to guarantee good spinning properties. This means that any clogging of filtration devices in the spinnpack must be avoided. The ratio of particle size to the diameter of the fiber has to be 1:10 at the maximum. In order to achieve an antimicrobial effect, a slow release of the silver ions must be ensured. With many types of fibers, good washfastness is desirable, especially in the sportswear sector. Here, thanks to the antimicrobial's incorporation into the fiber, good washfastness is given. The release of silver ions can be controlled by using an appropriate inorganic carrier. Various carriers are used in this type of application, for example zeolites, TiO_2 , ZnO , $\text{Zr}_3(\text{PO}_4)_2$, ceramics and glass. Controlled release of the silver ions is not the only factor that has to be balanced. At the same time it has to be ensured that the antimicrobial additive does not have any negative impact on the fiber properties, e.g. discoloration or impairment of mechanical resistance. The nature of the inorganic carrier for the silver ions is of the utmost importance for the quality of the fabrics manufactured. The differences are considerable and the choice of antimicrobial silver has to be made with great care.

Antimicrobial performance in polyamide

The antimicrobial active component of Sanitized[®] MB PA 99-57 is ceramic based silver. The product is incorporated into the fiber made of polyamide PA 6 during the melt spinning process. The resulting fabric was tested according to the Japanese test method JIS L 1902: 1998 (E) in the treated and unwashed state and after 5, 10 and 20 washings according to ISO 105/C01 at 40°C. The detailed results are given in table 7 and show the excellent antimicrobial performance.

It is interesting to observe that the antimicrobial performance is seen to be even better after washing than before. This can be explained by the effect that the spin finishing oil used in the fiber spinning process reduces the availability of the

Table 7

| | | | | |
|-------------------|-----|--------|---------|---------|
| untreated | 0h | cfu/ml | 4.2E+05 | |
| | 24h | cfu/ml | 4.3E+07 | |
| | | | | |
| original | 0h | cfu/ml | 6.0E+05 | |
| | 24h | cfu/ml | 2.0E+04 | |
| | | | | S = 3.3 |
| after 5 washings | 0h | cfu/ml | 6.0E+05 | |
| | 24h | cfu/ml | 0.0E+00 | |
| | | | | S = 6.8 |
| after 10 washings | 0h | cfu/ml | 6.0E+05 | |
| | 24h | cfu/ml | 0.0E+00 | |
| | | | | S = 6.8 |
| after 20 washings | 0h | cfu/ml | 6.0E+05 | |
| | 24h | cfu/ml | 0.0E+00 | |
| | | | | S = 6.8 |

$s = M_b - M_c$ (see section "Antimicrobial evaluations")

silver ions when the specimen is in the treated and unwashed state, thus diminishing the antimicrobial effect. During washing the spin finishing oil is removed and the antimicrobial performance is clearer in the test.

Human ecological safety of the antimicrobial treatment

Both silver and Triclosan are scientifically well documented and can be regarded as safe for the consumer in textile applications. Silver has been known for its antimicrobial effect for decades. Its use is permitted in many countries for the treatment of drinking water and in others for beverages and vinegar. In the EU it is permitted to use silver as a disinfectant for drinking water at concentrations up to 10 $\mu\text{g Ag/l}$.

Antimicrobial action of silver-based products

Silver ions show an oligodynamic action against microbes. It is described in the literature that silver possibly causes membrane interactions which disrupt phosphate absorption by the cell [9].

Silver may also be absorbed on the negatively charged surface of bacteria, or a reaction with enzymes may occur. The silver ions intervene in the energy-supplying process of the electron transport chain, where they inhibit cytochromes b and d as well as various flavoproteins [10].

Silver treatment is directed primarily against bacteria. Yeasts and molds are inhibited to a lesser extent [11]. Triclosan also covers bacteria to a greater extent than fungi.

Antimicrobial evaluations

The tests which were mainly used in the present study, namely the agar diffusion test according to AATCC Test Method 90-1970 [12] and the test according to Swiss Standard SN 195 920, which is often used in Europe [13], are practically the same; only the composition of the nutrient agar is slightly different in each test. In practice, this difference has no detectable effect whatsoever on the results. The procedure followed is as follows: sterile nutrient agar is poured into a Petri dish. The agar hardens on cooling. On top of this agar layer a second layer of agar is poured with the nutrient medium and the daily culture of the strain of test microbes under investigation. This batch of agar contains approximately 10^6 microbes per milliliter. A round piece of the textile specimen is placed on top of the hardened agar and fixed there. The plate is conditioned for 24 hours at 5°C. This is followed by incubation in an incubator, usually for

18 hours at 37°C. After incubation the specimens are assessed visually and under the microscope. The scale followed in this assessment is described as follows: The microbes grow onto the specimen and cover its underside completely or at least to a very great extent. This is designated full growth (shown in the results tables as: f). Such a specimen exhibits no inhibition whatsoever to the growth of this specific test microbe in this specific test. An antimicrobial finish is also considered to be completely inadequate when more than 5% but less than 50% of the undersurface of the specimen is covered with growth, which is interpreted as moderate growth (m). If under the microscope very little growth is to be seen on the agar at the spot where the textile specimen was incubated, and less than 5% of the undersurface of the specimen is covered with growth, this is termed weak growth (w). Here we find inhibition to the growth of the test microbe. This inhibition is interpreted as the limit of effectiveness of the antimicrobial finish with respect to the test microbe. - Here SN 195 920 deviates slightly from the above-described assessments. It calls "limit of effectiveness" the almost total lack of growth, describes "moderate growth" as the lack of a zone of inhibition and growth reduced by one half as compared with a control specimen, and considers "full growth" to be the case where no inhibition is seen and no zones of inhibition occur, compared with the control specimen. - If the agar under the specimen shows no growth whatsoever (0) or inspection with the naked eye can detect very distinct, mostly ring-like zones of inhibition around the specimen (numbers >0), the specimen is considered to exhibit very good protection against the growth of the test microbe. Any zones of inhibition are indicated in millimeters in the results, where the lowest value for a good antimicrobial finish in this test is zero.

The AATCC 147-1998 test is often used [14]. This test gives practically the same results as the above-described tests according to AATCC 90-1970 and SN 195 920, respectively. However it is easier to carry out with a small number of specimens, as the microbe suspension is spread over the nutrient agar in a zig-zag manner, the textile test specimen laid on it and conditioned, and then incubated. The assessment corresponds to the test standards described above.

Passing an agar diffusion test is a very hard condition for textile test specimens. In this test the specimen is exposed to about 10^6 bacteria per milliliter nutrient agar, i.e. the ideal nutrient medium, under absolutely ideal growth conditions for the bacteria. Fortunately, such ideal growth conditions for microbes are practically never encountered in actual practice.

An agar diffusion test can also be used for testing the protection against the growth of microfungi. The antimycotic values from the tests described above were worked out according to the Swiss Test Standard SN 195 921 [15]. The main differences from the test according to SN 195 920 are the use of another nutrient agar which is optimized for microfungi, the longer incubation time, which in the case of the pathogenic yeast *Candida albicans* ATCC 10231, for

example, lasts 4 days. The incubation lasts 5 days with *Aspergillus niger* EMPA 18 [16], a mold fungus which is standard for use in the test and which causes mildew spots in textiles, and the incubation time also lasts 5 days with *Trichophyton mentagrophytes* EMPA 334, the skin fungus which is usually tested.

As antimicrobial treatment with silver has a long history in Japan, the corresponding antimicrobial tests are carried out according to the Japanese industrial standard JIS L 1902: 1998 (E). Described is a test method for antimicrobials on textiles. The results give quantitative information on the antimicrobial performance of the treated textile. The procedure can be summarized as follows: A textile specimen measuring about 18 square mm is sterilized, placed into a vial and inoculated with a defined germ suspension of e.g. *Staphylococcus aureus* ATCC 6538 P or *Klebsiella pneumoniae* ATCC 4352. The concentration of inoculum is typically 1×10^5 cfu/ml. After incubation for 18 h at 37°C, the number of cfus is counted. The evaluation of the antimicrobial activity is made by using the simple equation:

$S = M_b - M_c$ where S is the activity value for the bacteriostat (or antimicrobial), M_b the common logarithm of number of active bacteria after 18-hour incubation on an untreated sample and M_c the common logarithm of the number of active bacteria after 18 hours incubation of the treated sample.

A significant reduction of cfus of at least 1-2 log units shows good performance for certain applications. A reduction of greater than 2 log units demonstrates a good bacteriostatic effect for all types of applications.

Conclusion

Different types of applications using different active ingredients have been described. The choice of antimicrobial application depends on the final use of the textile. In some cases the antimicrobial effect is needed only in the treated and unwashed state of the article and washfastness is not of interest. An example of this is antimicrobial protection of the product in its original state during storage, before use. In the field of functional clothing, however, washfastness and durability is a key factor. Sportswear in particular must meet the highest demands in this respect. Some textiles are used under extreme conditions and consumers expect them to keep their functional properties throughout the lifetime of the article. Here laboratory tests are indispensable for demonstrating the antimicrobial effect. The results have to be relevant to the particular requirements of the application field. Lab test conditions should be more severe than real life conditions. For this purpose an adequate and economical number of tests with standard microbes are used. They have to be representative for a number of different germs which are relevant in the special area of application.

Two different types of applications of antimicrobial finishing have been described. The classical procedure for applying the antimicrobial is still as an aftertreatment but the incorporation of the antimicrobial in the fiber spinning mass is becoming more and more popular. Besides all the positive aspects the consumer can benefit from, in the background there is the aspect of safety for man and the environment which certainly has to be paid attention to in the same manner. The given examples demonstrate some of the possibilities that really meet all requirements of a state-of-the-art antimicrobial treatment.

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Chapter 14

Durable and Regenerable Antimicrobial Textiles

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Antimicrobial textile materials have become necessary in many areas such as hospitals and institutional places as preventive measures against cross transmission of diseases. Whether the desired functions are durable is a major concern to textile researchers and users since the textiles are subjected to frequent laundry cleaning in applications. Durable antimicrobial functions can be achieved by a common practice that incorporates sufficient amount of biocides into the materials and then let them slowly released to the surface. However, the durability is limited, and more importantly the functions will be finally lost in the practice. Moreover, directly employing biocides into polymer materials may also generate unwanted side-effects. Thus, durable biocidal surfaces without leaching out agents would be a proper approach for the development of antimicrobial textiles. In this presentation, we will discuss our latest progresses in preparing durable and regenerable antimicrobial textiles by developing some innovative approaches in chemical finishing of textiles.

Biological safety is a major concern to both health care providers and patients in hospitals. Moreover, publics are facing a great challenge of infections from pathogens, most significantly from multidrug-resistant bacteria, which is commonly called nosocomial effect in hospitals. In 1992, a report estimated that nosocomial infections increased the annual cost of health care in the United States by more than \$4.5 billion and contributed to almost 80,000 deaths¹. Three years later, a study conducted in New York City reported that *Staphylococcus aureus* nosocomial effect was responsible for 13,550 cases of infections and led to 1,400 deaths². Another similar study conducted in 1417 Intensive Care Units (ICU) in Europe revealed that a 44.8% nosocomial infection rate of 10,038 patients, including 20.65 ICU-acquired infections. Most frequently reported microorganisms were *Enterobacteriaceae* (34.4%), *Staphylococcus aureus* (30.1%; of which 60% resistant to methicillin), *Pseudomonas aeruginosa* (28.7%), coagulase-negative staphylococcus (19.1%), and fungi (17.1%). More recently, drug-resistant *Staphylococcus aureus* was found responsible for fatal infections outside of hospitals, which brings more concerns to the popular spread of drug-resistant diseases. Textile materials, as good and common media for growth and transmission of microorganisms³, are possible means for the

spread of nosocomial effect inside and outside healthcare facilities. Thus, using antimicrobial textiles in these facilities could provide preventions from spread of drug-resistant diseases and protect or reduce healthcare workers and public from infections.

Antimicrobial Agents and Textiles

Antimicrobial textiles, which were mainly used for the purpose of preventing the materials from biodegradation, are not new at all to textile scientists conceptually. It, instead of protecting wearers but the materials, has created some confusion to customers by the claims of antimicrobial functions. It should be pointed out that antimicrobial may include biocidal and biostatic, two different properties. Biocidal function refers to killing microorganisms, while biostatic reaction indicates inhibiting growth of microorganisms. For protective purposes, biocidal functions, especially rapid and efficient inactivation of a broad spectrum of microorganisms, are required. As mentioned above, medical-use textiles, only acting as physical barriers to infectious blood and body fluid, cannot offer complete protection to health care providers. So, it is reasonable to believe that the combination of such biocidal properties to these tools should provide better protection to health care providers, and thus reduce their occupational risk greatly.

Table 1. Common antimicrobial agents and their limitations

| <i>Compound</i> | <i>Function</i> | <i>Limitation</i> |
|---|--------------------------------|---|
| Halogens (Cl ₂ , Br ₂ , and I ₂) | Oxidizing | Toxicity and skin irritation |
| H ₂ O ₂ | Oxidizing | Toxicity and skin irritation |
| Formaldehyde | Alkylating | Carcinogen and skin irritation |
| Alcohols | Dehydrating | High concentration, less effective on spores |
| Quaternary Ammonium salts | Affecting Permeability | Less effective and skin irritation |
| Phenols | Affecting permeability | Less effective and skin absorption |
| Heavy metals (Ag) | Sulfhydryl binding | Not effective against spores, water pollution |
| Antibiotics | Reproductive Enzyme inhibitors | Creating drug-resistance |

Antimicrobial material was first developed in 1867 by Lister who first demonstrated the relationship between fibrous materials and disease⁴. Since then, many innovative antimicrobial materials have been developed. In general, antimicrobial properties of textile materials can be obtained by two different

approaches, i.e. chemically or physically incorporating antimicrobial agents into fibers or fabrics. The antimicrobial agents can be antibiotics, formaldehyde, heavy metal ions (silver, copper), quaternary ammonium salts (with long carbon hydrogen chains), phenols, and oxidizing agents such as chlorine, chloramine, hydrogen peroxide, iodine, and ozone. The agents inactivate microorganisms via different mechanisms and with different limitations. Antibiotics kill bacteria by inhibiting their reproductive enzymes, which definitely creates concerns if used in textile materials. Quaternary ammonium salts and phenolic compounds damage cells by affecting permeable properties of microorganisms that usually results quite slow action. And oxidizing agents can rapidly inactivate microorganisms by chemical reactions with some functional groups of microorganisms, but most of are toxic and skin irritating (Table 1). Among them oxidizing inactivation of microbes is rapid, non-selective, and non-immutable to microorganisms.

Slow-Releasing Mechanism

The greatest challenge to the functional textiles, especially clothing materials, is durability of the antimicrobial functions, i.e. washfastness of the functions subjecting to repeated laundering. Durability of antimicrobial functions of textile materials can be grouped into two categories, i.e. temporary or durable functional fabrics. Temporary biocidal properties of fabrics are easy to achieve in finishing, but easy to lose in laundering, which is quite useful for disposable materials. Durable antimicrobial functions generally have been achieved by using a common technology, slow-releasing method, on certain textiles, mainly for preservation of the materials from biodegradation or odor reduction, not for protective purposes based on the functions.

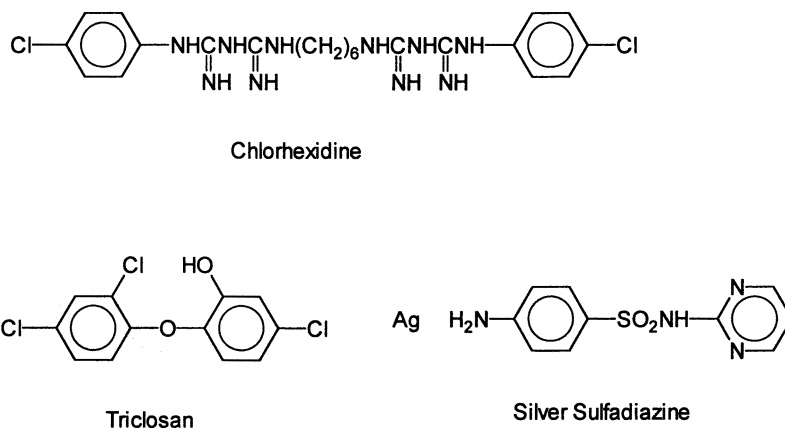


Figure 1. Some slow releasing antimicrobial agents

According to the method, sufficient antimicrobial agents should be incorporated into fibers or fabrics in a wet finishing process to provide prolonged usage. The

fabrics inactivate bacteria by slowly releasing the agents from the materials to the surface. However, the bactericides will vanish completely since they are impregnated in the materials without covalent bond linkages.

There are some successful examples of antimicrobial textiles developed based on this slow releasing concept. Durable antibacterial cellulose and cellulose blend fabrics combined with a zinc peroxide polymer were prepared by USDA⁵. Recently, in a similar approach, T. Vigo used magnesium peroxide in finishing of fabrics and achieved durable antimicrobial properties on the materials⁶. Polymers of

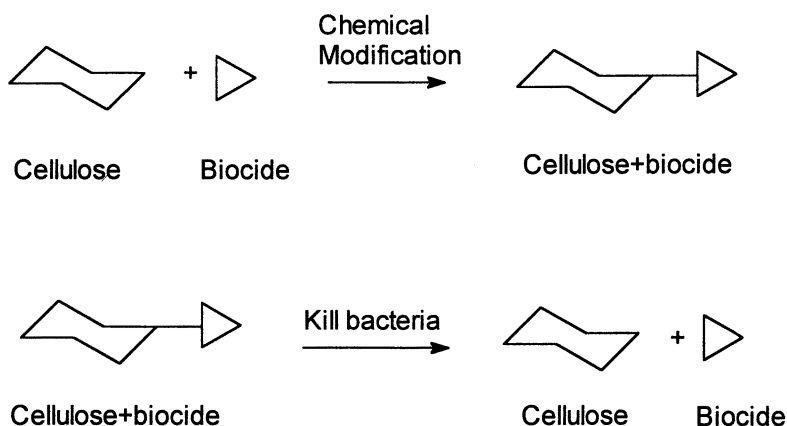


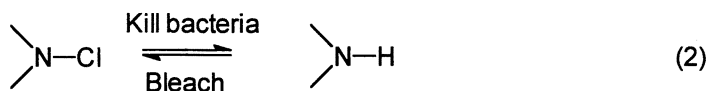
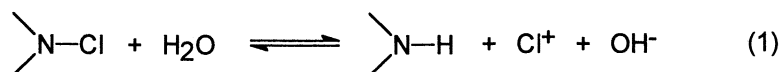
Figure 2. Slow-releasing mechanism (Reproduced from reference 16, copyright American Association of Textile Chemists and Colorists, AATCC)

hexamethylene biguanide hydrochloride⁷, quaternary ammonium salts⁸, and silver ions⁹ have been employed in treatment of textile products. Triclosan, a phenolic compound, has found applications in liquid or solid soaps, plastic products, and fibers as a bacterial inhibiting agent¹⁰. Nevertheless, the antimicrobial properties of these materials are not sufficient enough to execute quick kill to germs in contact, which is critically needed for protection of medical workers. For example, quaternary ammonium salts and Triclosan both will take more than 18 hours of contact time to exhibit their maximum functions, which therefore inhibits their applications in medical protective clothing. Beside the slow action against microorganisms, Triclosan was found to produce drug-resistant strains in microorganisms¹¹, which is also a major concern to using antibiotics in textile treatment¹². Beyond all of the problems related to the slow releasing treatment, the durability is limited and the function is non-regenerable as well (Figure 2). Obviously, in order to prepare durable protective antimicrobial textile materials slow-releasing mechanism is not a suitable method and a novel approach is in need.

Regeneration Principle and Durable and Regenerable Functions

In 1962¹³ Gagliardi proposed a model in making antimicrobial textiles, named regeneration principle. Although the model was presented over thirty years ago, there has been little reported success in textile and other related materials until recently. However, this principle has provided an important role in the design of this innovative functional finishing.

What are ideal protective antimicrobial textiles? It is commonly believed that the bio-protective clothing materials should possess the following features: 1) rapid inactivation of a broad spectrum of microorganisms; 2) non-selective and non-immutable to pathogens; 3) non-toxic and environmentally friendly; 4) durable to repeated washes; and easy to be recharged in laundering if rechargeable. If the functions are rechargeable, the recharging agents should be non-toxic, available at home, and compatible with our laundering chemicals such as detergents or bleaching agents. If regenerable properties are considered in selection of biocidal agents, only oxidative biocidal agents fit more closely into the requirements since redox reactions are reversible or regenerable. Bleaching chemicals such as chlorine and oxygen bleaches used at home are oxidizing agents.



After carefully examining the biocidal agents, it was found that halamine compounds are closest matches to the requirements. Halamine compounds are biocidal agents used in swimming pools¹⁴⁻¹⁵, similar to free chlorine, but safer than it because they are less likely to cause carcinogens (HCCl_3) in water. Halamines inactivate microorganisms mainly by oxidation mechanisms rather than biological functions, thus wide usage of them could result in less environmental concerns. If the halamine compounds can be covalently connected to polymers, a reversible redox reaction can then be played around on solid materials according to Equations 1 and 2. Covalent bonding between cellulose and the agents is not affected by inactivation of microorganisms and washing in water or chlorine bleach, thus providing a firm connection for biocidal sites on materials, while antimicrobial functions can be regenerated easily. The whole operation of modification of cellulose, activation or regeneration of halamine structures, and inactivation of microorganisms can be expressed by the regeneration principle (Figure 3)¹⁶⁻¹⁸.

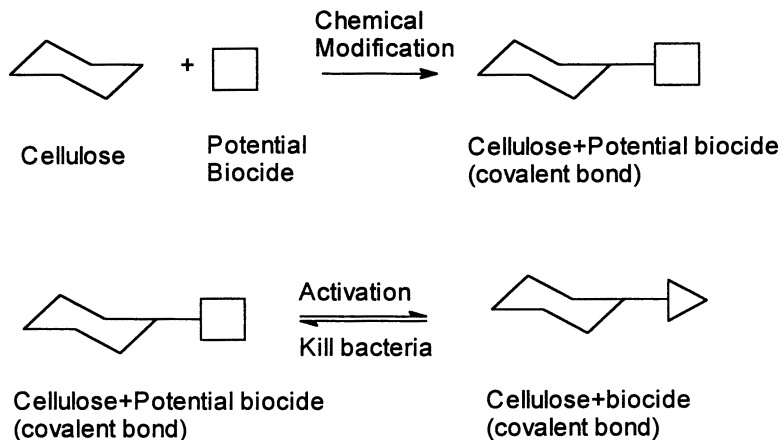
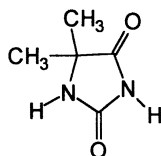
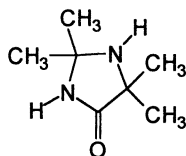


Figure 3. Regeneration principle (Reproduced from reference 16, copyright American Association of Textile Chemists and Colorists)

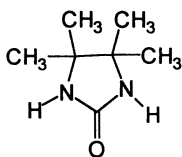
According to the mechanism of the biocidal function and regeneration process, chlorine solutions such as bleach solutions serve as both activation and regeneration agents of the biocidal functions. Chlorine bleach is one of the most frequently used home laundering chemicals. By using the laundering process, the potential biocidal groups grafted on cellulose, i.e. amide or imide N-H bonds in hydantoin rings, will be



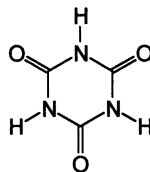
5,5-dimethylhydantoin



2,2,5,5-tetramethyl-4-imidazolidinone



4,4,5,5-tetramethyl-2-imidazolidinone



Triazine-1,3,5-trione

Figure 4. Halamine precursors

converted to biocidal halamine structures. It provides a convenient way for activation and regeneration of biocidal functions. Many of these halamine structures have been reviewed and investigated for water disinfection purposes¹⁴. Recent development of halamine polymers has brought in many applications of the chemical equations¹⁵.

Halamines that can achieve this durable and regenerable antimicrobial function are chlorinated products of 5,5-dimethyl hydantoin, 2,2,5,5-tetramethyl-imidozalidinone, and their monomethylol or dimethylol derivatives, shown in Figure 4. When chlorine replaces hydrogen on N-H bond, the N-Cl bond is stabilized by the vicinal methyl or carbonyl groups, which completely defers from another similar chemical, dihydroxyl-ethyleneurea (DHEU), while its derivative, dimethylol-dihydroxylethyleneurea (DMDHEU) has been employed in wrinkle-free treatment of cotton fabrics. DHEU will result an unstable halamine structure when chlorinated, distinctly different from the compounds listed in Figure 4 since it has a vicinal C-H to the N-Cl, which can quickly result in an elimination of HCl and forming C=N bond. Such a reaction not only causes losing of antimicrobial functions, but also generates HCl that damages cotton cellulose subsequently. Structural characteristics of stable halamine compounds were summarized by Worley¹⁴.

Table 2. Biocidal results of fabrics treated by 4% DMDMH

| <i>Microorganisms</i> | <i>Log reduction of microorganisms (Two minutes contact time)</i> | |
|--------------------------------------|---|--------------------------|
| | <i>Cotton</i> | <i>Poly/cotton 65/35</i> |
| E. coli | 6 | 6 |
| Staph. Aureus | 6 | 6 |
| Salmonell choleraesuis | 7 | 6 |
| Shigella | 6 | 7 |
| Candida albicans | 6 | 6 |
| Brevibacterium | 8 | 8 |
| Pseudomonas aeruginosa | 6 | 6 |
| Methicillin-resis. Staph. Aureus | 3 | 6 |
| Vancomycin resistant Enterococcus | 6 | 6 |
| MS/2 virus | 6 | 6 |

Notes: AATCC test method 100, contact time was two minutes. A 6 log reduction means total kill of the bacteria. A bleaching solution containing 0.01% Cl was used in activation and regeneration processes. Machine wash at 160 F for 30 minutes with 92 grams of AATCC detergent 124.

The stability of N-Cl bonds on halamines contributes to the durability and stability of antimicrobial properties on chlorinated fabrics, with evidence that the bleached fabrics could retain the antimicrobial properties for more than six months in conditioning room (at 21 °C and 65% relative humidity). After each laundering, the

fabrics, especially those treated with hydantoin derivatives, should be recharged by chlorine bleaching because of presence of predominant imide N-Cl bonds that can be washed off by detergents (reverse reaction in Equation 1). Adding amide or amine halamine structures can significantly improve washing durability of the antimicrobial properties based on the following stability order of halamine structure.

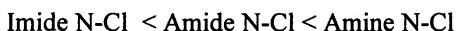


Table 2 The antibacterial properties of the finished fabrics were evaluated with Gram-positive and Gram-negative bacteria such as *Staphylococcus aureus* and *Escherichia coli*, fungus, yeasts, and virus following AATCC standard test method 100. These microorganisms represent a whole spectrum of pathogens that human bodies, particularly health care providers, are encountering every day. Based on characteristics of medical protection requirements, contact time of microorganisms on

Table 3. Durable and regenerable antimicrobial results of 6% MDMH treated fabrics (Reproduced from reference 16, copyright of AATCC)

| | <i>Fabric</i> | <i>Reduction of bacteria</i> | |
|-------------------------------|---------------|------------------------------|------------------|
| | | <i>E. coli</i> | <i>S. aureus</i> |
| After 10 washes and bleach | Cotton | 6 log | 6 log |
| | PET/cotton | 6 log | 6 log |
| After 20 washes and bleach | Cotton | 6 log | 6 log |
| | PET/cotton | 6 log | 6 log |
| After 30 washes and bleach | Cotton | 6 log | 6 log |
| | PET/cotton | 6 log | 6 log |
| After 35 washes and bleach | Cotton | 6 log | 6 log |
| | PET/cotton | 6 log | 6 log |
| After 40 washes and bleach | Cotton | 6 log | 6 log |
| | PET/cotton | 6 log | 6 log |
| After 45 washes and bleach | Cotton | 6 log | 6 log |
| | PET/cotton | 6 log | 6 log |
| After 50 washes and bleach | Cotton | 6 log | 6 log |
| | PET/cotton | 6 log | 6 log |

Notes: AATCC test method 100, contact time was 30 minutes. A 6 log reduction means total kill of the bacteria. A bleaching solution containing 0.01% Cl was used in activation and regeneration processes. Machine wash at 160 F for 30 minutes with 92 grams of AATCC detergent 124.

surfaces of fabrics was fixed at two minutes during testing, which was the shortest interval when a microbiological test can be managed properly. Two commonly used fabrics, pure cotton and polyester/cotton sheets, were treated by a finishing solution containing 4% of dimethylol dimethylhydantoin (DMDMH), and bleached in a diluted chlorine solution. The results, listed in Table 2, are reported in log reduction of microorganisms, with one log reduction referring to 90% kill and three log reduction meaning 99.9% kill. Comparing to other antimicrobial textiles, the new biocidal

fabrics exhibit superior properties as fabrication materials for medical workers and patients, owing to their rapid and effective inactivation of a broad range of microorganisms.

In addition, the outstanding biocidal properties of the fabrics are durable and regenerable in chlorine bleaching, a process commonly used in commercial laundering of medical-use textiles. Table 3 shows antimicrobial results of fabrics after repeated laundering and regeneration by bleaching. If the treated fabrics are only washed in water without adding detergents, their antimicrobial functions are not affected no matter whether they are soaked and stirred. Apparently, detergent solution may kick off chlorine atoms from halamine bonds or other additives in detergents may reduce halamine to chloride. Thus, after each laundry the fabrics are recommended to be bleached to recharge the antimicrobial functions. Chlorine bleaching is a required process in medical care industry as a routine sterilization procedure for used textiles, which certainly is very convenient. However, when this technology is applied to apparel products, recharging after each washing becomes a little burden to consumers. Recharging after a few washes or non-rechargeable functional materials would be more proper for apparel products.

Future directions

Functional textiles will be new trends in development of fabrics and clothing for next century. People expect to see more environmentally friendly and functional products that can provide benefits for health and safety and improve quality of life. Based on the needs of the functional materials, durable and refreshable antimicrobial synthetic materials or durable and regenerable antimicrobial fabrics may be suitable for different applications. Here, refreshable means the functions can be brought back by laundering without using a reversible reaction¹⁹. Materials used in carpets and non-laundry-able textiles are potential application for this category of functions. Overall, how to increase durability of functions on textiles, especially washing durability, will still be a difficult goal to achieve for textile scientist. More innovative methods and theories are anticipated for future research for development of new products.

Conclusions

Viewing major limitations of currently used functional modification methods, especially the slow-releasing mechanism widely employed in antimicrobial finishing of textiles, halamine derivatized textile materials were designed and prepared according to an innovative regeneration principle. Products developed based on the technology could demonstrate durable and regenerable antimicrobial functions, and could execute instant inactivation against a broad spectrum of microorganisms by contact without yielding drug-resistance. The unique properties of the products make them the best materials for medical-use and hygienic textiles.

Acknowledgements

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Chapter 15

Antimicrobial Properties of a Novel *N*-Halamine-Based Cellulose Treatment Process

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A novel method of permanently grafting antimicrobial *N*-Halamine compounds onto cellulose fibers has been developed. In AATCC tests (Method 100), treated fabrics showed potent, rapid biocidal efficacy, with up to 8 log reductions in challenge microorganisms evident after 2 minutes of contact. Efficacy was demonstrated against a wide variety of bacterial, fungal and viral organisms, ranging from pathogenic disease-causing agents to nuisance microbes responsible for odor generation or degradation of cellulose substrates. After exhaustion of the available chlorine atoms on *N*-halamines by interaction with microbial targets or bioburden, the biocidal capacity can be restored fully by exposure to a dilute hypochlorite rinse during routine laundering. These findings should permit practical applications in the development of antimicrobial textiles for such fields as healthcare, hospitality and sportswear, among others.

Introduction

Attempts to confer antimicrobial activities on cotton fabrics have been made over the years for several good reasons. These include protection of the fibers themselves against degradative effects of microbes on cellulosic polymers, for the benefits associated with control of bacteria and yeasts that generate volatile, odoriferous products in clothing that comes in contact with body fluids and skin cells of the user, and in order to prevent the survival and potential carriage of disease-causing agents that may contaminate cotton textiles. In the latter circumstance, cotton textiles may serve as inanimate vectors, conveying microbes so as to provide infection opportunities for them when other favorable situations arise. Effective antimicrobial efficacy in cotton can also be the basis for enhancing wound healing, by preventing access of potentially invasive organisms to lesions protected by the barrier function of cotton-based dressings.

In recent years the rationale for enhancing these functions in cotton textiles has acquired increased importance as the advantages of natural fibers in so many settings have become more apparent and more competitively successful in the commercial marketplace. Synthetic fibers are intrinsically much less likely to become subject to microbial degradative attack than cotton, and they often offer a much less hospitable microenvironment for the survival and transportation of bacteria and other flora than does cotton. As evidence has mounted for the incrimination of textiles in the dissemination of disease agents in healthcare contexts^{1,2}, and in food service areas, both institutional and domestic, so the need for enhanced protection of the fabrics and of the user/wearer has become more of an imperative. With these fabrics increasingly being based on cotton in pure or blended forms, new approaches to conferring high levels of antimicrobial action have been sought.

Instances have been coming to light which have made it clear that microbes can persist in cotton textiles for much longer than had previously been suspected, enabling them, for example, to survive on laboratory protective clothing for days after casual contamination^{3,4,5}. There is even evidence that, through such mechanisms, genetically engineered bacterial organisms could exit laboratories and enter environmental waste streams. Incidents of contamination of healthcare workers' protective clothing providing potential vector functions for antibiotic resistant bacterial pathogens have also surfaced. In yet another newly developed commercial context, bioburdens of microbes on cotton clothing have begun to assume importance in so called "clean rooms" used in preparation of electronic communications industry products, as standards of cleanliness required have become more and more demanding. In all these circumstances there is opportunity to enhance the performance of cotton textiles by conferring on them a broad spectrum antimicrobial action, especially if it can have the characteristics of being speedy, durable, and of sufficient breadth of

spectrum as to include human viruses. Concern about the latter in protective clothing worn by workers occupationally exposed to blood borne disease agents has led the US Occupational Safety and Health Agency (OSHA) to require these garments as hazardous and requiring special handling and laundering so as to avoid contamination of those involved in processing and cleaning them ⁶.

Novel Methods of Conferring Antimicrobial Activity on Cotton

There has been no shortage of efforts and methods for achieving this goal, though the resulting technologies have fallen short of the ideal of speedy, broad spectrum, durable, and preferably rechargeable efficacy.

Some of the historical background to this variety of approaches and an account of the most recent improvements have been reviewed in other components of this symposium publication. Here we characterize the biological performance of only that method based upon the procedure described by Sun and Xu ^{7,8,9} in a series of reports and US Patent #5,822,357 ¹⁰. In it, grafted heterocyclic ring compounds, hydantoin, serve as chlorine stabilizers covalently linked to the cellulose substrate, with the latter represented by cotton, wood pulp or even on wood surfaces themselves.

Chlorine, readily replacing H on available exposed N constituents of these anchored hydantoin, is able to participate in biocidal events triggered by interactions between Cl and oxidizable targets on the surface of microbes brought into close proximity or contact. Hydantoin is permanently affixed to the cellulose, and those Cl consumed by their participation in biocidal events can be replaced by exposure to dilute solutions of free Cl ^{7,8,9}. Therefore, textiles prepared in this way exhibit not only durable antimicrobial efficacy but also an unprecedented rechargeable feature. The antimicrobial properties reflect the power and speed of Cl, and in the experimental data reported here, these features are demonstrated to operate against a wide range of microbial organisms on woven and non-woven cotton based textiles.

Antimicrobial test data

Because the halogenated hydantoin is anchored and do not readily release free chlorine, conventional methods for demonstrating antimicrobial efficacy based on the diffusive release of free biocide into the local microenvironment—the so-called zone of microbial inhibition—cannot be applied. Inhibitory zones are minimal under circumstances where the challenge method depends on the slow release of biocides into an area populated by growing organisms, the net effect of which is to kill those which fall within a range of concentrations that is lethal. Retention of Cl on the grafted hydantoin demands that a test method be

applied for quantitation of efficacy that permits contact between the challenge microbes and the fiber surface for a sufficient time to allow lethal effects to be exerted, followed by recovery and enumeration of survivors. Such a test method is depicted diagrammatically in Figure 1, showing the sequence of steps involved in the AATCC Method 100 for contact biocides¹¹.

Examples of the kinds of results that can be achieved with challenge suspensions of bacteria and yeasts are shown in Figure 2 and Figure 3. In each instance an aqueous suspension of the challenge organisms was applied to swatches of cotton or cotton/polyester textiles grafted with hydantoin and either charged with Cl (test sample) or left uncharged (control). After 2 minutes of exposure at room temperature the surviving organisms were recovered and plated onto appropriate nutrient agar plates, incubated and counted. Recovery rates of these suspensions from non-halogenated grafted textiles were very high, often >95%. Recoveries from Cl charged swatches were undetectable in most instances, and based on the controls, log reductions in the numbers of viable challenge organisms were calculated. The speed and spectrum are remarkably evident in these experiments.

In order to assess how well this activity could be demonstrably conferred upon non-woven blended fibers the same grafting process was applied to a range of wood pulp cellulose/polyester blends, and the fiber mats challenged in a manner identical to that described for woven swatches. Challenge organisms included suspensions of MS-2 virus, a tough bacteriophage virus often used as a surrogate for mammalian enteroviruses in chemical disinfection tests. The results are shown in Table I, and include data on 55:45 wood pulp/polyester blends from two different commercial sources, as well as one sample of a rayon/polyester blend. High rates of kill were observed in all instances in uniformly short exposure periods (2 minutes).

Table I. Evaluation of antimicrobial efficacy of nonwoven textiles treated with the hydantoin grafting process

| <i>Product</i> | <i>Contact Time</i> | <i>Challenge Organism</i> | <i>Log Reduction of Challenge</i> |
|------------------------------|---------------------|---------------------------|-----------------------------------|
| 55:45 Wood Pulp:Polyester | 2 min. | <i>Staph. aureus</i> | 7 |
| | 2 min. | <i>E. coli</i> | 6 |
| | 2 min. | MS-2 Bacteriophage | 6 |
| 55:45 Wood Pulp:Polyester | 2 min. | <i>Staph. aureus</i> | 3 |
| | 2 min. | <i>E. coli</i> | 6 |
| | 2 min. | MS-2 Bacteriophage | 6 |
| 80:20 Rayon:Polyester | 2 min. | <i>Staph. aureus</i> | 5 |
| | 2 min. | <i>E. coli</i> | 7 |
| | 2 min. | MS-2 Bacteriophage | 6 |

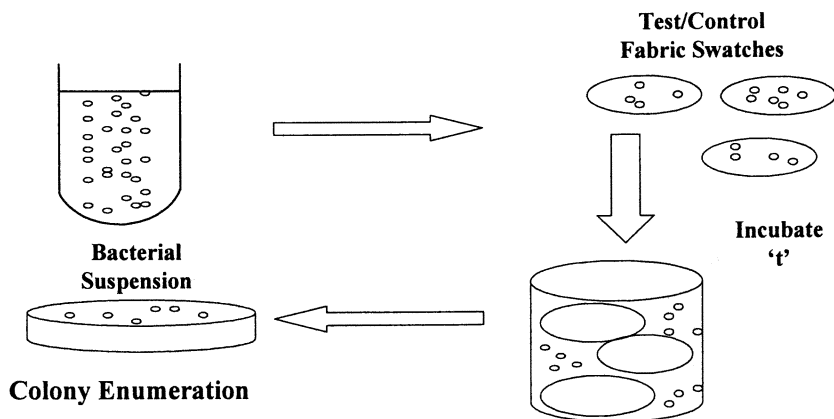


Figure 1. AATCC Method 100 Contact Biocidal Activity

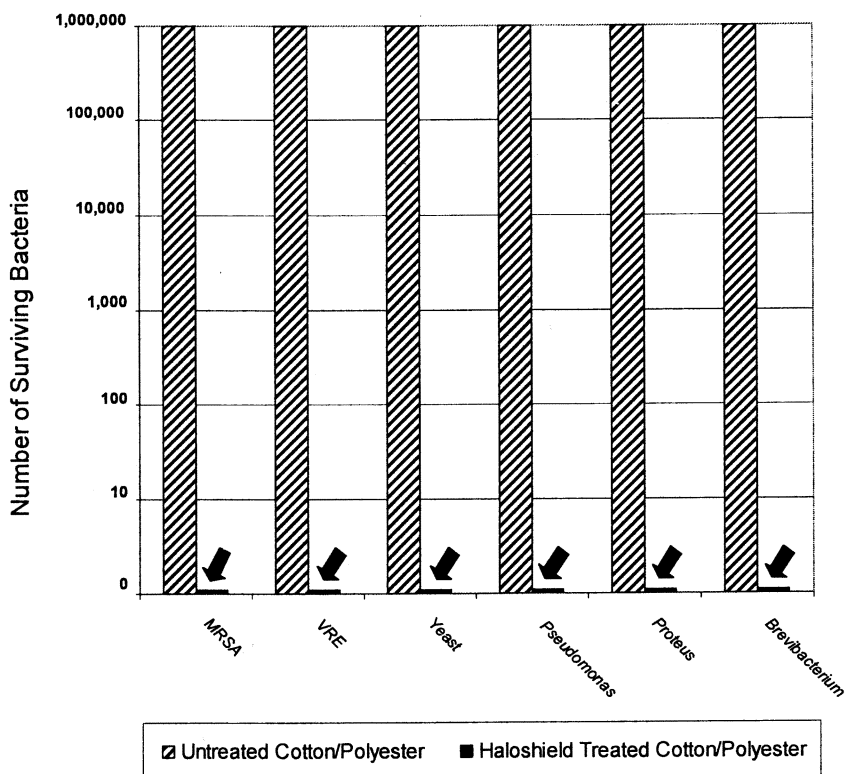


Figure 2. Efficacy of HaloShield @ Cotton/Polyester Fabric vs. Bacteria and Yeast. AATCC Method 100. Contact Time 2 Minutes

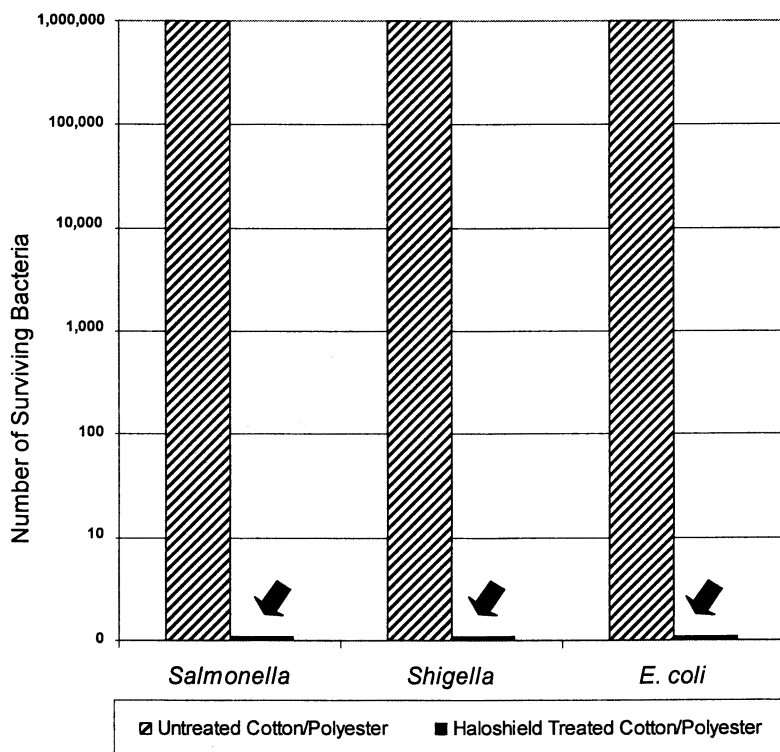


Figure 3. Efficacy of HaloShield® Cotton/Polyester Fabric vs. Food-Borne Bacterial Disease Agents. AATCC Method 100. Contact Time 2 Minutes

Additional data are shown in Table II for further commercially available cellulosic materials in a variety of formats, such as samples of pulp/polypropylene blends bonded to polypropylene backing, wood pulp alone, or treated cellulose in the form of utility wipes. Some of these materials were elements in the composition of diaper and incontinence pads. Yeasts, (*Candida albicans*), odor- generating bacteria (*Proteus mirabilis*), and pathogens (*Staph aureus*, *E.coli*) as well as MS-2 virus challenges all were killed to a very high degree after very short contact times, especially in comparison with commercially available antimicrobial preparations of comparable textile substrates.

Table II. Evaluation of antimicrobial efficacy of commercially available cellulose fibers treated using the hydantoin grafting (Haloshield®) Process

| Material | Contact Time | Challenge Organism | Log Reduction of Challenge |
|---|--------------|--------------------------|----------------------------|
| 70:30 Wood Pulp:Polypropylene (formed on a light weight polypropylene fabric) | 10 min. | <i>Staph. aureus</i> | 7 |
| | 2 min. | <i>Candida albicans</i> | 6 |
| | 2 min. | <i>Proteus mirabilis</i> | 6 |
| 70:30 Wood Pulp:Polypropylene | 10 min. | <i>Staph. aureus</i> | 7 |
| | 10 min. | <i>Candida albicans</i> | 6 |
| | 10 min. | <i>Proteus mirabilis</i> | 6 |
| Wood Pulp | 5 min. | <i>Staph. aureus</i> | 5 |
| 100% Cotton (tight weave) | 2 min. | <i>Staph. aureus</i> | 6 |
| | 2 min. | <i>E. coli</i> | 7 |
| | 2 min. | MS-2 Bacteriophage | 6 |
| Microfiber (cleaning cloth) | 2 min. | <i>Staph. aureus</i> | 6 |
| Rayon polyester (cleaning cloth) | 2 min. | <i>Staph. aureus</i> | 6 |
| | 2 min. | <i>E. coli</i> | 6 |

Microbes in nature are generally, of course, in a context of some kind of biological material, such as body fluids in the case of pathogens, urine in the case of odor-generating nuisance organisms, blood in the case of human leukemia viruses, and so on. In order to assess how significantly these antimicrobial effects would be influenced by the presence of bioburden in the suspension of organisms, challenges were prepared in concentrations of serum up to 5%, typical of circumstances used in many regulatory agency test protocols. Results are shown in Table III, and show that in the presence of these serum proteins, contact times are significantly prolonged to bring about the same degree of log reductions in viability, but these are still in the range of tens of minutes, very much faster than most commercially used antimicrobial textiles in the absence of serum bioburdens.

Table III. Evaluation of antimicrobial capacity of hydantoin treated cellulose in the presence of a bioburden

| Material | Bioburden | Contact Time | Challenge Organism | Log Reduction of Challenge |
|------------------------------|-----------|--------------|----------------------|----------------------------|
| 100% Cotton (tight weave) | 5% serum | 10 min. | <i>Staph. aureus</i> | 6 |
| | 1% serum | 10 min. | <i>E. coli</i> | 7 |
| | 5% serum | 20 min. | MS-2 Bacteriophage | 2 |
| 100% Cotton #400 | 5% serum | 20 min. | <i>Staph. aureus</i> | 6 |
| Polyester/Cotton #7049 | 5% serum | 20 min. | <i>Staph. aureus</i> | 6 |

Inhibition of odor generation by application of ammonia- generating bacteria to urine soaked non-woven hydantoin grafted cellulosic substrates, charged with Cl in the case of the test samples, was assessed using human “sniff” tests. Volunteers scored swatches incubated with urine and *Proteus*, after 4 hours, and the results are shown in Table IV. No ammonia was detectable in the vapor above swatches that had been charged with Cl prior to the incubation, illustrating the potential utility of this system in controlling objectionable urine ammonia production in pads and diapers.

Table IV. Qualitative smell test of textiles

| Number of Observations | Contact Time | Control Diaper Material ¹ | Treated Diaper Material ¹ |
|------------------------|--------------|---|--------------------------------------|
| 10 | 4 hours | Odor of stale urine, litter box smell; reactions ranging from gagging to coughing | Light chlorine smell |

1. Inoculated with *Proteus mirabilis* suspended in phosphate buffered water (pH 7) and filter sterilized cat urine. Placed in separated sterile petri dishes and wrapped with parafilm.

Conclusions

Few existing biocidal chemical systems lend themselves readily to covalent linkage to cellulose. Hence, most antimicrobial technologies depend on creation of reservoirs of impregnated chemicals which are released into microenvironments, rather than functioning by killing organisms on contact. Wash-out can occur on processing of re-useable textiles, leading to limited durability of efficacy of the treated fabric. Even fewer chemical biocides offer

an opportunity to add a recharge potential once the biocidal entity has been exhausted. The hydantoin graft described in publications by Sun and Xu^{7,8,9}, permits permanent incorporation of a rechargeable hydantoin, convertible to a biocidal surface by the simple expending of laundry rinsing with hypochlorite at low levels.

The biological performance illustrated above is remarkable for its speed, spectrum and potency, and offers many advantages in applications related to the principal needs for antimicrobial activity: preservation, odor control, prevention of carriage and dissemination of disease agents, and wearer protection. These features differentiate hydantoin grafted cotton from all other treatments. The efficacy reflects the power of Cl, and extends to mammalian viruses, even in the presence of high bioburden levels (data not shown).

Although the precise molecular mechanisms of action are not known, it is reasonable to infer from the results that Cl is irreversibly transferred to microbial target chemical structures in the course of the biocidal events when microbes are incubated with treated textile swatches. Since these Cl atoms are not readily released into the microenvironment, there would not likely be a risk of exposure of wearer skin to Cl, and there should correspondingly be little irritation or sensitization tendency associated with use of these textile products. Hydantoin themselves have a history of widespread use as cosmetic additives, and in chlorinated form, as halogen sources for recreational water disinfection¹². In these use patterns they have not exhibited sensitization capacities, suggesting once again that there will be little toxicological risk from their use in garments, sheets, diapers or other products involving intimate human skin contact.

Acknowledgements

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Chapter 16

Biodeterioration of Wool by Microorganisms and Insects

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Wool is stable through alpha-keratin peptide linkages and a highly crosslinked network of disulfide bonds. Microbes and insects can digest wool by secreting extracellular keratinolytic enzymes that catalyze the hydrolysis of peptide linkages releasing polypeptides and soluble sulfhydryl-containing amino acids that disrupt disulfide bonds. Ensuing mechanical damage opens the fiber's outer cuticle to attack of the inner cuticle layers and the lipid-rich complex of membrane cells (CMC), whereby the innermost structural elements, the cortical cells, become vulnerable to enzymatic digestion. Wool keratin can be the ideal substrate for enzymatic attack by proteases, esterases, lipases, and those that act specifically on disulfide bonds. The principal causes of accelerated microbial growth are prolonged high moisture, ambient temperature, and assimilable sources of nutrients for the specific organism. Enzymatic digestion is assisted if carbohydrates, fats, and additional sources of nitrogen are present in culture media, soaps, suint, and products of chemical attack on wool. Wool's heterogeneous and composite structure confounds the elucidation of the mechanisms of biological decay, consequently correlations among chemical and structural modifications are difficult. Biological decay can be documented with microscopy inspection to show damage to the edges of the distal scales on the cuticle and disaggregation with fiber fibrillation and fraying to the cortical cells. The role of wool's morphological fiber components (including constituent fatty acids of wool's lipids as sole sources of carbon and energy) to resist microbiological attack by bacteria, fungi, and mildew is examined, as are chemical modifications to protect wool from insect attack.

[†] Mention of brand or firm name does not constitute an endorsement by the Department of Agriculture above others of a similar nature not mentioned.

Introduction

For 12,000 years, wool, the “Golden Fleece,” has provided not only warmth and comfort, but has been and is a vital commodity in world trade. With the advent of wool spinning in 3500 B.C. men could expand the frontiers of exploration, thereby spreading civilization, with sheep at their side to provide food and clothing suitable for changing climates. Throughout history, the sheep industry spread from Central Asia to Europe by way of Greece, to Rome, Persia, North Africa, and to Europe where all contributed to improvements in the breeds. The Spaniards who colonized California and New Mexico introduced the first sheep into America in 1519. By 1698 wool production had become a colonial initiative and essential enterprise for export and trade. Soon spinning and weaving became acts of patriotism to allay the threat of British imperialism. By the 19th century millions of sheep grazed in the Southwest where Spanish sheep were mixed with the sheep from Eastern herds brought by pioneers seeking grazing lands in the West and Northwest. Although wool’s current position in various end-use markets is low as a percentage of fiber production worldwide, it is stronger than the overall data might suggest because wool is positioned at the quality, high value end of the market. In these sectors wool’s position is stronger than in the market as a whole.

Wool’s physico-chemical structure combines strength, elasticity, and resiliency with hygroscopicity for durability and comfort. As wool absorbs moisture, heat is generated, causing a net slowing of the transmission of body heat; hence, wool is warm in winter. (1) Wool fiber consists of a thin outer covering of overlapping, scales for moisture penetration into inner spindle-shaped cortical cells at the fiber core. These cortical cells are longitudinally divided into distinct sections, their relative proportions and arrangement varying with fiber diameter. (2) They exhibit differential behavior to chemical and physical stimuli, and are responsible for wool’s crimp that contributes to warmth and insulation. Paradoxically, wool provides coolness by slowing heat transmission from the atmosphere to the body and trapping body heat for slow cooling through evaporation. The complex nature of wool and its unique properties suggest a myriad of end uses that continue to inspire scientific investigations.

The hydrophobic nature of the surface of wool keratin fibers suggests that a waxy or oily lipid material is present. Apparently and observably, the surface and bulk portions of wool exhibit different mechanical and chemical properties. Lipids, present both in external grease originating from sebaceous gland secretions (wool grease) and in internal structural material, were proposed to originate as lipoprotein membranes of once living follicle cells (3). There is evidence of amide and ester covalent linkages between lipid and protein in the protein membranes (4). The three major lipid classes in wool with their approximate amounts are: sterols (40%) consisting of cholesterol and desmosterol (2:1), polar lipids (30%) consisting of ceramides and glycosphingolipids, cholesterol sulfate, and fatty acids (25%) consisting of stearic, palmitic, 18-methyleicosanoic acid, oleic, and myristic acids (4). Only small amounts of phospholipids have been found. The composition of raw wool from fine Merino and coarse Lincoln breeds is reported in Table I.

Table I. Composition of Raw Wool (5)

| <i>Component</i> | <i>Merino</i> | <i>Lincoln</i> |
|---------------------|---------------|----------------|
| Moisture | 16.8 | 17.1 |
| Grease ^a | 20.2 | 6.7 |
| Suint ^b | 9.3 | 2.3 |
| Sand and dirt | 7.2 | 5.1 |
| Clean fiber | 46.5 | 68.8 |

^aAn ester of high molecular weight fatty acids – a saponifiable portion contains esters of cerotic, stearic, and palmitic acids with some free fatty acids, and an unsaponifiable portion containing cholesterol (C₂₇H₄₅OH).

^bDried perspiration consisting of a mixture of potassium salts of organic acids (oleic, palmitic, and acetic).

Wool is a member of the alpha-keratin family of proteins, as are horn, fingernails, skin, and feathers. Its fiber diameter ranges from 18 microns (fine-grade) to 40 microns (coarse-grade). The morphology of wool is highly complex because its composite structure includes an outer layer of grease that sometimes exceeds the weight of protein, and a covalently bound surface layer surrounding differentiated cellular structures. Each fiber is made up of three cellular structures: the cuticle, cortex (90% by weight), and the medulla (hollow cells that are absent in fine wool fibers) (6). Cuticle cells or scales are flat, rectangular, plate-like structures that are arranged to overlap in a manner similar to roofing tiles, to completely cover the cortex. They compose the outermost regions of the wool fiber and can be removed by ball milling, grinding, scraping, slicing, or enzymatic treatment. Within each cuticle cell there are three isolatable layers: the exocuticle A-layer, (100°A thick) being outermost that forms sacs on the outer surface, the exocuticle B-layer that is more readily digested by enzymes, and the innermost layer, the endocuticle (7).

During the keratinization process, the living cell membranes form a new structure commonly referred to as the Cell Membrane Complex (CMC) that exists underneath the cuticle and between all regions in assemblies of keratinized tissues, for example, within the intercellular spaces between the underlying cortical cells of the wool fiber. Essentially, the cuticle and cortical cells are held together by a continuous, chemically-resistant proteinaceous CMC membrane. Under transmission electron microscopy two regions were identified as comprising the CMC: an intensely stained gamma-layer consisting mainly of protein and a beta-layer containing lipids. (8). The lightly crosslinked protein material is referred to as “globular protein cement.” The lipid component is susceptible to enzymatic attack. Although only 3% by weight of fiber, it has an inordinate effect on mechanical and chemical properties. Current thinking is that the adhesive strength of the CMC derives from intercellular linkages formed between intermediate filament proteins of neighboring cells bridged by proteins remnant from the membrane keratinization process (9). The CMC contains a similar amino acid composition as wool but with lower cysteine content (1.3% compared to 10% for whole wool and 11.9% for the epicuticle), thus, it contains less

crosslinked disulfide bonds (10). Consequently, it is easily swollen and easily modified.

Underneath, the cortical cells (100 microns long and 4 microns thick) are arranged lengthways along the fiber axis as closely packed elements that develop near the base of the fiber follicle. They elongate, keratinize, and form cross-links through the amino acid, cysteine. The differentiation of the cortex into ortho- meso-, and para cells leads to a bilateral structure (one side referred to as ortho-cortex and the other as paracortex) that is responsible for the crimp in wool fibers. Whiteley and Kaplin have shown that in the fine (Merino sheep) fibers, those with low-crimp frequency differ from those with high crimp frequency by the substitution of more mesocortex for paracortex on the central paracortical side of the fiber (11). In Figure 1, the mesocortex is shown at the boundary of the bilateral structure as a highly ordered component that is differentiated from the paracortex. In a wool follicle the paracortex is found on the inside of a crimp curvature and is thought to be richer in cysteine amino acids, thus it reacts differentially to environmental changes (12).

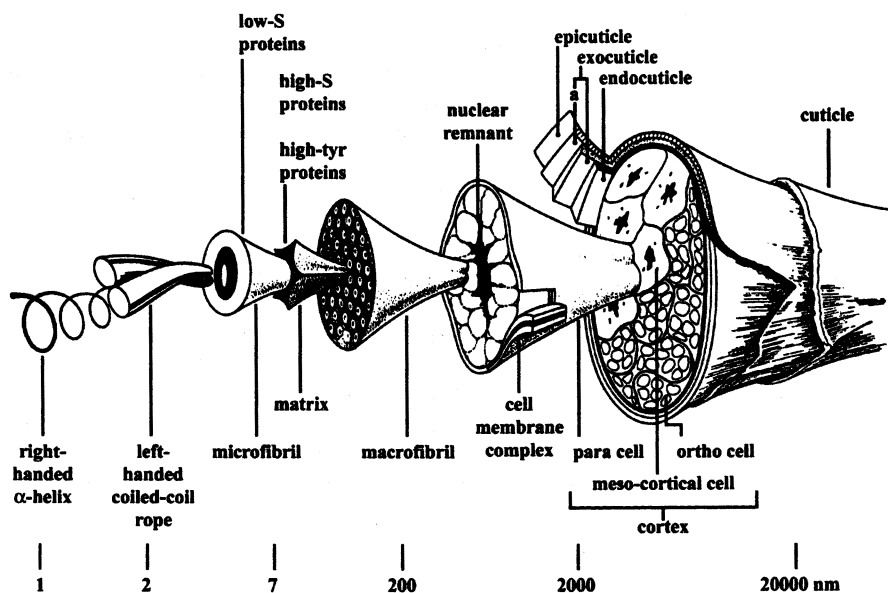


Figure 1. Cross-section of a Merino wool fiber showing the structure at progressive magnifications (Produced by H. Roe from a drawing by Dr. RDB Fraser, in Freughelman, M., *Mechanical Properties and Structure of Alpha-keratin Fibres*, University of South Wales Press, Sydney, Australia, 1997).

At a higher level of order, the proto-, micro-, and macrofibrillar structures of wool are made up of the cortex cells containing spindle-like crystalline proteins surrounded by the amorphous CMC matrix that is rich in high-sulfur and high

glycine/high tyrosine globular proteins. The assemblies of microfibrils, rich in low-sulfur proteins containing lysine, leucine, and aspartic and glutamic acids, favor the alpha-helix configuration (13). Each microfibril can be approximately 7nm in diameter and at least one micron long (14, 15). The protein chains are associated through hydrogen bonding and polar forces of intramolecular attraction and cystine disulfide groups are associated through covalent bonds of intermolecular attraction. Protofibrils pack to form microfibrils that build to form higher-ordered macrofibrils. A cortical cell, composed of a bundle of 500-600 microfibrils as the primary structural element in keratin fibers, has been described as "a bundle of keratin intermediate filaments, embedded in keratin-associated proteins" (16). These filaments aggregate in discrete domains to form the microcrystalline structure of alpha-keratin wool fibers. In 1950, X-ray data confirmed that wool's molecular architecture is one of associated helical configurations. It was postulated that "there are 3.6 to 3.7 amino acid residues per turn of the helix and the nitrogen atom of each planar peptide is bonded to the oxygen atom of the third peptide beyond it in the chain by a hydrogen bond with length 2.79°A" as shown in Figure 2 below (17).

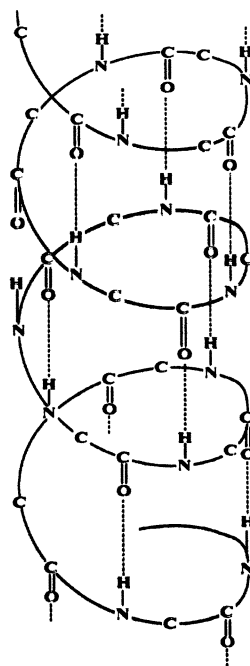


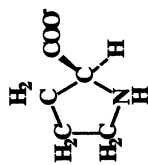
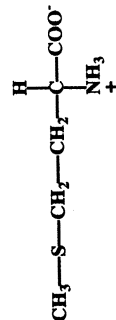
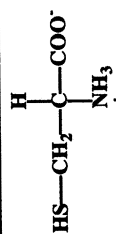
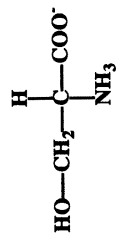
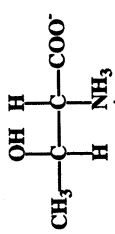
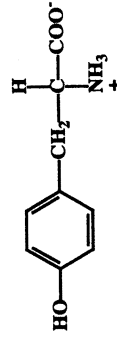
Figure 2. Helical molecular arrangement of wool (*The International Wool Secretariat, Heusden, The Netherlands*)

Wool keratin is of amphoteric nature, exhibiting acidic and basic functionalities that contribute to ionic forces of attraction or salt linkages between helical chains, shown in Figure 3 below. Through hydrolysis wool decomposes into the various amino acids shown in Table II.

**Table II. Amino Acid Composition of Wool Keratin
(Molecular Weight 68,000 kD) (18,19)**

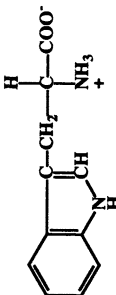
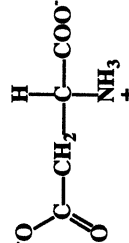
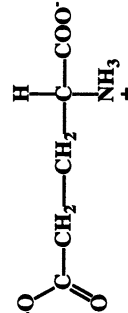
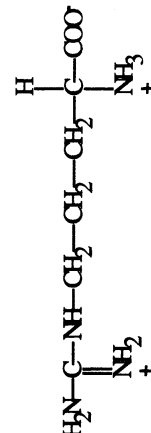
| <i>Amino Acids</i> | <i>Mol. Wt.</i> | <i>% of Wool (g/100g)</i> | <i>Chemical Nature</i> | <i>Structure</i> |
|---------------------|-----------------|-------------------------------|----------------------------|---|
| Glycine (Gly) | 75.07 | 6.50 | NP ^a | $\begin{array}{c} \text{H} \\ \\ \text{H}-\text{C}-\text{C}-\text{O}-\text{O}^- \\ \\ \text{N H}_3^+ \end{array}$ |
| Alanine (Ala) | 89.10 | 4.40 | NP | $\begin{array}{c} \text{H} \\ \\ \text{CH}_3-\text{C}-\text{C}-\text{O}-\text{O}^- \\ \\ \text{NH}_3^+ \end{array}$ |
| Valine (Val) | 117.15 | 4.72 | NP | $\begin{array}{c} \text{H} \\ \\ \text{CH}_3-\text{CH}-\text{C}-\text{C}-\text{O}-\text{O}^- \\ \quad \\ \text{CH}_3 \quad \text{NH}_3^+ \end{array}$ |
| Leucine (Leu) | 131.18 | 11.30 | NP | $\begin{array}{c} \text{H} \\ \\ \text{CH}_3-\text{CH}-\text{CH}_2-\text{C}-\text{C}-\text{O}-\text{O}^- \\ \quad \\ \text{CH}_3 \quad \text{NH}_3^+ \end{array}$ |
| Phenylalanine (Phe) | 165.19 | 3.75 | aromatic NP | $\begin{array}{c} \text{H} \\ \\ \text{C}_6\text{H}_5-\text{CH}_2-\text{C}-\text{C}-\text{O}-\text{O}^- \\ \\ \text{NH}_3^+ \end{array}$ |

Continued on next page.

| Proline (Pro) | 115.13 | 6.75 | cyclic NP |  |
|-----------------------------|----------|--------------------|-------------------------|--|
| Methionine (Met) | 149.21 | 0.71 | thiol NP |  |
| Amino Acids | Mol. Wt. | % of Wool (g/100g) | Chemical Nature | Structure |
| Cysteine (Cys) ^c | 121.16 | 12.72 | sulfhydryl NP |  |
| Serine (Ser) | 105.09 | 9.41 | hydroxyl P _b |  |
| Threonine (Thr) | 119.12 | 6.76 | hydroxyl P |  |
| Tyrosine (Tyr) | 181.19 | 5.80 | phenolic P |  |

Continued on next page.

Table II. Continued

| | | | | |
|------------------------|--------|-------|-----------------|---|
| Tryptophan (Trp) | 204.23 | 0.70 | indole NP |  |
| Aspartic Acid (Asp) | 133.11 | 7.27 | acidic (-) P |  |
| Glutamic Acid (Glu) | 147.13 | 15.27 | acidic (-) P |  |
| Arginine (Arg) | 174.20 | 10.40 | basic (+) P |  |

| <i>Amino Acids</i> | <i>Mol. Wt.</i> | <i>% of Wool (g/100g)</i> | <i>Chemical Nature</i> | <i>Structure</i> |
|--------------------|-----------------|-------------------------------|----------------------------|---|
| Lysine (Lys) | 146.19 | 3.30 | basic (+) P | $\text{H}_3\text{N}^+-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{C}(\text{H})-\text{COO}^-$ |
| Histidine (His) | 155.16 | 0.70 | imidazole P | $\begin{array}{c} \text{H} \\ \\ \text{HC}=\text{C}-\text{CH}_2-\text{C}-\text{COO}^- \\ \quad \quad \\ \text{HN}^+ \quad \text{NH} \quad \text{NH}_3^+ \\ \quad \quad \quad \\ \quad \quad \quad \text{H} \end{array}$ |

^aNonpolar; ^bPolar; ^cCysteine forms Cystine, (-NH-CH(CO)-CH₂-S-S-CH₂-CH(CO)-NH-) disulfide groups through intermolecular crosslinking as shown in Figure 3.

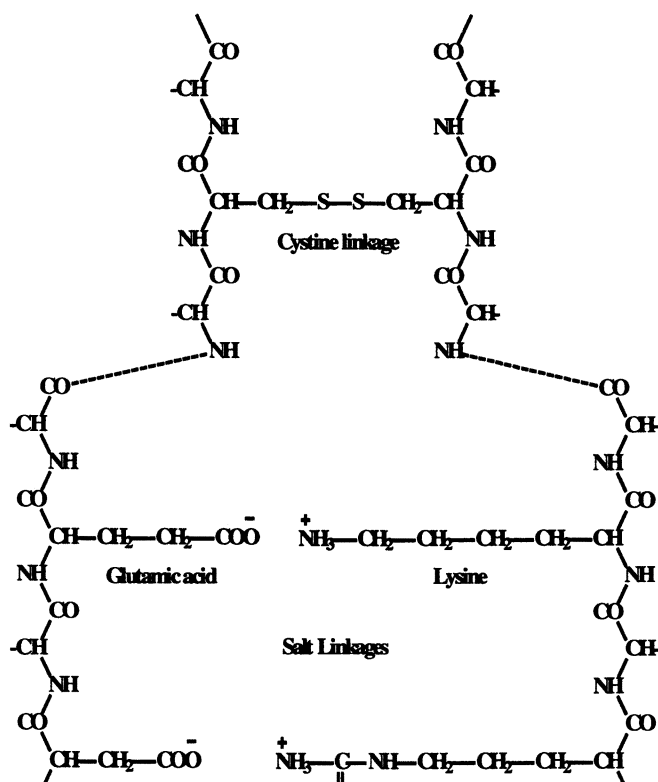


Figure 3. Molecular Structure of Wool (Von Bergen, W., *American Wool Handbook: Text and Reference Book for the Entire Wool Industry*, NY: Textile Book Publishers, 1948).

Wool's chemical composition is 50% carbon, 22-25% oxygen, 16-17% nitrogen, 7% hydrogen, and 3-4% sulfur (19). The salient features of wool's composition leading to its unique set of physical and chemical properties are the following: a great number of protein configurations enable great flexibility; a large number of highly polar peptide linkages give rise to inter- and intramolecular bonding and high reactivity; large side chains pendant from the polypeptide backbone prevent close packing of the protein molecules leading to a high amorphous content accessible to the environment; forces of association through side chains including intramolecular hydrogen bonding and intermolecular covalent bonding through cystine's disulfide linkages contribute stability and strength. Reagents that alter the stability of the disulfide bond will alter and destroy wool's physical structure (19).

Microbial Contamination

Wool as fleece on the sheep, and through many stages of manufacture and wear, is subjected continually to contamination by microorganisms. Pathogenic organisms may be contained within this microorganic flora, as well as nonpathogenic microbes, capable of multiplying rapidly under favorable conditions. The problem becomes visually apparent when wool develops mildew staining that can accompany strength loss or other forms of deterioration. Mildew will form in wool storage when bales become wet or in fabric storage when occluded starch or glue becomes wet. A comparison of the amounts of mold and bacteria found in wet and dry wool has been reported as shown in Table III.

Table III. Bacterial and Mold Counts per gram in Wool (19)

| | <i>Raw Wool</i> | <i>Shaken Wool</i> | <i>Scoured Wool Wet</i> | <i>Scoured Wool Dried</i> |
|----------------------|-----------------|--------------------|-------------------------|---------------------------|
| Molds | 2,700 | 36,000 | 300 | 300 |
| Bacterial, all types | 1,200,000 | 17,000,000 | 65,000,000 | 3,400,000 |
| Bacterial spores | 190,000 | 210,000 | 100,000 | 110,000 |

Shaking spreads molds and bacteria and results in a wider distribution of spores over the fiber surfaces. In the case of molds, scouring removed substantially most of the contaminant; but led to higher bacterial counts because of redistribution over a greater area. Only scouring followed by drying with heat was effective in inactivating bacterial contamination. Modern nitrogen scours with ethoxylate detergents at 65C have been found to kill most organisms before they reach the peroxide bath (20)

In simulated storage studies, the mean numbers of bacteria and fungi in 30 scoured wools were recorded before and after storage in TableIV.

**Table IV. Number of Micro-organisms on Scoured Wool
(Mean Counts /gram) (21)**

| | <i>Before Storage</i> | <i>After Storage</i> |
|----------|-----------------------|----------------------|
| Bacteria | $10^3 - 10^4$ | 10^9 |
| Fungi | $<10^2$ | 10^2 |

From this study it was established that under favorable conditions, the presence of more than 10^4 and 10^3 bacterial counts per gram of wool can cause biodeterioration whereas damage from fungal attack was less acute.

Microbial contamination includes bacteria (parasitic or saprophytic one-celled organisms that form mycelial-producing colonies) and fungi (any of a group of thallophytic plants comprising the molds, mildews, rusts, smuts, mushrooms, etc.). In

textile terms, "mildew" is defined as "a superficial growth produced by certain species of spore-forming fungi on textile materials that may lead to discoloration, tendering, and variation in dyeing properties" (22). Its growth is formed by a warm and moist, but not too wet, confined atmosphere. Soaps and sizings are conducive to its growth that is manifest by colored areas. The fungi are airborne, infecting, spore-bearing organisms that exist primarily as filamentous hyphae (23). Species of *Penicillium* and *Aspergillus* can form bright multicolored stains on tops, yarns, and fabrics. Typically they excrete extracellular proteolytic enzymes and break down wool fibers into easily absorbable, metabolizable, and diffusible nutrients so that degradation products can sustain microbial growth.

Mildew is produced by the attack of molds, fungi, and bacteria. Molds produce a musty smell and localized discolorations on the surface of materials that ultimately leads to loss of strength. Relative humidity, percentage and type of residual materials that are not completely washed out in textile processing can induce or inhibit the growth of these microorganisms. Bacteria, like fungi can digest insoluble organic matter by secreting exoenzymes, then absorbing the solubilized nutrients. There is risk of biodegradation from microorganism growth when more than 1000 bacteria or 1000 fungi per gram of wool are present. Thus it is important to examine the role of auxiliary products used in the various stages of textile manufacture in supporting microbial growth.

The species, *Penicillium*, *Aspergillus*, *Actinomyces*, *Chrysosporium*, and *Scopulariopsi* fungi are known to degrade wool. They appear as bright, multicolored stains from mold growth that live on impurities such as fatty oil and soap residues that are left behind in processing, Solubilized keratin and body fluid containing residual nitrogenous matter also contribute to mold growth (24) Keratinophilic (wool colonizing) fungi, *Chrysosporium keratinophilum* and *Malbranchea anamorph of Uncinocarpus reesi*, *Aspergillus fumigatus*, *Aspergillus. flavus*, and *Scopulariopsis candida* were isolated from soil samples and were shown to utilize wool lipids and fatty acids such as cholesterol, myristic acid, palmitic acid, and linoleic acid as a sole source for carbon and energy after 10 to 15 days at 25C (25). In the case of further colonization of wool keratin, it was found that *Chrysosporium keratinophilum* and *Malbranchea sp.* softened and weakened the fiber whereas *Aspergillus fumigatus*, *Aspergillus flavus*, and *Scopulariopsis. candida* had no effect on wool's mechanical properties (25).

The important bacteria that degrade wool are *Proteus vulgaris*, *Bacillus megathertium*, *Bacillus mycoides*, *Pseudomonas pycyanes*, *Pseudomonas fluorescens*, *Bacillus mesentericus vulgatus*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus putrificus*, and *Bact. Alkaligenes faecalis*. All are active within a temperature range of 15C to 50C with maximum activity at 25C to 37C. Optimum pH ranges for their activities are 7.6 to 8.2. They are strongly retarded by slight acidity (26). Under these conditions, bacterial attack readily occurs with relative humidity of 95%, equivalent to 25-30% regain in wool and becomes prevalent when wool's regain is over 40%.

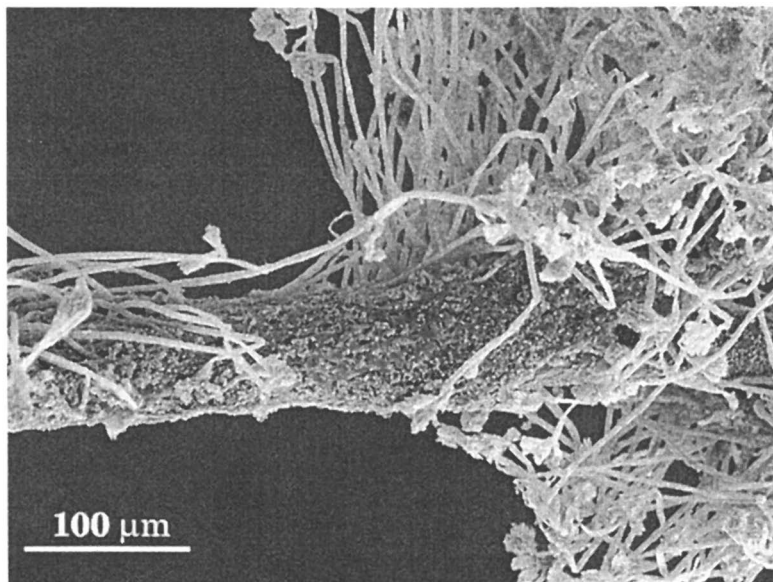
Humidity plays an important role in the spread of fungi and bacteria. Interestingly, grease in raw wool fibers contains chemical components known to have relatively high natural resistance to microbial attack (27). In degreased wool,

experience has shown that at relative air humidities up to 50% there is little risk of fungal attack. However, at 75% and above, relative humidity and temperatures of 15C to 40C, fungi can propagate at a fast rate. Ideal conditions for mildew growth are 95% relative humidity and a pH range of 6.5 to 8.5. After being subjected to these conditions, degraded wool can be characterized microscopically from cortical cells that split and detach in the paracortex half of the fiber while the orthocortex side remains resistant and intact (28). While molds affect the surfaces of wool, certain proteolytic bacteria and fungi rapidly attack the CMC between the cortical cells. Mildew fungus produces filamental hyphae and spores that attack cuticle cells. Once infected, they begin to lift off and curl up, sometimes freeing cortical cells. At the fundamental level, the higher the degree of disulfide crosslinking, the more resistant keratin is to microbiological attack (29).

Intact wool that is free of mechanical and chemical damage, with structurally sound scales, and intercellular matrices to hold cortical cells together, can be either completely resistant to attack or degraded by the proteolytic enzymes pepsin, trypsin, chymotrypsin, and papain that can cause structural damage. In one case, clean wool with structural integrity was degraded to 2% of its weight within 72 hours with papain (30). Pepsin and chymotrypsin can attack mechanically damaged wool that has the enzyme-resistant exocuticle removed or damaged. Trypsin cannot digest intact keratin consisting of ortho- and para-cortex unless the disulfide bonds of the remaining 90% of wool keratin become ruptured through oxidative or reductive processes (24). It can however, cut a peptide bond only when an arginine or lysine residue is on the carboxyl side of the peptide bond, causing damage to the exocuticle, the more vulnerable endocuticle, and the associated cortex. Free amino acids are the ultimate degradation products formed by protease digestion of keratin (24). If only 10% by weight of the fiber were solubilized enzymatically, there would be severe disruption of fiber structure so that it would have no useful life. Interestingly, when the sulfhydryl groups of reduced wool are reoxidized to disulfide groups, wool regains its stability to enzymatic attack but when converted to bis-thio ether groups by reaction with aliphatic dihalide, enzymatic attack is enhanced (19).

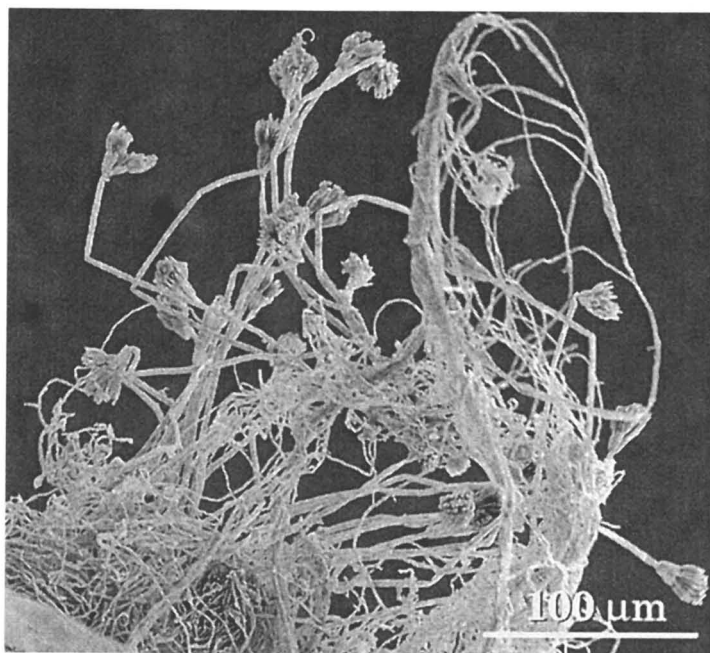
Filamentous fungal species in *Aspergillus* section *Flavi*, more commonly known as the *Aspergillus flavus* group, are of economic importance because many isolates produce the potent carcinogenic aflatoxin in foods (including peanuts and corn) and seeds yet the species possesses metabolic versatility that can have positive impacts. They are generally known as storage fungi but can cause ear rots in the field where it can grow at temperatures higher than 90F, with grain moisture as low as 16%. *Aspergillus flavus* has been isolated from the mycoflora of the hair of goats (31). The alkaline protease isolated from *Aspergillus flavus* has been used as a depilation agent to replace the lime-sulfide process for the removal of hides and skins (32).

We found colonies of *Penicillium aculeatum* in spent onion skin dyebaths containing dyed wool and *Aspergillus flavus* in red henna dyebaths containing the dyed American wool fiber. Before dyeing the fiber we washed it in successive steps with nonionic detergent at 40C. Scanning electron photomicrographs showed the presence of residual bacteria on the washed wool, as shown in Photograph 1. From the onion skin dyebath, we observed bacterial growth that enveloped the fibers in a

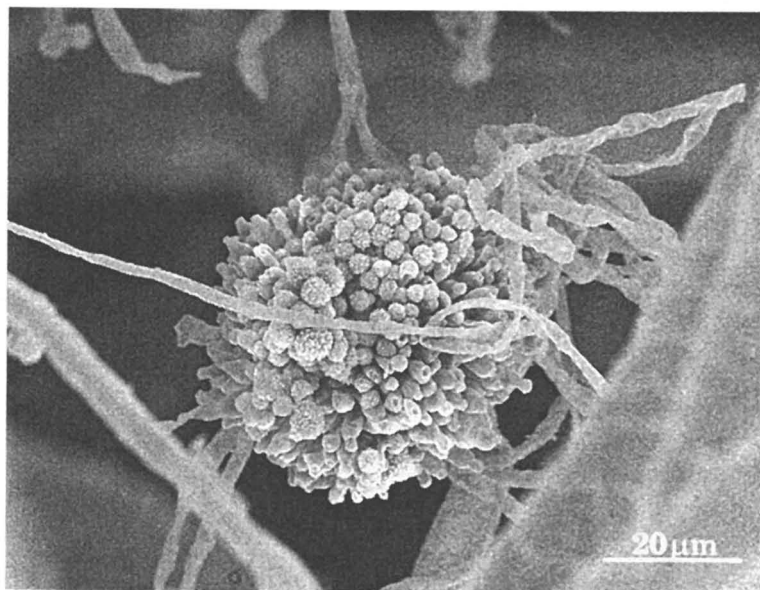


Photograph 1a

Photograph 1 (a-h): *Penicillium aculeatum* growth found on wool fibers, found in residual onion skin dyebath (a-b) and *Aspergillus flavus* growth cultivated from residual red henna dyebath (c-h): (a) - evidence of advanced bacterial growth and mycelia development with fungal conidiophores; (b) - detail of hyphae with radiating conidial heads arising from phialides, and typically splitting into several poorly-formed columns; (c) - cluster of conidiospores arranged in chains at the end of conidiophores that are attached to the phialides; (d and e) - evidence of reproduction by the species with the appearance of cleistothecia (closed ascocarps) or fruiting bodies that are lodged within the cuticle layer; (f) - obvious damage by penetrating fruiting bodies that are lodged within the cortical cells, aligned along the fiber axis; (g and h) - detail of damage to the cuticle that exposes the underlying cortical cells.

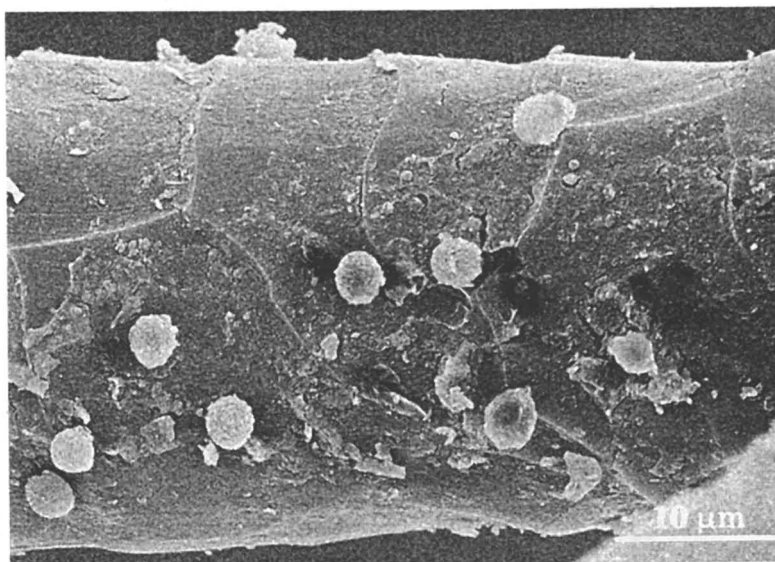
Photograph 1. *Continued.*

b

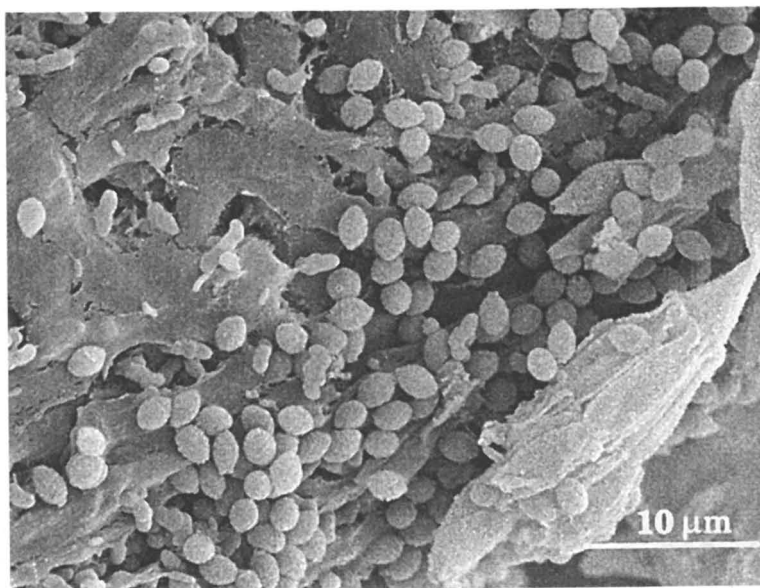


c

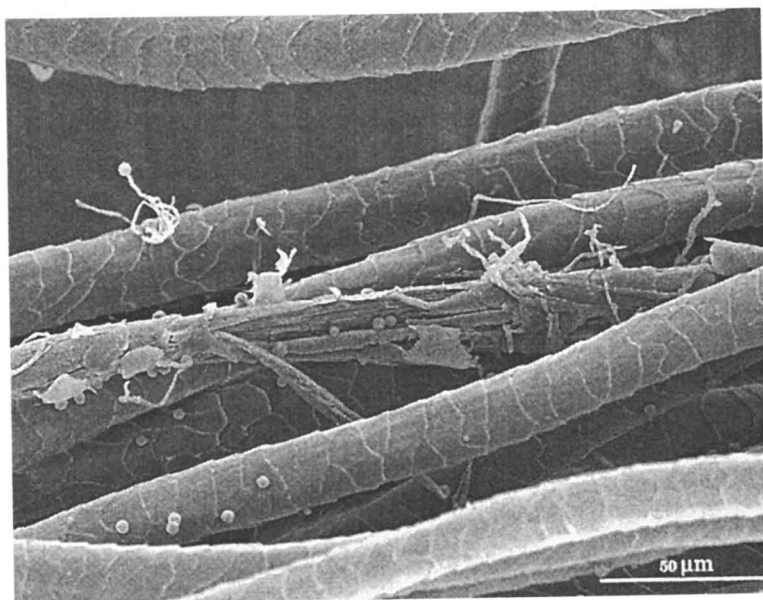
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Photograph 1. *Continued.*

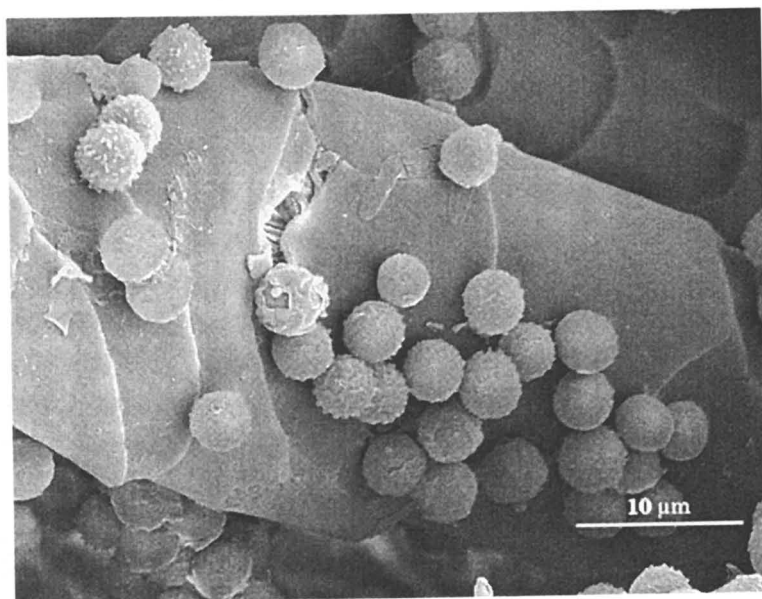
d



e

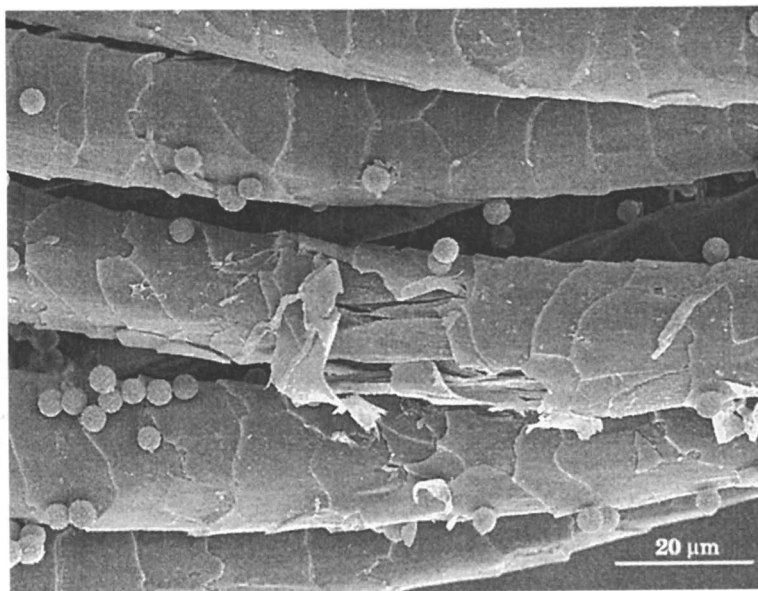
Photograph 1. *Continued.*

f



g

Continued on next page.

Photograph 1. *Continued.*

h

biofilm and accompanying mold growth with conidiophores characteristic of *Penicillium aculeatum*, shown in Photograph 1 (a, b). From dyed wool in the spent henna dye bath, we extracted *Aspergillus flavus* and isolated colonies on the various culture media: potato malt, yeast malt, brain heart infusion, and Czapek agars. Characteristic conidiophores, coarsely roughened with a globose vesicle containing phialides comprise the thallus which consists of branching, filamentous thread-like growths called hyphae, shown in Photograph 1 (a-c). The fertile hyphae extend into the air, forming and discharging spores. The vegetative hyphae burrowed into the wool fiber, digesting and absorbing nutrients as shown in Photograph 1 (d, e). Individual colonies from the red henna dye bath were concentrated on yeast agar and used for inoculation of commercial wool fabric (TF 523, Testfabrics, Inc., West Pittston, PA) that had been subjected to sequential water and petroleum ether extractions followed by sterilization in 70% ethanol/ water (v/v). The fabric was air-dried, inoculated with the microorganism, and incubated in a chamber at 30C. After five days there was evidence of a bluish-green with sulfur-yellow powdery mold. A few fibers were removed from the fabric using aseptic techniques and were transferred to a sterile vial containing a few milliliters of 2.5% glutaraldehyde and 0.1M imidazole solution for scanning electron microscopy preparations. The micrographs showed damage caused by infesting hyphae and spores in Photograph 1 (f, g). In Photograph 1 (h) there is damage to the cuticle cells, evident from the lifting and partial removal of sections of the fiber's scales, thereby exposing the cortical cells.

Insect Infestation

While the presence of disulfide crosslinks renders wool resistant to attack by the vast majority of insects, there is a small group of moth and beetle species that feed on wool during their larval stages. The mid-intestine of larvae able to digest wool contains an enzymatic secretion that reduces the disulfide bonds in the keratin, leaving it susceptible to enzymatic digestion that dissolves the fiber completely. For the larvae of the common webbing clothes moth, *Tineola bisselliella*, two digestive enzymes, cystine reductase and cysteine lyase/desulfydase, are known to be powerful reducing agents. They may or may not be present in the gut of the larvae at pH 9.8-10.0 (33). When larvae from this webbing clothes moth were held on staple sections for two weeks, damage assessment showed that the amount of insect feeding and growth differed significantly by differences among sheep and by whether feeding occurred on weathered or nonweathered wool (34). Keratin can be digested by some larvae from the *Lepidoptera*, *Tinea*, *Tinei*, and *Oecophoridae* families of moths and by some beetle species such as *Coleoptera*, *Dermestidae*, *Attagenus*, *Anthrenus*, and *Anthrenoceros* as well as several hundred species of *Mallophaga*, bird lice. The insect species most damaging to textiles include the webbing clothes moth, *Tineola bisselliella*, the case-bearing clothes moth or "fur" moth, *Tinea pellionella*, the brown house moth, *Hofmannophila pseudospretella*, the white-tip clothes moth or "tapestry" moth, *Tichophaga tapetzella*, and the four types of carpet beetles; the common carpet beetle, *Anthrenus scrophularie*, the furniture carpet beetle, *Anthrenus vorax*, the variegated carpet beetle, *Anthrenus verbasci*, and the black carpet beetle, *Attagenus piceus* (19). Damage from moths can be deduced from deposits of debris found on the fabric from webbing, cast skins, and moth wings. In North America the black carpet beetle, *Attagenus piceus*, is a major pest. In northern Europe, *Attagenus peillio* is important; and in Australia, *Anthrenoceros australis* is an important species. Damage from the carpet beetle is more pronounced as bitten edges and gnawed-out areas where the bite marks can be seen through the cuticle into the cortex of some fibers. Microscopic evidence of gross damaged areas in wool fiber has included sometimes swollen and raised cuticle separated from the cortex, loss of cell membrane leading to separation of cortical cells, and preferential damage to regions such as cortical cells rather than to the cuticle cells and wherever there is least cystine content (35, 36). Physical damage is dependent upon species and nature of infestation. For studies on the digestion of wool by insects, refer to the reports of Waterhouse (1952-1958) and Lewis (1975) (37, 38). A comprehensive review of the growth of microorganisms can be found in the articles of McCarthy and Greaves, 1988 (39).

Investigation of keratin digestion by insects using amino acid and gel electrophoresis analyses showed that peptides and free amino acids were found in larval feces. Cysteine and histidine were found in moth excreta whereas cysteine and arginine were found in beetle excreta. Analyses of the amino acids from gut juices enabled investigations to evaluate various mothproofing agents (40). Damage caused by carpet beetles is more than half the total amount of insect damage to wool textiles. Thus an effective mothproofing agent must control infestation by the fully-grown larvae of both moths and beetles. Some microorganisms and insect species known to

cause the deterioration of keratinaceous materials are found in Table V as well as a representative group of Bacterial enzymes that have been cultivated for commercial availability to improve the properties of wool, for example, shrink-resistance (41, 42, 43, 44).

Assay and Efficacy Testing

Microbes

The various methods used to determine biodeterioration and the efficacy of biocides in textile applications include deliberate infection or infestation followed by damage assessment and reduced test duration. Extended incubation periods are required. A substrate can be inoculated with a microbial species or unknown biodeteriogenic organisms on a pure or mixed culture/agar plate (24). Evaluation can be carried out by visual assessment, color change, weight loss, loss in tensile strength and, in mildewed specimens, microscopical inspection of hyphae and spores. Bacteria can also become evident by inspection of small, globular, opaque, discontinuous material. Typical methods of assay include: p-nitrophenyl acetate, fluorescein diacetate, ergosterol, urease activity, and adenosine triphosphate (ATP) luminescence (45). Standard Test Methods to evaluate biological properties include the following: Antibacterial Activity Assessment of Textile Materials: Parallel Streak Method; Test Method 147-1998, Assessment of Antibacterial Finishes on Textile Materials; AATCC Test Method 100-1993, Antifungal Activity, Assessment on Textile Materials: Mildew and Rot Resistance of Textiles; AATCC Test Method 30-1993, Antimicrobial Activity Assessment of Carpets; AATCC Test Method 174-1993 (45), ASTM G21, DIN 53931, BS 6085, NFS 41-600, and SNV 195921.

Insects

Mortality testing is carried out where textile samples are enclosed with a number of larvae for fourteen days. Weighings before and after exposure are taken and visual damage is recorded (46). Results can be reported according to standard test method: Insects, Resistance of Textiles to; AATCC Test Method 24-1994 (47), or similar test methods such as ASTM D 582, D1116-65, IWTO-9-62, and IWTO-14-64, and ISO 3998.

Antimicrobial Agents

Antimicrobial agents are selected for their effectiveness against bacteria and microfungi at low concentrations. Preferably, they are colorless and odorless, active for the life of the textile, and have low mammalian toxicity at the concentrations used. Other criteria for selection include: inexpensive and easy to apply, resistant to sunlight, durable to refurbishing, will not impact negatively on fabric handle or strength, are compatible with textile auxiliaries used in processing, and do not sensitize the textile to light or other damage. Often called biocides, antimicrobials are used to reduce the growth of microbiological organisms. For example, fleece on the

Table V. Representative Microorganisms and Insects Known to Digest Wool

| Wool Colonizing Organisms | | Commercially- available Proteolytic Enzymes | <u>Lepidoptera</u> (Moths) | <u>Coleoptera</u> (Beetles) |
|---------------------------|-----------------|--|---|---|
| Fungi | Bacteria | | | |
| Penicillium | Proteus | Pepsin | | Dermestidae Family (Hide Beetles) |
| Aspergillus | Bacillus | Chymotrypsin | Oecophoridae Family (House Moths) | Attegenus spp. (Fur Beetles) |
| Chaetomium | Pseudomonas | Papain | Endrosia sarcitrella | megatoma A. piceus A. pello |
| Acremonium | Corynebacterium | Trypsin | | |
| Actinomyces | Penicillium | Esperase | Tineidae Family (Cloths Moth) | |
| Chrysosporium | Enterobacter | Alcalase | Tineola bisselliella | Anthrenus spp. (Carpet Beetles) A. verbasci A. flavipes A. vorax A. museorum A. scrophulariae |
| Scopulariopsis | Flavobacterium | Savinase | | |
| Malbranchea | Acinetobacter | Pronase | Tinea dubiella | |
| Trichophyton | Alcaligenes | Neutrase | Tinea translucens | |
| Keratinomyces | Staphylococcus | Newlase | Tinea pelliionella | Anthrenoceros A. Australis |
| Arthroderma | Aeromanos | | Trichophaga tapetzella | |
| | Serratia | | | |
| | Streptomyces | | Hofmannophila pseudospretella | |
| | Chromobacterium | | | |
| Nannizia | | | | |
| Ctenomyces | | | | |
| Cladosporium | | | | |
| Fusarium | | | | |
| Rhizopus | | | | |
| Mucor | | | | |
| Sepedonium | | | | |
| Micosporum | | | | |
| Bird Lice: Mallophaga | | | | |

backs of sheep sometimes developed a condition referred to as “pink rot” that was attributed to a bacterium isolated from the deteriorated wool and identified as *Bacillus vulgatus* (48). *Bacillus subtilis* and *Bacillus mesentericus* attack the CMC causing disaggregation and fibrillation of the cortical cells held within it (49). Wool materials most susceptible are those that had been subjected to alkali, acid, peroxide bleaching, chlorination, or boiling water.

Early methods to prevent mildewing were developed during World War II for textiles that would serve in environments with high humidity and temperatures where mildew attack would be prevalent. Various antiseptics such as the sodium salt of salicylanilide, sodium pentachlorophenate, pentachlorophenol, and p-nitrophenol can inhibit the growth of fungal spores. They were developed to treat wool during processing (50). Although these treatments were relatively effective for imparting resistance to biological deterioration, they were only semi-durable. Inevitably, the most severe test, soil burial, was carried out to determine the effectiveness of active agent applied to the fabric when stored. More practical evaluations involved subjecting the treated fabric to laundering where alkali and other formulation additives as well as heat plus mechanical action could be used to evaluate durability.

Early attempts to control microbial growth on industrial wool products involved treatment with resin-bonded 1% copper-8-quinolinolate (51). In the processing of wool textiles various finishing processes imparted a measure of protection against mildew damage. For example, chlorination processes used to pretreat wool for shrinkproofing were effective as well as acidic substances used in carbonization and flameproofing that inhibited bacterial growth because of low pH (52). Commercial mildew proofing formulations were applied by exhaust, padding, or spraying to fiber, yarn, fabric, or garments. In the 1970's the following products were effective: o-benzyl-p-chlorophenol, 5,5'-dichloro-2,2'-dihydroxydiphenylmethane, 2,4,4'-trichloro-2'-hydroxydiphenylether, sodium hypochlorite, sodium dichloroisocyanurate, pentachlorophenol, pentachlorophenyl laurate, quaternary ammonium compounds, chlorinated phenols and their fatty acid esters, and organo tin compounds where proper handling for safety was required.

Specific biocide formulations used in the wool textile industry included copper or zinc naphthalene, hydroxyquinolatedithiocarbamate, n-butyl tin oxides, halogenated phenols, amines, aryl-hydroxides, bis-quaternary ammonium compounds, benzyl trialkylammonium chlorides, bis-hydroxychlorosulfides, and sodium or zinc salts of mercaptobenzothiazole and pyridinethioatone salt. Where wet applications were not practical, solvent systems were used. Another option was autoclaving at 100-150C for 5 minutes or tenting the fabric and passing it through a drying oven at this temperature (52, 53). Home removal methods relied upon hot solutions of sodium dithionite reducing agent (53).

The world of biocides and antimicrobials is highly regulated, thus there is a preponderance of modifications of existing products in response to continuous environmental pressure. Antimicrobial bis-quaternary surfactants (quaternary ammonium halide) compounds known as “quat salts” are widely used in the textile industry for surfactant, detergency, and antimicrobial efficacy. They are fairly effective against *B. pumilus*, *S. aureus*, *E. coli*, and *Ps. Aeruginosa*. A new generation, bis-quats, includes NN'-bis (n-dodecyl-N,N-dimethylglycine) cystamine dihydrochloride that is effective against *B. pumilus* and *S. aureus* bacteria and NN'-

bis (N-dodecyl-N,N-dimethylglycine)1,4-diaminobutane dihydrochloride, proven to protect wool against *S. aureus* (54). A biguanide-derived quaternary ammonium biocide, poly(hexamethylene biguanide) hydrochloride, was found to impart antibacterial character to cotton and wool/cotton blended textiles to prevent the growth of *Staphylococcus aureus* (55, 56).

Insect-proofing Agents

The ideal mothproofing agent would be odorless, toxic only to larvae, and durable or permanent through textile finishing, wear, and refurbishing and be fully exhausted on application. An early mothproofing agent was the textile dye, Martius Yellow (C.I. Acid Yellow 24, Chemical Constitution 10315) that originated with Martius in 1864 (57, 58). It was produced in one case by reacting 1-naphthol-2,4,7-trisulfonic acid with nitric acid. Other naphthol-derived dyes were not as successful. Inspired by this early success, other agents such as camphor and naphthalene, with high vapor pressures at room temperature, were used but found to be ineffective detergents because protection was afforded for only as long as vapors were emitted and because carpet beetles can become immune to them. Soon chlorinated organic compounds such as paradichlorobenzene were developed. Dichlorodiphenyltrichloroethane (DDT) applications were permanent but its toxicity and resistance led to other approaches. Other insect-proofing compounds included tri-(octyldecyl)methylammonium chloride, C₁₂-C₁₈ linear and branched alkylbenzenesulfonates, organotin compounds, carbamates and pyrethroids.

A summary of various compounds and approaches to preventing microbial growth and insect infestation is found in Table VI below.

The insect-resist agents, Eulan and Mitin are derived from chlorophenylide, and diphenylurea, respectively. Dieldrin and pyrethroids are insecticides. Dieldrin is inexpensive, highly toxic, and persists in the environment; thus its use has decreased. (61) Synthetic pyrethroids are a series of insecticides related in molecular structure to the natural insecticide contained in the pyrethrum plant. The most common is *permethrin* (3-phenoxybenzyl (1RS)-cis, trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate) is sold under the trademarks, Perigen (Wellcome) and Mothproofing Agent 79 and Vickers SMA-V (Shell). Other pyrethroid insecticides are *cyfluthrin*, (R, S)-alpha-cyano-4-fluoro-3-phenoxybenzyl (1RS)-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate. They have high activity as insecticides and exhaust onto wool at a low level of add-on, 0.015 – 0.02% (% vol./wt.of fiber) at relatively low cost and environmental impact (62, 63). Sulfanilamide, an antimetabolite, although not fast to washing, is an effective protectant against moths and carpet beetles. It is structurally similar to Eulan BL. It contains no chloro groups and binds the secreted enzymes of insects, not to regenerate them but to inhibit their metabolic cycle. Some sulfonilamides such as Eulan BL contain chlorine and can act as organochlorine poisons to protect wool against moth and beetle larvae (63).

The chemical structures of old commercial products utilized in the past for effective mothproofing are shown in Figure 6 below:

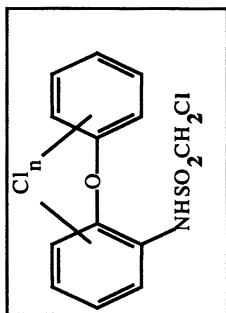
Table VI. Various Treatments and Methods Used in the Past to Prevent Microbial Growth and Insect Infestation

| Early Semi-durable Antimicrobial Treatments | Biocides and Antimicrobials | Mothproofing Agents |
|---|---|--|
| Resin-bonded 1% copper-8-hydroxyquinolinolate | NN'-bis (n-dodecyl- N,N-dimethyl glycine) cystamine dihydrochloride | Martius Yellow (C.I. 10315, Acid Yellow 24) |
| Chlorination, Carbonization Flameproofing | 1,4-Diaminobutane dihydrochloride Poly(hexamethylene biguanide) hydrochloride | Camphor and Naphthalene (ineffective) Dichlorodiphenyltrichloroethane (DDT) |
| Antimicrobial 1970's Agents | | Tri-(octyldecyl)methylammonium chloride C ₁₂ -C ₁₈ linear and branched Alkylbenzenesulfonates |
| Dichlorophen (5,5'-dichloro-2:2'-dihydroxydiphenylmethane Pentachlorophenyl laurate | Biocides Used in the Wool Textile Industry Copper or zinc naphthenate | Organotin compounds |
| Quaternary ammonium | Hydroxyquinolinolate, dithiocarbamate | Carbamates |
| Chlorinated phenols and their fatty acid esters | n-Butyl oxides | Pyrethroids |
| Organo-tin compounds | Halogenated phenols, amines, arylhydroxides Bis-quaternary ammonium chloride Benzyl ammonium chloride Bis-Hydroxychlorosulfide Mercaptobenzothioate Pyridinethiolate | Benzyl 2-methyl-2-phenylpropyl ethers (REF) 5-hydroxy-2-methyl-1,4-naphthoquinone |
| | Some Biocides Used in Textile Processing | |

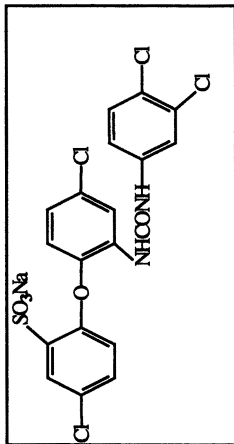
Table VI. *Continued*

| Early Semi-durable Antimicrobial Treatments | Biocides and Antimicrobials | Mothproofing Agents |
|--|--|---------------------|
| | o-Benzyl p-chlorophenol | |
| | 5,5'-Dichloro-2,2'- dihydroxydiphenyl sulfide | |
| | 2,4,4'-Trichloro-2'- hydroxydiphenylether | |
| | 2-hydroxy-2',4,4'- trichlorodiphenyl ether | |
| | Sodium hypochlorite | |
| | Sodium dichloroisocyanurate | |
| | Home Laundering | |
| | Polyhexamethylene biguanide (PHMB) (59) | |
| | Chlorinated phenoxide (CPC) | |

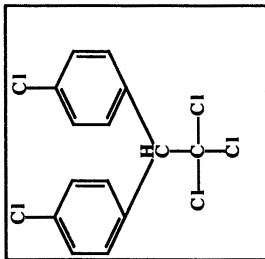
Other more common aromatic or cyclic chlorinated compounds were found under various trademarks are: 4,4'-dichloro-3,3'-trifluoromethyl diphenylurea (Mitin LP, Ciba-Geigy); N-5-chloro-2-(2-sulfo-4-chlorophenyl-N'-3',4'-dichlorophenyl urea, (Mitin FF), polychloro-2-(chloromethylsulfonamido)-diphenyl ethers, (Eulan WA New, Eulan U33, Molantin P), and hexachloro-epoxy-octahydro-endo-exo-dimethano-naphthalene (Dieldrin).

EULAN WA
(1928)

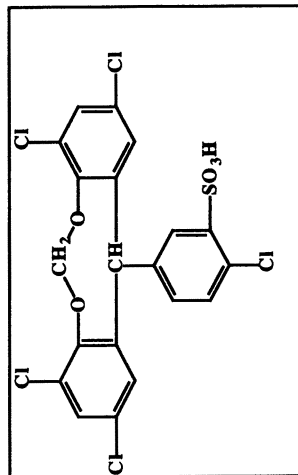
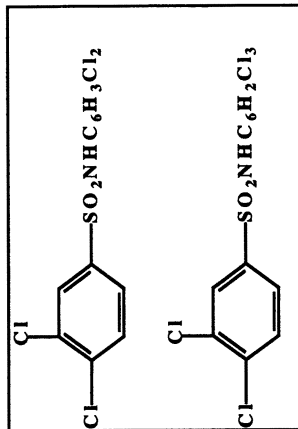
polychloro-2-
(chloromethyl-sulfamido)
diphenyl ether

MITIN FF
(1938)

dichlorodiphenyl
-ether & dichlorophenyl
groups linked by urea

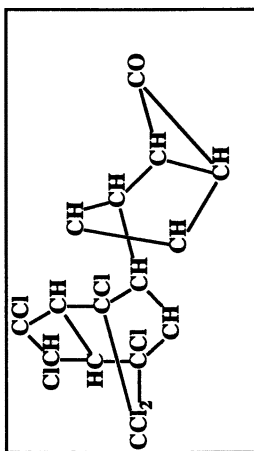
DDT
(1940)

dichlorodiphenyl
trichloroethane

Triphenylmethane derivatives
(1950's)**Chlorinated Sulfonamides**
(1950's)

DIELDRIN

(1950's)



hexachlorodimethanonaphthoxirene

**QUATERNARY
PHOSPHORUS CPDS**
(1950's)

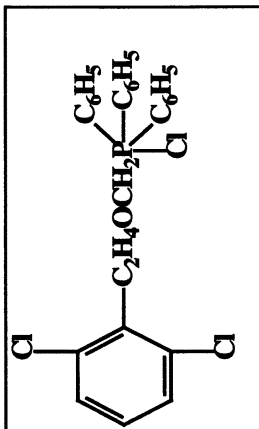


Figure 6. Early Mothproofing Agents

In the 1980's Pyrethroid ester compounds, shown in Figure 7, were developed. They were modeled on pyrethrin, the natural insecticide found in *Chrysanthemum cinerariaefolium*, the pyrethrum daisy plant. Unlike natural pyrethroids, synthetic pyrethroids are stable to photolysis and hydrolysis. They are more concentrated to exhibit greater unsecticidal activity, and yet are low in mammalian toxicity, although fish toxicity is a concern. However, at a low yet effective application level of 0.02% by weight of wool, these moth-proofing agents remain effective as mothproofing agents, thereby minimizing the hazard and providing a more economical alternative to other insecticides (64,65).

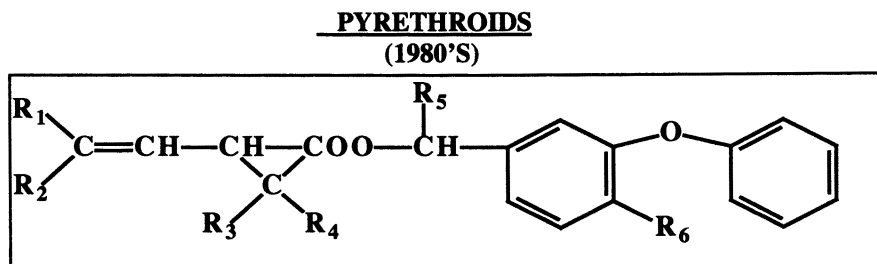
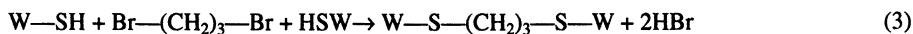
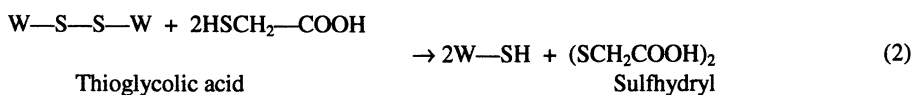


Figure 7. Synthetic Pyrethroid modeled on pyrethrin: natural insecticide found in *Chrysanthemum cinerariaefoli*

The Use of Crosslinking Agents to Impart Insect Resistance

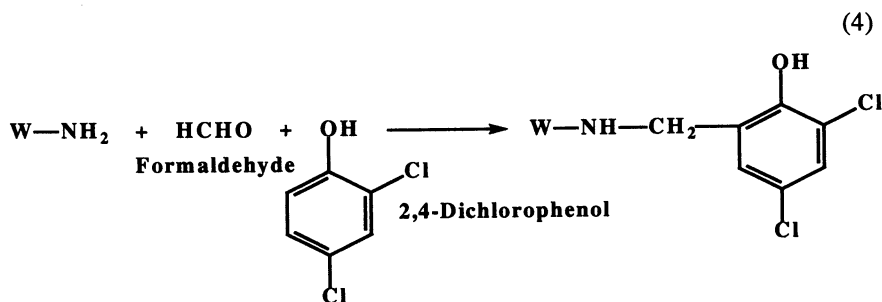
Because it was believed that wool was digested in the midgut of the larvae by highly reducing substances that broke wool's disulfide (-S-S-) bonds allowing subsequent proteolytic enzyme digestion, reducing agents and bifunctional crosslinking agents were used on wool to convert disulfide bonds to bis-thioether crosslinks (Wool-CH₂-S-(CH₂)_n-S-CH₂-Wool) in the hope of conferring permanent mothproofing (66). Cystine disulfide is reduced to cysteine residues (-NH-CH(CO)-CH₂-SH) by larvae protease and rapid proteolysis proceeds (67). Attention was therefore drawn to chemical modification of new or damaged wool through the functionality of wool's amino acids to form new crosslinks by reaction with bi- or multifunctional reagents, thereby producing wool that was stable but unpalatable to insects. Crosslinking to produce new crosslinks in wool that are more stable than those in native wool has important implications not only for mothproofing but for stabilization to alkalis, oxidizing and reducing agents, and light degradation.

Hartsuch described modifications through wool's -NH₂ groups and -S-S-linkages by crosslinking with formaldehyde (Eq. 1) or benzoquinone, by the addition of an amino group to a ketone (5) The reduction of disulfide bonds with thioglycolic acid and the subsequent formation of new crosslinks between two adjacent chains by dihalogen compounds (Eq. 2, 3) is shown below:



An alternate approach for mothproofing is with formaldehyde to modify wool's amino acid side-chain's with guanidine and hydroxyl groups that might have specificity for larvae proteolytic enzyme (68).

A different approach is to chemically bond toxic compounds to wool. For example, chlorophenolic compounds modify wool for toxicity through covalent bonds by reaction with formaldehyde (69):



Crosslinking with formaldehyde is by the addition of an amino group to a ketone. For example, the inhibition of protease keratinolytic reduction can occur through crosslinking with glyoxal. In this case, two adjacent wool molecules are crosslinked through their terminal amino groups. Another method of crosslinking is through the reduction of wool's disulfide linkages with sodium hydrosulfite or thioglycolic acid, followed by the addition of ethylene dibromide (or other halogenated alkene) to reform the crosslink not with the -S-S- bond but with a stronger new bond, -S-CH₂-CH₂-S-. Alternatively, the reaction of acrolein with reduced wool to form -S-CH₂-CH₂-S- crosslinks as well as crosslinks between reduced wool's -SH thiol and -NH₂ amino groups is a possibility. These reactions are shown in Figure 8 below.

Conclusions

Wool's complex morphological and chemical structures confound the study of its biodeterioration by microorganisms and insects. Although microorganisms differ in

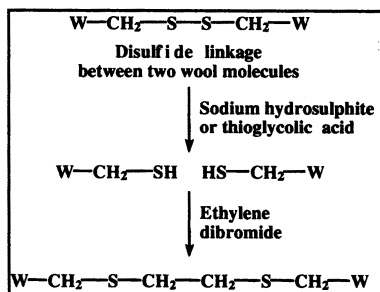
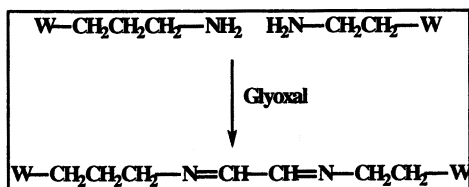
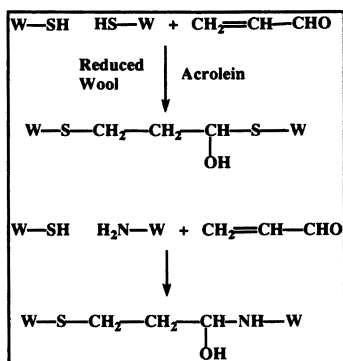
ALKYL DIHALIDE**GLYOXAL****ACROLEIN**

Figure 8. Crosslinking to inhibit protease keratinolytic reduction

their mechanisms of attack (for example, the hyphae of a fungus compared to the bite-marks of an insect) similar enzymatic degradation pathways can prevail. Wool is a fertile substrate that bears inherent and occluded substances to feed fungi, bacteria, and insect larvae. Under optimum conditions for growth; high humidity, moderate temperature and neutral to slightly alkaline pH, that are the normal conditions for textile use and care, microbiological degradation becomes visually apparent in the formation of filmy, powdery residues that are discontinuous over the surface of a textile. Microscopically there is evidence of fruiting bodies exuding filamental tentacles or hyphae to erode wool's scales in order to permeate the underlying cortex through the cell membrane complex. Supplementary degradation by enzymatic digestion releases more nutrient to support the fungi or invading organism, thus wool loses its structural integrity and becomes weakened. The same mechanism of attack and digestion prevails in the case of deterioration by the larvae of moths and carpet beetles. Interestingly, wool which has been damaged mechanically or chemically in processing, wear, or refurbishing becomes most vulnerable to enzymatic attack of wool's crosslinking sulfhydryl groups, demonstrating the pivotal role of the cuticle as the dominant barrier to insects and microorganisms.

Early attempts to provide microbial and insect resistance relied on the application of temporary antiseptics or durable resin-bonded organic salts to provide a barrier to infestation. More environmentally safe compounds such as quaternary ammonium salts proved effective against various microbial species. Overall the most efficient and effective method for preventing the growth of fungi and bacteria is in the control of ambient conditions for storage at the various stages of fiber, yarn, fabric, and garment fabrications, that is, a dry environment not to exceed 55% to 65% relative humidity, temperatures not exceeding 25C, and slightly acidic to neutral pH conditions.

Symptomatic solutions to counteracting mildew and insect infestation have employed various natural and synthetic insecticides that are selected for their specificity for the targeted species. Application methods for their maximum efficiencies must be specified. Some are noninvasive to humans and provide toxicity only to larvae while others are regulated compounds that could provide environmental risk. The broad range of development in this area includes chemical modification of wool's disulfide crosslinks and side-chain hydroxyl and amine groups. Applied topically or by infusion, certain aromatic or cyclic compounds are available under recognized trademarks. In recent years these approaches have been eclipsed by the use of pyrethroid insecticides such as permethrin. They can be used alone or coadded to conventional formulations.

Although the above discussion casts the microbiological properties of wool in a dim light with wool as the fertile breeding ground for contamination, in-depth studies of the growth of bacteria and fungi on wool merit investigation for the possibility of engineering new end-uses for wool. On the other hand, wool's biodegradability

provides a natural advantage over most synthetic fibers as we strive to maximize usage of zero-land-fill materials in the new millennium.

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