Thermal Crosslinking of Collagen Immobilized on Poly(acrylic acid) Grafted Poly(ethylene terephthalate) Films

BHUVANESH GUPTA,[1,*] JÖNS HILBORN,[1] CHRISTOPHER PLUMMER,[1] ISABELLE BISSON,[2] PETER FREY[2]

[1] Polymer Laboratory, Department of Materials, Ecole Polytechnique Fédérale de Lausanne, Switzerland

[2] Laboratory for Pediatric Urology, CHUV, Lausanne, Switzerland

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ABSTRACT: Graft polymerization of acrylic acid onto plasma-treated poly(ethylene terephthalate) (PET) films was used to prepare surfaces suitable for collagen immobilization by dip-coating. Such surfaces could be used as matrices for smooth muscle cell cultures in tissue engineering. Contact angle measurements showed that plasma-treated and grafted PET films undergo considerable surface reorganization during storage under ambient conditions. However, after collagen immobilization the contact angle remained relatively stable. The amount of collagen initially attached to the film surface increased with increasing poly(acrylic acid) graft density, but subsequent washing in water led to significant collagen loss. This loss could nevertheless be substantially reduced by thermal crosslinking of the collagen in the range 110–130 °C. Atomic force microscopy (AFM) observations suggested that the washed crosslinked collagen has a very similar structure to that of the un-crosslinked collagen. © 2002 Wiley Periodicals, Inc. J Appl Polym Sci 85: 1874–1880, 2002

Key words: poly(ethylene terephthalate); acrylic acid; plasma graft polymerization; immobilization; contact angle; thermal crosslinking

INTRODUCTION

In the human body, cells adhere to the extracellular matrix, which consists mostly of proteins such as collagen, laminine, and fibronectin. Synthetically produced surfaces functionalized with these proteins therefore often represent suitable substrates for the *in vitro* culturing of cells. Collagen has proven to be particularly useful in this respect. It is biodegradable and biocompatible, and is widely used for wound dressing and related surgical applications because it provokes no or only a very weak immune response.[1] A recent application of collagen is tissue engineering in which human cells adhere to and proliferate on a collagen scaffold, which may subsequently be implanted into the patient.[2] This application opens up new possibilities for reconstructive surgery by tissue replacement and tissue reinforcement without significant risk of an immune response.

Crosslinking of collagen is important where an insoluble biomaterial is required.[3–6] Crosslinked collagen-based foams have great potential for tissue engineering because of their biocompatibility,

Correspondence to: J. Hilborn, Polymer Chemistry Programme, Materials Chemistry, Ångström Laboratory, University of Uppsala, SE-75121 Uppsala, Sweden (joens.hilborn@epfl.ch).

**Permanent address:* Department of Textile Technology, Indian Institute of Technology, New Delhi-110016, India

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dimensional stability, and high porosity, which promote cell growth and vascularization of the developing tissue. Depending on the extent of crosslinking, such scaffolds may show a high level of mechanical strength and proteolytic resistance, although their resorption rate and biocompatibility are also strongly influenced. Crosslinking of collagen has been carried out in a number of ways, including thermal treatment, chemical treatment, ultraviolet (UV) exposure, and gamma irradiation.[4–8] Chemical treatment with glutaraldehyde has been found to be effective in reducing immunogenicity while increasing the response to enzymatic degradation.[9] However, postimplantation depolymerization and glutaldehyde release can lead to cytotoxicity during cell growth.[10] Thermal crosslinking, on the other hand, requires long treatment times and leads to partial denaturation of the collagen. However, the denaturation is generally insignificant, and thermal crosslinking has the advantages of reduced cytotoxicity and a moderate resorption rate.[11, 12]

Despite its usefulness as a substrate for cell culture, the collagen offers only limited possibilities for forming complex shapes with a wide range of properties as compared, for example, with synthetic biodegradable fabrics produced from poly(lactic acid) (PLA) and its copolymers. PLA fibers, whose classical use is in suture threads, can be knitted into fabrics and structures displaying a combination of high ductility, high compliance, and tuned porosity. However, their surface chemistry is inappropriate to cell attachment and tissue culture. Our ultimate aim is therefore to develop techniques for the surface modification of synthetic polyesters to overcome this problem. We have therefore carried out plasma treatment of poly(ethylene terephthalate) (PET) films to activate them for subsequent graft polymerization of acrylic acid.[13,14] The grafted surfaces can then be used to immobilize collagen for subsequent seeding of human smooth muscle cells and urothelial cells.[15,16] It was found that PET surfaces with low graft levels of poly(acrylic acid) (PAA) grafts ($\sim$0.4 μg/cm^2) with a complexed collagen content of $<$1 μg/cm^2 were the most appropriate for cell culture. At higher graft levels of PAA, the cells grew less well. It was suggested that when the collagen content became too high, large areas of the noncomplexed collagen detached from the film surface during cell seeding, exposing the cells to the PAA layer locally and hence to a low pH environment. This low pH environment, in turn, leads to the cell degeneration. The collagen stability on the PAA-grafted PET surface is needed to achieve proper cell growth. Although this problem could be avoided by controlling the PAA graft level, higher levels of collagen immobilization remain desirable because they would provide for more flexibility in controlling the long-term properties of the surfaces. Thermal crosslinking of the surface-complexed collagen has therefore been investigated as a means of improving the stability of relatively thick collagen coatings on PAA-grafted PET films and to assess its potential for use as cell scaffolds based on knitted polyester fabrics.

The urinary tract is exposed to a variety of injuries; some of them appear during fetal development and some of them represent acquired diseases. All these injuries may lead to urinary organ damage or loss, requiring surgical reconstruction with nonurological tissues, for example tissues derived from the intestinal tract. However, these methods are prone to severe complications. With the help of tissue engineering, functional new tissue is produced by transplantation and subsequent growth of urothelial and bladder smooth muscle on synthetic biodegradable and biocompatible polymer matrices. For this reason polymer surfaces, modified with collagen immobilization and additional collagen crosslinking, have been evaluated regarding adherence and growth of human smooth muscle cells.

EXPERIMENTAL

Materials

The poly(ethylene terephthalate) (PET) films used in this study were supplied by Goodfellow, England, and were 23 μm in thickness. Acrylic acid, Mohr's salt, acetic acid, sulfuric acid, and sodium hydroxide were supplied by Fluka. Toluidine Blue O was supplied by Aldrich. Deionized water was used in all experiments, and the acrylic acid was purified by distillation under reduced pressure.

Vitrogen (acidified soluble bovine collagen type I [97%] and type III [3%]; Cohesion, Palo Alto, CA) was used. Phosphate buffered saline (PBS), trypsin, and Ham's F10 nutrient mixture used for cell cultures were supplied by Sigma.

Graft Polymerization

Graft polymerization of acrylic acid on PET films was carried out by plasma exposure method.[14]

The PET films were treated with argon plasma in a plasma reactor operating at 13.6 MHz, gas pressure of 0.4 mbar, and argon flow rate of 50 sccm, at a power of 80 W.[13] The plasma treatment time was 60 s. Immediately after the treatment, oxygen was introduced into the chamber and maintained at atmospheric pressure for 1 h. Graft polymerization of acrylic acid onto plasma-treated PET films was carried out in 2×16-cm^2 size glass tubes under nitrogen atmosphere. Care was taken to keep the transfer time of the treated film from the plasma chamber to the grafting reaction tube within 20–25 min. A film was placed in the reaction tube containing aqueous acrylic acid solution of required concentration. Nitrogen was bubbled through the solution to remove oxygen. The tube was then placed in a constant temperature water bath for a specified period. After the grafting reaction, the film was taken out of the tube and Soxhlet extracted with water overnight to remove any homopolymer adhering to the surface. The film was finally dried under vacuum at 40 °C. After the grafting reaction, the films were Soxhlet extracted with water overnight to remove any homopolymer adhering to the surface. The graft density was determined by the colorimetric method with Toluidine Blue O staining using a UV spectrometer (Perkin Elmer UV/VIS/NIR spectrometer Lambda 900).[14]

Contact Angle Measurements

The contact angle of PET films was determined as described elsewhere.[13] A water droplet of 10 μL was placed on the film surface and the diameter was measured after 20s. Six measurements were made at different places on the film surface and averaged. Prior to the measurements, the films were stored in the dark under vacuum. The collagen immobilized films were washed with water at pH 3.0 for 1 h and dried under vacuum before the contact angle measurements.

Atomic Force Microscopy (AFM)

The film surfaces were investigated using a Park Scientific Instruments Autoprobe CP Scanning Probe Microscope. Intermittent contact mode imaging in air was used throughout to limit deterioration of the film surface as far as possible.

Collagen Immobilization

The immobilization of collagen onto the grafted PET films was carried out by the dip-coating method.[17] The vacuum dried grafted films were immersed in a 1.5-mg/ml collagen solution in water (pH 3.0) for 24 h at 22 °C. Subsequently, films were air dried for 1 h under ambient conditions. Washing of immobilized films was carried out for 1 h in water at pH 3.0 with stirring.

The collagen content at the film surfaces was measured by a ninhydrin method.[16] The films were immersed in 4.4 N H_2SO_4 for 12 h at 100 °C to hydrolyze the protein. The resulting solution was cooled and neutralized with 5 N sodium hydroxide solution. Then, 400 μL of the hydrolyzed solution was added to 2.5 mL of ninhydrin and heated at 100 °C for 15 min to give a violet-blue color. The optical density of the solution was measured using a spectrophotometer at a wavelength of 570 nm. The collagen content was calculated from a standard calibration plot.

Crosslinking

Thermal crosslinking of the collagen immobilized on PET films was carried out under nitrogen flow in the temperature range 25–130 °C. A treatment time of 3 days was used for all the samples.

Smooth Muscle Cell Culture

Samples of smooth muscle were obtained from the bladder of children undergoing open surgery. Tissue specimens were digested into a suspension of individual cells using 100 μg/mL collagenase type II (Gibco). They were then plated in Nunc culture flasks and incubated with 5% CO_2 in air at 37 °C. When cell confluence was reached, the cells were detached by incubation with trypsin. Then, 1.2×10^5 viable cells/cm^2 were seeded on the surface-modified polymers. The cultured cells were examined daily by phase contrast microscopy to assess growth and morphology.

RESULTS AND DISCUSSION

Contact Angle Measurements

The influence of the storage time on the contact angle is summarized in Figure 1 for plasma-treated PET films; PET-g-PAA films with graft densities (gd) of 0.4 and 11 μg/cm^2 PAA; and washed collagen-coated PET-g-PAA films with a graft density of 0.4 μg/cm^2 PAA. The plasma-

treated and grafted (0.4 $\mu g/cm^2$) films showed a considerable increase in contact angle, reaching equilibrium within 2 weeks. Similar contact angle enhancement has been seen by Johnsen et al. in the plasma grafted polyethylene films.[18] However, in our system the collagen-coated surface did not show any change in contact angle even after long storage times in our samples.

Both the plasma-treated and the PAA-grafted surfaces are dynamic in nature and undergo surface reorganization, the extent of which depends on the graft density.[13,14] The increase in the contact angle is thought to be due to driving forces arising from interfacial tension between the polar surface and the hydrophobic bulk layers. The polar groups at the surface tend to migrate towards the bulk of the matrix and nonpolar species tend to migrate to the top to minimize the interfacial free energy. Films with higher graft densities showed less increase in contact angle with time probably because of an increase in the size of the PAA domains, which lowers the mobility of grafts within the surface of the material. Similarly, the lack of surface reorganization in the collagen-coated films could be attributed to the stabilizing effect of the interactions between the collagen and the PAA, to be discussed in the next section.

Collagen Immobilization

As shown in Figure 2, the amount of collagen at the surface of the dip-coated films increased with increasing PAA graft density. This result is indicative of electrostatic interactions between the positively charged collagen and the negatively charged acid functional groups associated with the PAA. Washing of the immobilized films for 1 h nevertheless led to a substantial decrease in the collagen content, suggesting that only ~50% of the collagen initially present was strongly bound to the surface.

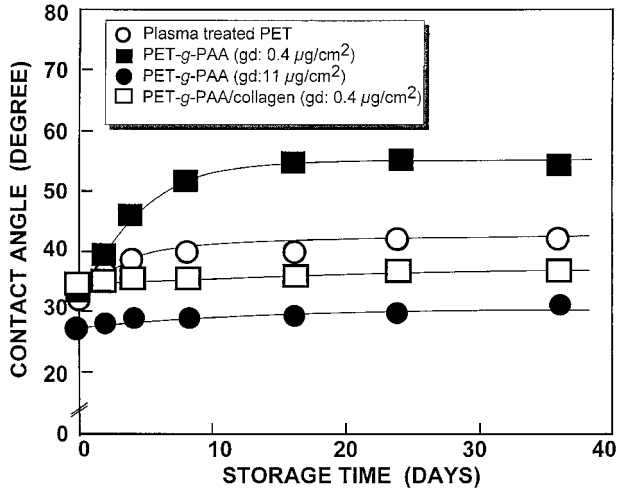


Figure 1 Variation of the contact angle with the storage of PET films.

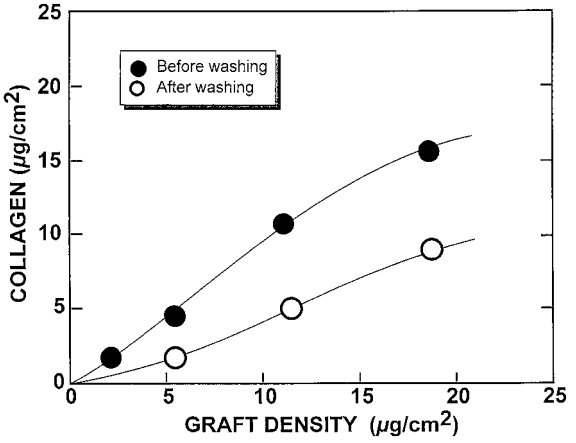


Figure 2 Variation of the collagen content with the graft density on PET films.

AFM images of samples were taken to study the morphological variations arising out of the washing of the films. The AFM images of a collagen-coated film with a PAA graft density of 11 $\mu g/cm^2$ before and after washing for 1 h are shown in Figure 3. After collagen immobilization, the film showed a highly characteristic surface texture consisting of undulating fibrous structures of the order of 100 nm in thickness, which persisted after washing.

Collagen Crosslinking

As pointed out in the previous section, a significant amount of the deposited collagen was removed from the surface of the grafted films after washing in water. It follows that variations in pH and contact with other electrolytes, such as those found in cell culture media or *in vivo*, will result in further detachment of collagen. Thermal crosslinking was therefore used in an attempt to stabilize the collagen layers. The influence of the crosslinking temperature on the stability of the collagen layers during washing is shown in Figure 4. Heat treatment in the temperature range 90–130 °C was particularly effective in preventing collagen loss during washing. Because carboxyl and amino groups are present in native collagen, they may undergo a thermally

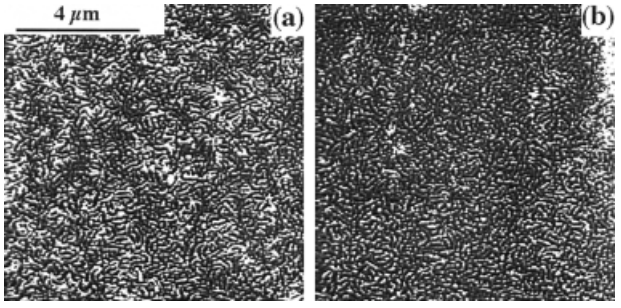


Figure 3 Intermittent contact mode AFM phase images of collagen immobilized PET-g-PAA films with 11 $\mu g/cm^2$ PAA (a) before washing and (b) after washing for 1 h.

induced condensation reaction.[6] It is also reasonable to assume that some of the collagen amino groups react with the carboxyl group of the PAA brushes to form amide linkages, thus providing an anchor. As a result, a continuous three-dimensional structure may be formed at the PET surface, leading to a dynamically stable modified surface layer.

The influence of the washing time on collagen loss from the crosslinked layers is shown in Figure 5. There was relatively little collagen loss on washing for up to 144 h under the present experimental conditions, suggesting good hydrolytic stability. Although a certain portion of the collagen chains presumably remained uncrosslinked and were removed by the water extraction, the total collagen loss was reduced from 55% without heat treatment to ~16% after thermal crosslinking following washing for 144 h.

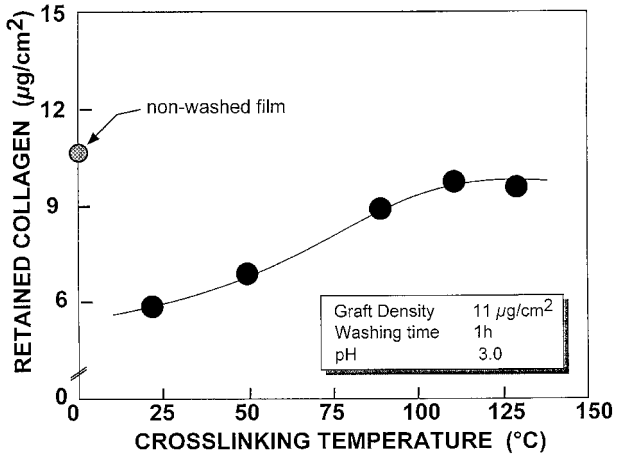


Figure 4 Variation of the retained collagen with crosslinking temperature.

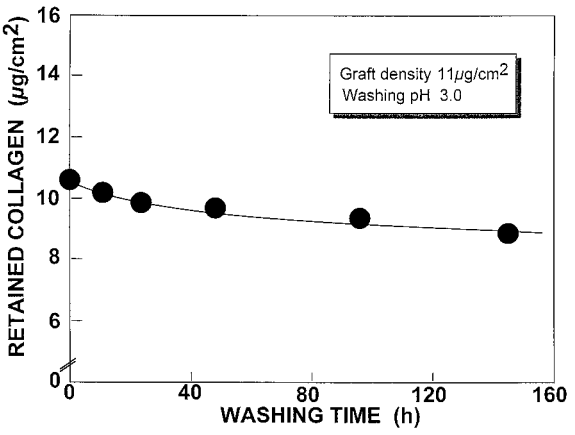


Figure 5 Variation of the retained collagen with washing time.

Atomic Force Microscopy (AFM)

Images of crosslinked collagen layers after different washing times are shown in Figure 6. Immediately after thermal crosslinking, the films showed relatively little surface roughness, although some fine fibrillar texture remained visible at the film surfaces (Figure 6(a)). However, after washing, the coarse fibrillar structure characteristic of the non-heat-treated collagen coated films returned, presumably as a result of rehydra-

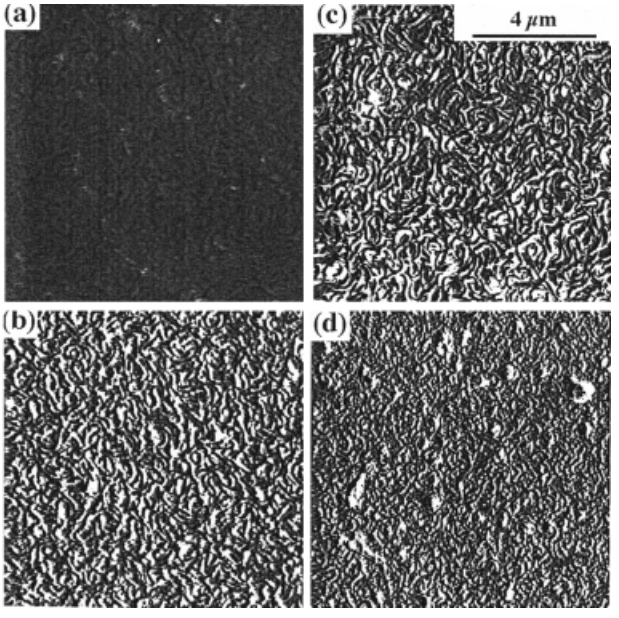
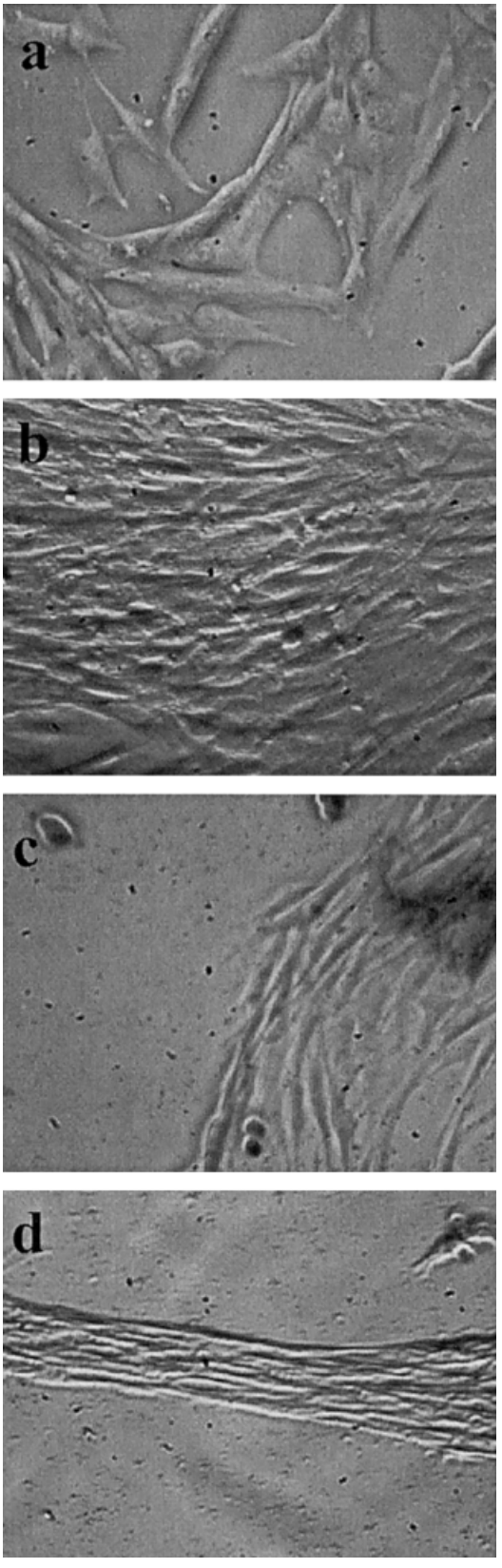


Figure 6 Intermittent contact mode AFM topographical images of thermally crosslinked, collagen-immobilized PET-g-PAA films: (a) unwashed; (b) washed for 12 h; (c) washed for 48 h; (d) washed for 95 h.



tion of the surface layer. As suggested by the results in Figure 6, prolonged washing may also have resulted in somewhat finer textures than short washing times.

Smooth Muscle Cell Cultures

PET-g-PAA films with a graft densities of 11 $\mu g/cm^2$ PAA, modified with collagen immobilization and subsequent collagen crosslinking, were investigated as matrices for smooth muscle cells. Preliminary results show that these matrices were apt to allow adherence and growth of human smooth muscle cells (Figure 7(a) and (b)). Before full cell confluence was reached, patchy detachment of the cell layer could be observed. (Figure 7(c) and (d)).

CONCLUSION

In the absence of collagen, surfaces with low PAA graft densities undergo rapid reorganization accompanied by a rapid change in surface energy similar to that in ungrafted plasma-treated films. The immobilization of collagen on PAA-grafted PET surfaces leads to an increase in the hydrophilicity that is stable over long storage periods because of polyionic crosslinking. The total amount of collagen immobilized by complexation with the PAA at the film surfaces increases with increasing PAA graft density. However, ~50% of the collagen appears to be only weakly adsorbed and can be removed by washing. Thermal crosslinking of the collagen layer at ≥110 °C was shown to be effective in reducing collagen loss during washing. The heat treatment apparently results in a collapse of the fibrillar structure of the collagen layer present after dip-coating. However, this structure is restored on washing in water. Such surfaces may be used in tissue engineering.

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Figure 7 Human smooth muscle cells grown on control costar cell culture plates, and PET-g-PAA films modified with collagen immobilization and subsequent thermal collagen crosslinking: (a) 1 day culture; (b) 3 day culture; (c) and (d) patchy detachment of the cells could be seen before 100% confluence was reached.

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