Journal of Industrial Microbiology (1995) 15, 391–396

Prevention of biofilm formation by polymer modification

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Bacterial biofilm formation on synthetic polymers plays an important role in industry and in modern medicine, leading, for example, to difficult-to-treat infections caused by colonized foreign bodies. Prevention of biofilm formation is a necessary step in the successful prophylaxis of such infections. One approach is to inhibit bacterial adherence by polymer surface modification. We have investigated polymer modification by glow discharge treatment in order to study the influence of the modified surface on bacterial adherence. Surface roughness, surface charge density and contact angles of the modified polymers were determined and related to the adherence of *Staphylococcus epidermidis* KH6. Although no influence of surface roughness and charge density on bacterial adherence was noticed, a correlation between the free enthalpy of adhesion (estimated from contact angle measurements) and adherence was observed. There seems to exist a certain minimum bacterial adherence, independent of the nature of the polymer surface. Modified polymers with negative surface charge allow for bacterial adherence close to the adherence minimum. These polymers could be improved further by the ionic bonding of silver ions to the surface. Such antimicrobial polymers are able to prevent bacterial colonization, which is a prerequisite for biofilm formation. It is suggested that modification of polymers and subsequent surface coupling of antimicrobials might be an effective approach for the prevention of bacterial biofilm formation.

Keywords: biofilm; prevention; polymer modification; glow discharge treatment

Introduction

Bacterial adherence to solid surfaces is a naturally occurring phenomenon. Many bacterial species live and grow under conditions in which they are attached to natural surfaces [26], often growing embedded in their own organic polymer matrix as biofilms. Adherence of bacteria to and subsequent biofilm formation on synthetic surfaces such as industrial or medical material surfaces is also well known but may have undesirable effects. For example, marine bacteria can readily adhere to solids and cause fouling [5]. Industrial polymers susceptible to bacterial adherence may undergo changes in their properties and degrade due to enzymatic attack by microorganisms [19]. In medicine, bacterial adherence and colonization of devices, usually associated with biofilm formation, play an important role in the development of dental plaque formation and of device- or implant-related infections [7,30].

Adhesion of microorganisms to mammalian cells is supposed to be the first important step in the development of many infectious diseases. Also, there is evidence from many investigations that bacterial adherence to medical devices (mostly made from synthetic polymers) is the first and most important step in the pathogenesis of foreign body-associated infections. Subsequent colonization, production of extracellular substances ('slime', 'glycocalix') and involvement of host factors (cells, proteins) lead to the formation of a compact matrix (biofilm) on a biomaterial surface. Generally, biofilm formation protects embedded microorganisms against host defense mechanisms and antibiotics [4,13,14], and can lead to their undesirable persistence and survival on industrial polymers such as pipes, tubes and medical devices and implants. Biofilm formation may therefore explain the difficulties in treatment of most foreign body infections and their chronic, long-lasting nature.

In the past ten years, prevention of biofilm formation has been the subject of a number of investigations. The aim of these studies has been to prevent the undesirable effects of biofilm formation, eg fouling or degradation of industrial polymers, and to prevent foreign body infections [23]. The majority of these investigations has dealt with prevention of foreign body infections by incorporation of antibiotics or other antimicrobial agents, such as metal salts, into medical devices. Only a few studies have reported modification of polymers to obtain anti-adhesive or anti-infective surfaces, and even fewer concern bacterial cell modulation or blocking of adhesion factors. Other strategies proposed recently are the destruction of the biofilm by enzymes followed by the administration of antimicrobials to destroy the released cells, and electrical enhancement of antibiotic penetration [1,20].

Some of these investigations on prevention of foreign body infections have centered directly on the inhibition of biofilm formation [1,3,24,27], eg by using silver alloy-coated devices or by using enzymes together with antibiotics for the destruction of biofilms. Recently, Costerton *et al* [20] found that when an electric field was applied together with antibiotic administration, the killing of biofilm-embedded *Pseudomonas aeruginosa* by antibiotics was dramatically enhanced by tobramycin. It is assumed that penetration of the antibiotic through the biofilm is facilitated by this technique, offering new perspectives on the prevention and therapy of foreign body infections.

Our group has been involved in the development of stra-

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Received 17 November 1994; accepted 27 May 1995

tegies for the prevention and control of foreign body infections. Currently, we are following two main approaches [18]:

1) Modification of medical polymers by physico-chemical methods to obtain anti-adhesive devices [17,22].
2) Incorporation of antibiotics or other antimicrobial substances into polymers to obtain anti-proliferative, colonization-resistant devices [11,15,16].

In this article, we focus on our studies dealing with surface modification of polymers. The aim is to achieve surfaces to which bacteria do not adhere or to which microbial adherence is greatly reduced. This could be accomplished by changing the surface properties of a polymer in a way such that altered interactions occur with bacteria and/or with the biological environment. For example, it is well known that bacteria show lesser adherence to hydrophilic, water-swollen polymers and to surfaces to which a preferential adsorption of blood protein albumin occurs [17]. We wanted to evaluate the specific influence of polymer surface properties on bacterial adherence *in vitro*.

An elegant and versatile method to achieve polymers with varying surface properties is surface modification by the glow discharge technique ('plasma'-technique), where the surface of a synthetic material is exposed to a glow discharge under reduced pressure [8,12,29,33,34]. Using this method, the surface of most polymers can be modified in a variety of ways without changing bulk properties like mechanical stability and elasticity.

Depending on the nature of the glow-discharge gas that is used, the polymer surface may be modified in different ways. Treatment with polymerizable gases ('plasma-polymerization') results in the formation of a dense coating of a 'plasma'-polymer on the polymer surface. Plasma-induced grafting, on the other hand, leads to the formation of a coating consisting of mobile polymer chains. In this process, glow discharge is used to generate radicals inside the surface of the synthetic material. Following the glow discharge reaction, a polymerizable monomer is transferred into the system and reacts with the polymer, creating graft chains.

We describe here the influence of surface properties of modified materials created by glow discharge treatment on the adherence of *Staphylococcus epidermidis* KH6, as a prerequisite for the development of anti-adhesive or anti-infective polymeric materials.

Materials and methods

Polymers

The unmodified polymers used were polyethylene, polypropylene, polyethylenterephthalate (all Hoechst AG, Germany), polyetherurethane 'Walopur 2201 U' (Wolff Walsrode, Germany) and poly(tetrafluorethylene-co-hexafluorpropylene) 'Teflon FEP' (Du Pont Deutschland GmbH, Bad Homburg).

Bacteria

S. epidermidis strain KH6 was isolated from a patient with catheter septicemia. The strain is a strong slime producer and serves as a reference strain for adhesion experiments in our laboratory.

Glow discharge treatment

To obtain modified polymers, the polyurethane was treated using the glow discharge technique. The polyurethane was extracted in ethanol and dried under reduced pressure prior to use. The plasma reactor (Softal, Hamburg, Germany) was designed for the treatment of films with a size of 100 mm × 100 mm. The high frequency generator used for glow discharge operates at a frequency between 20 and 30 kHz.

Plasma polymerisation was done with gas mixtures of CO_2 and C_2H_4 or CO_2 and C_4F_8. In plasma-induced grafting, monomers like acrylic acid, butyl acrylate and methyl vinyl acetamide were grafted onto polyurethane films which had been treated with oxygen in a glow discharge. The liquid monomers were distilled and stored under reduced pressure prior to use.

After modification the films were cleansed with water and finally dried under reduced pressure. The surface properties were varied by the change of glow discharge conditions—gas flow rates, pressure, or grafting time.

Coating with silver

An acrylic acid-grafted polyurethane was incubated for 30 min in a saturated aqueous solution of silver nitrate at room temperature to obtain a device with potential antimicrobial properties. The sample was then washed ten times with small portions of distilled water and dried under reduced pressure. The same method was applied to a native polyurethane film. Measuring the silver uptake and release by atomic absorption spectroscopy revealed that all of the unbound silver had been removed.

Free enthalpy of adhesion

On the basis of contact angle measurements it is possible to calculate the free enthalpy of adhesion. Contact angles of the surfaces were determined using a Lorentzen & Wettre (Stockholm, Sweden) goniometer. At least 30 measurements were performed for each sample and the mean value was taken as the final result. Water contact angles in air (sessile-drop technique) were determined with triple-distilled water. Contact angles of samples in an aqueous environment were examined with an air bubble or an octane drop by the method of Hamilton [9,10].

Contact angles were measured on a bacterial layer of *S. epidermidis* KH6. Bacteria were cultured in tryptic soy broth (Difco, Germany) for 18 h at 37° C, harvested by centrifugation and washed three times in phosphate-buffered saline (PBS, pH 7.2, 0.0023 mol L^{-1} NaH_2PO_4, 1 mol L^{-1} Na_2HPO_4, 0.15 mol L^{-1} NaCl). Cells were resuspended in PBS and collected on a cellulose triacetate filter according to a method described by Busscher *et al* [2]. Contact angles on the bacterial substrata were examined in an aqueous environment using an air bubble or an octane droplet. Thus the effect of drying was excluded, and the contact angles remained constant for at least 3 h. The free enthalpy of adherence, ΔG (often referred to as 'free energy of adherence'), can be calculated by knowledge of the interfacial tensions between solid and bacteria, γ_{SB}; solid and

liquid medium, γ_{SL}; and bacteria and liquid medium, γ_{BL} (Equation 1) [6].

$$\Delta G = \gamma_{SB} - \gamma_{SL} - \gamma_{BL} \quad (1)$$

We used the 'harmonic mean' approach [31,32] to calculate interfacial tensions from surface tensions of the single media and their polar and disperse components, which were calculated from contact angle data [21,28].

Roughness

Surface roughness of the polymers was determined with a Perthometer (Feinprüf GmbH, Göttingen, Germany), where the surface is scanned by a laser beam focused on the surface. The change in focus gives an image of the surface roughness. Ten successive distances of 1.25 mm were examined and the mean value of roughness was taken as the final result.

Surface charge

The negatively charged carboxyl groups of the acrylic acid grafted onto the polyurethane films were determined by adsorption of a cationic dye. The dye, Astrazon Blue F2RL (Bayer AG, Leverkusen, Germany), was purified by filtration and subsequent removal of the solvent (acetone). A buffered dye solution (0.05% in PBS) was used to stain the treated surface of the polymer samples (surface area about 1 cm^2) at pH 7.2 for 10 min. After rinsing the samples with 50 ml of water in small portions, the adsorbed dye was removed with 5 ml of 84% acetic acid. Photometric measurement of this extract was performed at a wavelength of 591 nm using a Zeiss Q II photometer (Zeiss Oberkochem, Germany).

Bacterial adherence to modified films

Bacterial adherence of *S. epidermidis* KH6 to the surface-modified polymer films was measured using the bioluminescence assay described by Ludwicka *et al* [25]. Bacteria were cultivated as described above and resuspended in PBS. The modified surface of a 12-mm diameter polymer disc was cleansed with ethanol and distilled water and the disc was then incubated in 2 ml PBS (pH = 7.2) usually containing 10^8 colony forming units (cfu) per ml at room temperature. After an adherence period of 3 h with gentle shaking, the disk was washed three times with PBS and then treated with 100 μl 2% trichloroacetic acid to extract bacterial ATP. The extract was diluted with 400 μl TRIS-acetate/EDTA buffer (pH = 7.75, 0.1 mol L^{-1} TRIS-acetate, 0.002 mol L^{-1} EDTA). A 50-μl aliquot was taken, to which 400 μl TRIS-acetate/EDTA buffer and 200 μl ATP monitoring reagent (LKB Wallac, Finland) were added. The ATP monitoring reagent converts ATP to AMP and light, which was measured in a bioluminometer (LKB Wallac, Finland). Light emission is proportional to the ATP concentration, and by establishing a standard curve for bacterial concentration vs ATP content, the percentage of adherent bacteria (in relation to the concentration of the initial bacterial solution) per cm^2 surface was estimated. The mean value of nine measurements per sample was taken as the final result. Adherence results were standardized to the concentration of bacteria in suspension and to the surface area of the polymer disk and expressed as % ml cm^{-2*}. There was a linear correlation between adherent bacteria per cm^2 and bacterial concentration in the range of 10^5–10^8 cfu ml^{-1}. For the measurement of each modified polymer sample, a standard curve was established and the unmodified polymer film was measured simultaneously. Background ATP levels were constant for the different materials and were subtracted from the sample ATP values.

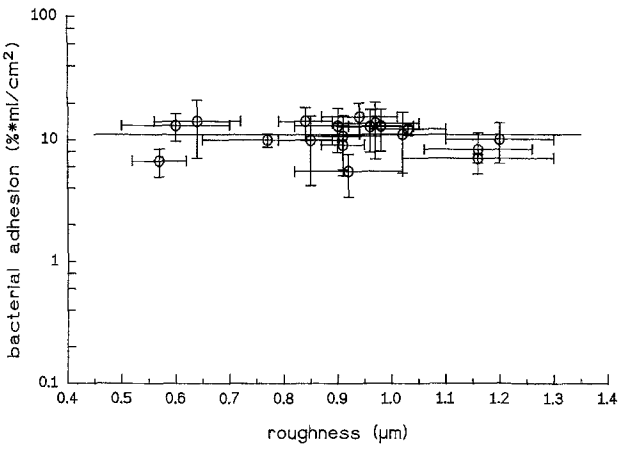

Figure 1 Influence of biomaterial roughness on bacterial adhesion. Adhesion values were standardized with respect to bacterial concentration and sample area

To study the antimicrobial efficacy of silver-containing samples, the number of viable adherent bacterial cells on their surfaces was determined. Bacterial adherence was carried out with 10^5 cfu ml^{-1} in TRIS-acetate/EDTA buffer (pH = 7.75). Adherent bacteria were detached from the polymer in 10 ml PBS by ultrasonication (Branson Sonifier, USA, 2 × 45 s at 80 watts). Ultrasonication did not affect the viability of *S. epidermidis* KH6. The number of adherent colony forming units (cells) was determined by viable count on blood agar.

Results

Figure 1 shows the results of bacterial adherence measurements as related to the surface roughness of the samples. Within the observed range of 0.4–1.4 μm, bacterial adherence was independent of surface roughness.

Although no correlation between surface hydrophilicity as determined by contact angles (results not shown) and adhesion was found, a marked influence of the free enthalpy of adhesion on the measured bacterial adherence was observed for uncharged polymers (Figure 2). For negative free enthalpy values, bacterial adherence decreased with increasing free enthalpy. In the case of positive free enthalpy adherence of *S. epidermidis* KH6, a constant value of about 1% ml cm^{-2} was determined.

Polymer samples with negative surface charge showed reduced bacterial adherence compared with uncharged surfaces having the same calculated enthalpy of adhesion (Figure 3). However, the negative charge density did not

* $\dfrac{\text{Number of adherent bacteria (cfu)} \times 100}{\text{Surface area } (cm^2) \times \text{concentration of bacteia in suspension (cfu } ml^{-1})}$

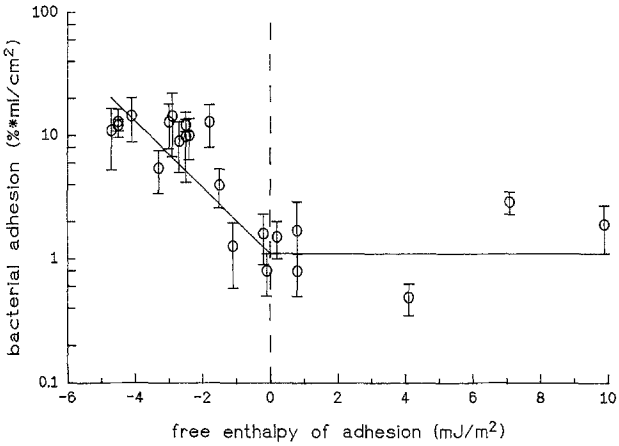

Figure 2 Correlation between bacterial adhesion and calculated free enthalpy of adhesion. Adhesion values were standardized with respect to bacterial concentration and sample area

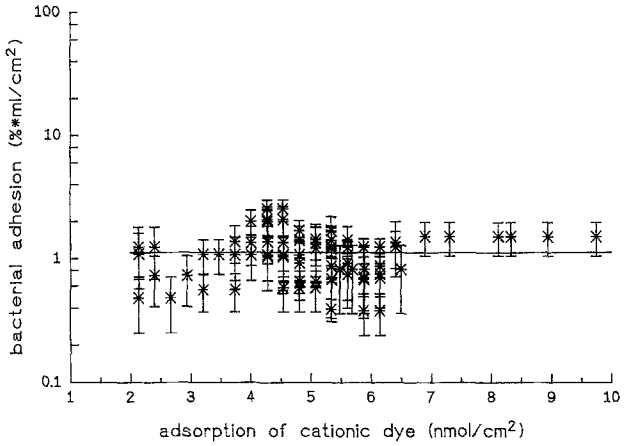

Figure 4 Influence of charge density of polymer, measured as amount of cationic dye absorbed by the film, on bacterial adhesion. Adhesion values are standardized with respect to bacterial concentration and sample area

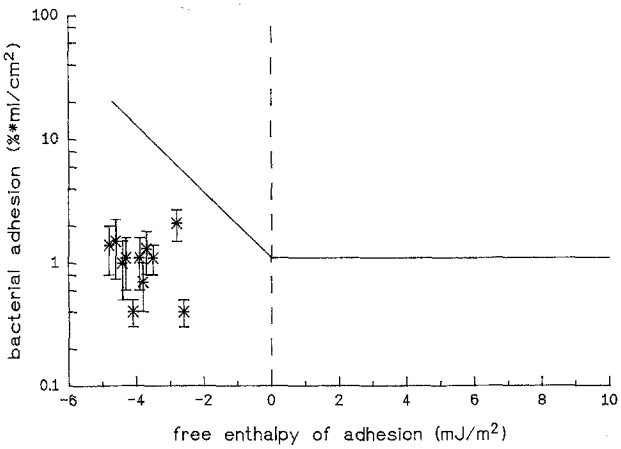

Figure 3 Influence of surface charge on bacterial adhesion depending upon calculated free enthalpy of adhesion. Adhesion values were standardized with respect to bacterial concentration and sample area. Line: Bacteral adhesion of polymer surfaces without charge. Stars: Bacterial adhesion on polymer surfaces with negative charge

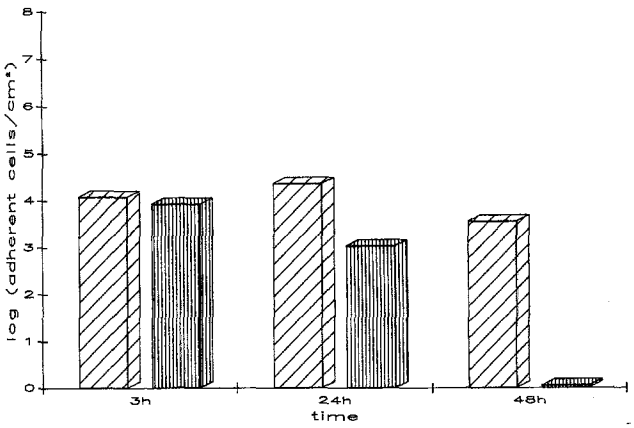

Figure 5 Adherent viable bacteria on an acrylic acid-grafted polyurethane film, with or without surface-bound silver, with respect to adhesion time. ▨ modified polymer; ▥ modified polymer with surface bonded silver

influence bacterial adherence (Figure 4). All samples had adherence values of about 1% ml cm^{-2}.

Experiments with an antimicrobial agent (silver ions) bonded to acrylic acid-modified, negatively-charged polymers revealed that the maximum silver uptake in the surface was about 23 $\mu g\ cm^{-2}$. Three $\mu g\ cm^{-2}$ could be washed out with water because this amount was absorbed by the polyurethane backbone itself and therefore was not ionically bonded. In the case of silver-containing modified films the number of adherent bacteria was dramatically reduced compared to the native modified film; after 48 h of adherence no viable bacteria could be detached from the surface (Figure 5).

Discussion

The development of materials with anti-adhesive or antimicrobial properties is a promising approach to prevention of biofilm formation. For the design of anti-adhesive surfaces, it is necessary to know how bacterial adherence will be influenced by the physico-chemical surface properties of the polymer. We used the glow discharge technique to modify a wide range of polymers used for medical devices. With the aid of contact angle measurements of both the modified materials and of the bacterial cell surface (in our case *S. epidermidis* KH6) it was possible to calculate surface interaction parameters and the free enthalpy of adhesion. Attempts were made to determine whether a correlation exists between physico-chemical surface parameters and bacterial adherence in order to draw conclusions as to how anti-adhesive surfaces could be manufactured.

From the physico-chemical point of view, bacterial adherence to polymers can be regarded as the adherence of a particle to a solid surface in a liquid environment. Thus, adherence is controlled by a change of free enthalpy of adhesion. Adherence is thermodynamically favored if the free enthalpy of adhesion is negative and decreases with increasing free enthalpy values. This behavior was confirmed by our results obtained with samples having negative enthalpy values. No adherence should occur if the free enthalpy of adhesion is positive (the bacterium needs

energy to adhere). In contrast to this we found that *S. epidermidis* KH6 shows a constant adhesion value of about 1% ml cm^{-2} on modified polymers with positive values of adhesion enthalpy.

Our results suggest that *in vitro* there seems to exist a certain *minimum number of adherent bacteria*, independent of the free enthalpy of adhesion and the nature of the polymer surface. As most of the bacteria in a liquid medium are negatively charged, a negatively-charged polymer surface should repel bacterial cells. This was demonstrated for negatively-charged, modified polymers, which showed less bacterial adherence than uncharged modified polymers. However, all negatively-charged samples showed a minimum number of adherent bacteria of about 1% ml cm^{-2}. These observations may have implications for the development of strategies to avoid bacterial adhesion and biofilm formation on polymers, since it might be impossible to create a polymeric surface to which no bacterial adherence will take place.

Nevertheless, there are some materials that show very low adherence, eg negatively-charged surfaces. Such polymers with a reduced tendency for bacterial adherence might be useful materials with respect to prevention of adhesion, colonization and biofilm formation since in the practical situation the bacterial load is much less than in our *in vitro* experiments. Thus, very few bacteria should adhere to such modified surfaces in comparison with materials currently used.

Further, glow discharge-modified polymers with negative charge are able to bind ionically antimicrobial agents such as silver ions. Such antimicrobial materials are able to eliminate initially-adherent bacteria very effectively within 48 h. The delay in activity is probably due to the time needed for the silver ion to diffuse into the bacterial cell. The silver-containing surfaces reduced adherent viable bacteria from initially 10^4 cm^{-2} to zero, thereby preventing bacterial colonization which is a prerequisite for biofilm formation. Such a system with surface-bonded silver shows high activity for a long time and its antimicrobial action is restricted to the polymer surface, so that development of resistance or systemic toxicity might not occur.

In conclusion, we have demonstrated for the system tested (glow discharge-modified polymers/*S. epidermidis* KH6), that by polymer modification alone bacterial adherence to a native polymer surface cannot be prevented. Based on our investigations on bacterial adherence to polymers with different physico-chemical properties, it must be hypothesized that there exists a certain minimum of bacterial adherence on a polymer surface which cannot be reduced. Work is in progress to determine if this hypothesis holds true for other *S. epidermidis* strains and for other bacterial species. A practical consequence from our results is that it is possible—eg by introducing negatively-charged surface groups into polymers—to develop polymers with reduced bacterial adherence close to the minimum level of adherence. These polymers might be of potential value for medical and industrial applications. Such materials can further be improved by surface-coupling of antimicrobial agents like silver. Thus, modification of polymers together with subsequent bonding of antimicrobials seems to be an effective strategy to prevent bacterial colonization and biofilm formation.

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