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Binding of salivary proteins and oral bacteria to hydrophobic and hydrophilic surfaces in vivo and in vitro

B. Lassen[1]), K. Holmberg[1]), C. Brink[2]), A. Carlén[3]) and J. Olsson[3])

[1]) Institute for Surface Chemistry, P.O. Box 5607, S-114 86 Stockholm, Sweden
[2]) Berol Nobel, S-444 85 Stenungsund, Sweden
[3]) Department of Cariology, Faculty of Odontology, University of Göteborg, P.O. Box 33070, S-400 33 Göteborg, Sweden

Abstract: Chemical modifications of mineral surfaces were performed in order to gain insight into what surface properties are decisive of the accumulation of dental plaque. A non-charged, hydrophilic surface was made by two consecutive plasma polymerizations, firstly with allyl alcohol, secondly with acrylic acid, followed by adsorption of a poly(ethylene glycol)-poly(ethylene imine) adduct. A strongly hydrophobic surface was obtained by plasma polymerization of hexamethyldisiloxane. Ellipsometry was used to monitor protein interaction with the surfaces. The hydrophilic surface gave very little adsorption of both a model protein, IgG, and of saliva proteins. The hydrophobic surface, on the other hand, adsorbed high amounts of both types of proteins. In vitro adhesion of an oral bacterium, *S. mutans*, as well as in vivo studies, gave the opposite result, the hydrophobic surface giving less adhesion and less plaque accumulation than the hydrophilic surface. A tentative explanation of this behavior is that the saliva proteins that bind to the hydrophobic surface adsorb in an unnatural conformation which does not favor bacteria adherence.

Key words: Protein – adsorption – surface modification – hydrophilization

Introduction

It is by now an established fact that inert, protein-repelling materials can be obtained either by surface grafting with a non-charged, hydrophilic polymer, such as poly(ethylene glycol) (PEG), or by transformation of the surface into an extremely low energy layer, for instance a perfluorinated surface. Both types of surfaces show promise in a variety of biomedical products. Surfaces with low tendency to adsorb poteins and cells are of interest for implants and catheters, in solid phase diagnostics and in biosensor technology, just to mention a few applications [1–3].

The two types of surfaces mentioned above are clearly very different and the mechanisms by which they prevent proteins to stick are likely to be of different origin. A densely packed and firmly attached PEG layer rejects proteins because of the unfavorable entropy change that results upon compression of the conformationally-random, heavily hydrated PEG chains by protein adsorption. Adsorption could either be by interpenetration to provide contact with the surface, or it could be directly on top of the PEG layer. It can be shown that both events are thermodynamically unfavorable. This explanation of the protein-repelling effect of PEG chains is analogous to steric stabilization of suspended particles by nonionic surfactants. In addition, the PEG chains as such, being strongly hydrophilic and free of charges, will give minimum driving force for adsorption since both hydrophobic interactions and double-layer attractive forces are absent [4]. The protein-repelling character of the low energy surface, on the other hand, may be seen as a wetting effect: spreading on very low energy surfaces is energetically unfavorable for all molecules, including proteins.

The two major oral diseases, caries and periodontitis, are both caused by bacteria colonizing in

dental plaque. Plaque formation is believed mainly to be due to microbial adhesion and growth [5]. Thus, ways to modify the tooth surface to render it more inert in terms of adsorption from aqueous solution should be of interest for prevention of oral disorders. We have investigated hydrophilization and hydrophobization of dental surfaces in order to minimize biofouling. We have earlier demonstrated that hydrophilization using either PEG derivatives or uncharged polysaccharides is an effective way of preventing adhesion of salivary proteins and binding of oral bacteria in vitro, also in the presence of saliva [6]. Attempts to employ similar routes of hydrophilization in vivo have not been successful in preventing plaque formation, however [7]. We have also showed in a preliminary set of experiments that strongly hydrophobic surfaces were less effective than the PEG surface in preventing protein and bacteria adhesion in vitro but more effective in in vivo experiments [8].

These somewhat contradictory results gave rise to the present study in which glass objects were transformed either into a non-charged, hydrophilic surface by dense grafting of PEG chains or into a low energy surface by plasma polymerization of a low molecular weight siloxane. The two types of surfaces were then compared with regard to protein adsorption and bacterial adhesion in vitro. The same surface modifications were also made on porcelain crowns and evaluated with regard to dental plaque accumulation in vivo.

Materials and methods

Preparation of hydrophilic surface

Plasma polymerization of allyl alcohol followed by acrylic acid was carried out on polished silicon wafers (Wacker Chemie, FRG). The plasma polymerization system consisted of a glass vessel with an inner diameter of 150 mm and a length of 650 mm. The vessel is equipped with a rotary vacuum pump, a radio frequency power generator working at 125–375 MHz, and a gas flow control system. Two external copper electrodes, 30 mm wide, are wrapped around the glass vessel at a distance of 70 mm from each other and connected to the power generator. The applied effect for the polymerization of allyl alcohol is 20 W and the pressure before plasma ignition is 20 mtorr. After 4 min reaction the reactor was evacuated and subsequently charged with acrylic acid. Polymerization was carried out at an effect of 10 W and with an initial pressure of 26 mtorr.

The poly(acrylic acid) modified surface was treated with a graft copolymer consisting of poly(ethylene glycol) (PEG) chains attached to a poly(ethylene imine) (PEI) backbone. The PEG-PEI adduct was synthesized by reaction of diglycidyl ether of PEG 4000 (5.0 g; development product from Berol Nobel, Sweden) and PEI (0.43 g; Polymin SN from BASF, FRG) in 0.05 M carbonate buffer at 45 °C for 3 h. The surface treatment was performed with a 10% solution in carbonate buffer, pH 9.5 of the adduct for 5 h at 40 °C. The pH was then adjusted to 7.0–7.5.

Preparation of hydrophobic surface

Hexamethyldisiloxane (Aldrich) was plasma polymerized on polished silicon wafers using the same equipment as described above. The effect applied was 10 W and the pressure before plasma ignition was 17 mtorr.

Contact angle measurement

Wettability of the PEG surface and the polyhexamethyldisiloxane surface was determined by measuring advancing contact angles of sessile water drops at the solid-air interface using a goniometer equipped with an eye-piece.

Measurement of protein adsorption by ELISA

Adsorption of IgG was measured on small glass tubes with an inner diameter of 10 mm using a modified ELISA method. The glass tubes were subjected to the same surface treatment as described above for the silicon wafers. After thorough washing and rinsing the tubes were incubated in swine IgG (Dakopatts, Denmark; 0.02 mg/ml in 0.05 M phosphate buffer, pH 7.0) for 1 h at 20 °C. The tubes were rinsed, first with distilled water, then with 0.1 M phosphate buffer, pH 7.0, containing 1.0 M NaCl and 0.2% Tween 20, and finally with distilled water. The washed tubes were incubated in peroxidase-conjugated antibodies (rabbit anti-IgG from Dakopatts, diluted to a final concentration of 10 μg/ml in the

above-mentioned phosphate buffer) for 30 min at 20 °C. Rinsing was repeated as above and the substrate, consisting of 8 OPD tablets of 1,2-phenylenediamine hydrochloride (Sigma, USA), containing 10 mg substrate per tablet, dissolved in 24 ml 0.1 M citrate buffer, pH 5.0, was added together with 10 µl of 30% (v/v) hydrogen peroxide. The oxidation was stopped after 3 min by addition of 5 M sulfuric acid (0.25 ml/ml OPD solution). The absorbance was recorded at 495 nm. The values given are means of triplicate runs.

Measurement of protein adsorption by ellipsometry

Adsorption of saliva constituents on the surface modified silicone wafers was monitored in situ by ellipsometry using a flow cell with a total volume of 0.054 ml. The ellipsometer was a Rudolph Research null ellipsometer, model Auto EL III, equipped with a 632.8 nm He–Ne laser light source. The flow rate was kept at 2 ml/min, giving laminar flow in the cell. The temperature was kept constant at 20 °C. The saliva used was parotid saliva, collected using Lashley cups into an ice-chilled tube. The saliva was collected 16–20 h before the measurements, immediately diluted 1:1 with either PBS or a Ca/Mg buffer (1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 1 mM K_2HPO_4, 1 mM KH_2PO_4 and 50 mM KCl) and stored at 4 °C before use.

Prior to measuring, the surface was equilibrated in the buffer used. The buffer solution was then replaced by saliva, diluted 1:10 with the same buffer. The solution was circulated in the cell for approximately 90 min (exact time given in Figs. 2 and 3) and the cell was then rinsed with buffer for 10 min. A 0.1% solution of sodium dodecyl sulfate (SDS) was then circulated in the cell for approximately 5 min followed by rinsing with distilled water.

Average refractive index, n_f, and mean optical thickness, d (in nm), of the surface film were calculated using the McCrackin program to evaluate the ellipsometric data [9]. From these values the surface concentration, Γ, (in ng/mm^2) was calculated according to de Feijter's equation for a transparent film (10):

$$\Gamma = \frac{d(n_f - n_m)}{\mathrm{d}n/\mathrm{d}c}$$

n_f is the refractive index of the film; n_m is the refractive index of the bulk; dn/dc is the refractive index increment of the solute.

For proteins dn/dc is 0.18 (11) and for SDS dn/dc is 0.17 (12). The value of surface concentration obtained by de Feijter's equation has been found to be within an error limit of ±15% [10, 13].

Measurement of bacterial adherence

Streptococcus mutans strain Ingbritt [14], metabolically labeled with 35-S, were allowed to adsorb on the same type of glass tubes as had been used in the ELISA experiments. The tubes had been surface modified as described above for the silicon wafers. The tubes were incubated for 60 min at 37 °C in parotid saliva, diluted 1:1 with PBS. The tubes were rinsed gently with PBS and then incubated in a suspension of 5×10^8 *S. mutans* cells. After 60 min at 37 °C the tubes were rinsed gently in PBS and transferred to a vial containing 10 ml of scintillation liquid (OptiPhase 'HiSafe' II Wallac, Kabi-Pharmacia, Sweden). Counting of radioactivity was performed in a 1215 Rackbeta liquid scintillation counter (Wallac, Kabi-Pharmacia). The procedure has been described in detail elsewhere [7].

In vivo experiments

Dicor crowns were hydrophilized and hydrophobized as described above for the silicone wafers and evaluated with respect to plaque build-up on a patient who needed replacement of crown restorations on the upper medial incisors. In the same experiment, of 5 days duration, plaque accumulation was investigated both on the hydrophilized and on the hydrophobized surface. The crowns were luted with temporary cement and care was taken not to touch the buccal surfaces of the modified Dicor crowns. The patient performed no oral hygiene during the test period. All teeth were disclosed with erythrosine (Diaplac Rondell, Astra, Sweden) and polished free from visible plaque with a rubber cup and a polishing paste (RDA, mesh 170). After the 5 days test period the teeth were again disclosed with erythrosine and photographs were taken in the front and premolar regions. Crowns and natural tooth surfaces were subjected to a gentle air/water spray

in order to remove any loosely bound material and new photographs were taken.

Results and discussion

Surface modification

Adsorption of the graft copolymer of PEG and PEI has previously been found to be an efficient way to obtain a dense and firmly attached PEG layer on hydrophobic surfaces, such as polystyrene [15]. In order to improve anchoring of the PEI backbone, the plastic surface is first made anionic by some kind of surface treatment, e.g., acid etching. Such PEG modified surfaces display very low tendency to adsorb proteins.

We have previously attempted to transform glass and other mineral surfaces to protein-repelling materials by the same type of surfaces treatment. This has invariably given poor results, however, presumably due to formation of hydrogen bonds between PEG ether oxygens and silanol groups in the mineral surface. Such positive interactions, which are known to occur [16], may cause the hydrophilic chains to lay flat on the surface rather than to stretch out into the bulk phase. Protein repulsion due to steric interaction will, of course, not be effective unless the PEG chains strive away from the mineral surface. Our rationale for a plasma polymerization step prior to adsorption of the PEG-PEI adduct was to bury the silanol groups under a thin organic layer. Since the layer should be negatively charged in order to give maximum interaction with the highly positively charged PEI, attempts were made to plasma polymerize acrylic acid directly on the surface. The poly(acrylic acid) coating obtained proved to have too low adhesion to the substrate to be of practical use, however. As an alternative route two consecutive plasma polymerizations were performed, firstly with allyl alcohol, secondly with acrylic acid. The layer obtained was 530 Å thick, according to ellipsometry, and it showed no tendency to come loose from the underlying mineral surface.

Adsorption of the PEG-PEI adduct to plasma modified glass or silicon wafer was made from aqueous solution [15]. The steps involved in the surface modification are schematically illustrated in Fig. 1. The low contact angle obtained (Table 1) is indicative of a high density of PEG chains on the surface. An even better indication of an effective PEG grafting is the low values of protein adsorption obtained (see below).

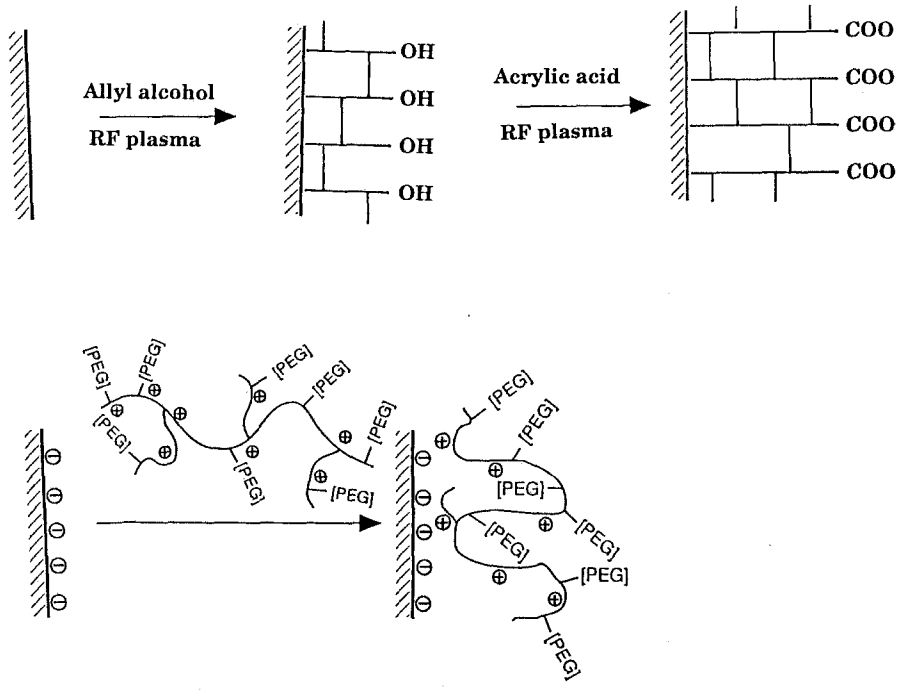

Fig. 1. PEG modification of a mineral surface.

Table 1. Advancing contact angles of distilled water on glass modified by PEG and by hexamethyldisiloxane (HMDSO)

Sample	Contact angle (°)
PEG treated	<10
HMDSO treated	90

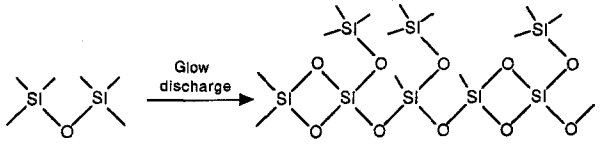

Fig. 2. Plasma polymerization of hexamethyldisiloxane.

Plasma polymerization was also used to prepare materials of very low surface free energy. The monomer used was hexamethyldisiloxane which has proved to give silicone-like surface layers in previous experiments [17]. Figure 2 illustrates the reaction. The high contact angle obtained by this treatment (Table 1) shows that the surface obtained is very hydrophobic. The polysiloxane layer thickness was not determined in this experiment but is typically in the range 200–300 Å.

Protein interaction studies

As can be seen from the ELISA results shown in Table 2, the PEG coating reduces IgG adsorption to less than 7% of the amount adsorbed on untreated glass. The hydrophobic surfaces, on the other hand, give an increase in protein adsorption. A higher amount of adsorbed protein on hydrophobized mineral surface than on unmodified material is in accordance with Elwing's results with surfaces of varying degree of wettability [18].

Ellipsometry is a useful technique for analysis of the amount of protein adsorbed on the surface, as well as for studying the kinetics of adsorption and desorption. It has recently been shown, both by ellipsometry and by reflectometry, that salivary film formation on solids is a very fast process, starting immediately after exposure to saliva. Hydrophobic surfaces, such as methylated silica, adsorb higher amounts than hydrophilic mineral surfaces [19, 20]. Figure 3 shows the interaction of dilute saliva with the surface of untreated silicone wafer. As is clearly seen, the initial adsorption is very fast and a plateau value of 3–3.5 mg protein per m^2 wafer surface is attained after about 1 h. The adsorption pattern is typical for proteins and is indicative of a fast initial step which is followed by reorientation in the adsorbed layer allowing more proteins to reach the surface [21, 22]. The plateau value corresponds to a rather dense monolayer of adsorbed proteins. The two buffers used for dilution give almost the same results.

Table 2. Adsorption of IgG on untreated glass and on glass modified by PEG and by hexamethyldisiloxane (HMDSO) expressed as optical density at 495 nm (OD 495 nm)

Sample	OD 495 nm
Untreated glass	0.261
PEG treated glass	0.018
HMDSO treated glass	0.471

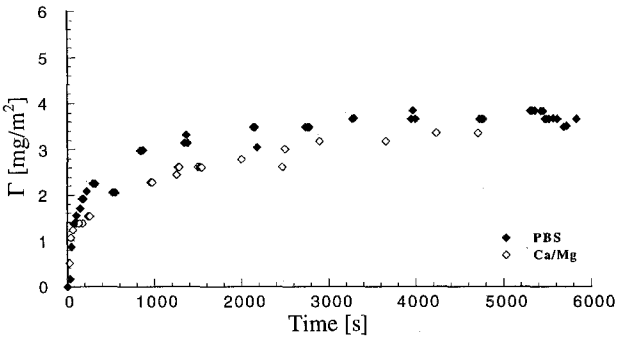

Fig. 3. Adsorption of saliva constituents on untreated silicone wafer as a function of time.

Figure 4 shows the interaction of the same saliva with the hydrophilic PEG surface. No adsorption of saliva constituents can be detected when the sample is diluted in PBS buffer and only a very small amount is seen when the Ca/Mg buffer is used.

Adsorption of saliva constituents on the hydrophobic siloxane surface is shown in Fig. 5. Adsorption is strong from both of the buffer solutions with a more rapid initial rate of adsorption and a higher plateau value than on the untreated surface (Fig. 3). The plateau value obtained, 4–5 mg/m^2, is indicative of a densely packed monolayer of adsorbed protein and is in the same range as has been found in a recent study using ellipsometry on hydrophobic methylated silica [19]. Addition of the anionic surfactant SDS after 6000 seconds leads to rapid desorption (data not shown).

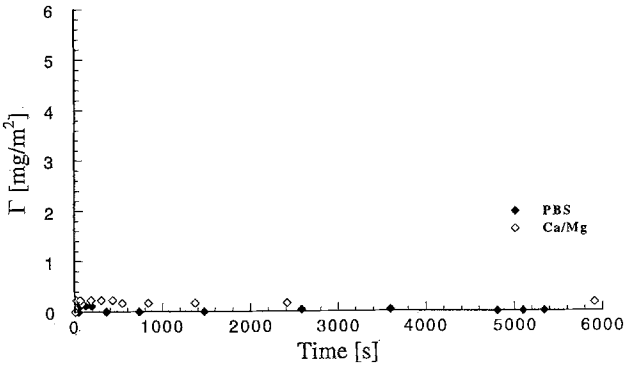

Fig. 4. Adsorption of saliva constituents on PEG modified surface as a function of time.

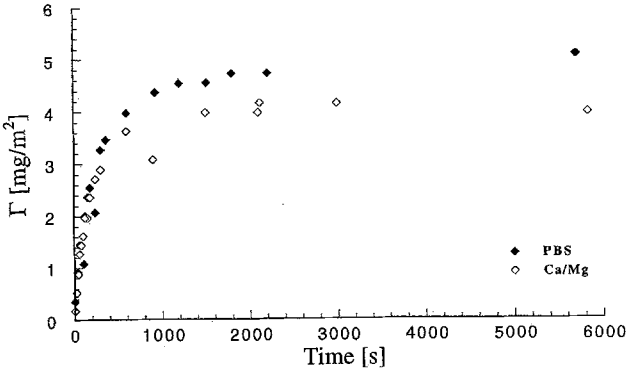

Fig. 5. Adsorption of saliva constituents on hexamethyldisiloxane modified surface as a function of time.

Thus, the results obtained with ELISA using a model protein (IgG) and with ellipsometry using parotid saliva are consistent and show that proteins are effectively repelled by the PEG surface and strongly attracted by the polysiloxane surface. It is particularly interesting that the route employed for PEG grafting of glass and silicone wafers, i.e., consecutive plasma polymerization of allyl alcohol and acrylic acid followed by adsorption of the PEG-PEI adduct, is an efficient procedure in terms of protein rejection. PEG modification of such materials seems not to have resulted in surfaces with low protein-adhering characteristics before.

Bacterial adherence studies

It has been demonstrated by Christersson et al. that the level of adsorption of oral bacteria in the

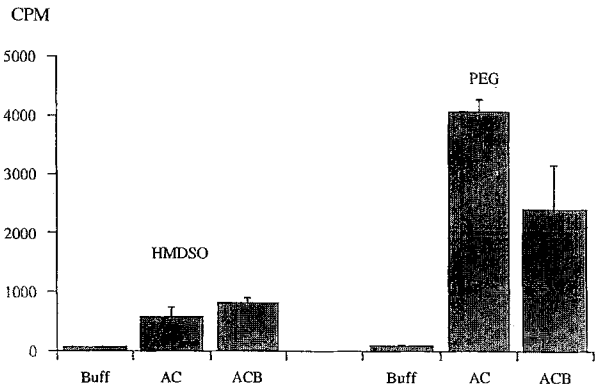

Fig. 6. Binding of 35-S labeled *S. mutans* (given in counts per minute, CPM) to PEG modified surfaces (PEG) and to hexamethyldisiloxane modified surfaces (HMDSO). Experiments were carried out in buffer and in the presence of saliva from two donors, AC and ACB.

presence of saliva depends on the critical surface tension of the substrate [23, 24]. Surfaces of medium critical surface tension (30–38 mN/m) retained higher numbers of microorganisms than both higher and lower energy surfaces. Incorporation of fluorine atoms into hydroxyapatite crystals leads to a lowering of the critical surface tension and also to reduced plaque accumulation [25]. Hence, the character of the underlying surface is vital to adhesion of oral bacteria in vivo, in spite of the fact that the microorganisms are believed not to bind directly to the mineral but to the proteinaceous film which is rapidly formed on all surfaces exposed to saliva. It was suggested that the initial organization of salivary constituents on the surface is related to the critical surface tension and that subsequent events of bacterial adherence are largely controlled by the properties of the proteinaceous film [26].

The PEG surfaces used in this work are of relatively high surface free energy [3], as is human tooth enamel, although absolute values for the latter material are difficult to determine [27]. Unlike tooth enamel the PEG grafted surfaces are non-charged, a fact which minimizes double-layer attractions with molecules in solution. In addition, the PEG chains, being attached in one end only, have a very high mobility, a property which is advantageous for protein rejection from entropic reasons.

Figure 6 shows adherence of the oral bacterium *Streptococcus mutans* on surface modified glass walls. Measurements were made both in the

absence and in the presence of saliva. *S. mutans* is a relevant bacterium to study since it is believed to be associated with the development of caries. The results obtained are unexpected. Whereas neither surface attracts much bacteria in the absence of saliva, both surfaces, and in particular the PEG surface, bind considerable amounts of bacteria in the presence of saliva. The mediating effect of saliva constituents on bacterial adhesion is well known and has been observed by us earlier in similar experiments [6]. The surprising observation made here, however, is that the PEG treatment results in a more bacteria-attracting surface than the siloxane treatment. This is counter to what would be expected from the results from adsorption of saliva constituents (mainly proteins), as displayed in Figs. 4 and 5. Since practically no *S. mutans* binds in the absence of saliva, adhesion of bacteria directly on top of the dense PEG layer can be excluded. The results imply that the small quantity of saliva constituents that does adhere to the PEG layer is enough to efficiently mediate binding of bacteria. Even if many more saliva proteins stick to the hydrophobic surface, the bacteria-mediating effect is much less pronounced.

One may speculate that the reason behind the higher amount of bacteria on the hydrophilic surface than on the hydrophobic surface is that the saliva constituents which promote binding of bacteria are present in a more natural conformation on the PEG surface than on the low energy polysiloxane surface. It is known from studies of saliva constituents at the air-water interface that accumulation of oral proteins at the surface is a fast process which is followed by a stage of rearrangement and increased adsorption, leading to a plateau value of surface tension of around 43 mN/m [28]. The hydrophobic interactions that govern such events may involve interactions with hydrophobic patches located on the exterior of the saliva molecules, as well as with hydrophobic domains originally located in the interior of the biomolecule but exposed as the conformation is changed during the adsorption process [29, 30]. Proteins adsorbing on the polysiloxane surface can be expected to behave in an analogous manner and interact via the hydrophobic domains, directing all the polar groups away from the surface. Protein interaction with mineral surfaces, such as glass or teeth, on the other hand, will mainly take place by electrostatic attraction forces and the presence of divalent ions, such as Ca^{2+}, in saliva is likely to assist binding by forming bridges between negative sites on both biomolecule and solid surface. Thus, proteins adsorbed on the two types of surfaces will induce very different surface characteristics which may strongly influence the adhesion properties. It is well known that proteins interacting with very hydrophobic surfaces often lose their biological activity due to changes in conformation [31, 32]. If this explanation is correct, i.e., only very small amounts of proteins adsorbed on PEG modified surfaces are enough to cause bacteria to adhere, then the prospects for hydrophilization as a means of eliminating bacterial adherence on teeth are not promising.

An alternative explanation of the poor effect of the PEG layer in the presence of saliva is that the hydrophilic chains are rapidly degraded by the action of hydrolytic saliva enzymes. The protein repelling effect of PEG is known to be very molecular weight dependent, and it is likely that degradation of the chains to molecular weights below around 1000 would result in an almost complete loss of effect [4]. We intend to investigate the susceptibility of PEG to saliva enzyme catalyzed cleavage in a future work.

Plaque formation in vivo

Neither of the two routes of surface modification, hydrophilization with PEG or hydrophobation with hexamethyldisiloxane, led to any marked difference in plaque accumulation compared with the control, a non-treated Dicor crown surface. After 5 days without oral hygiene both the test and the control surface showed a thin, even layer of plaque along the gingival margin. Also, a large portion of the buccal surface was covered with a relatively strongly staining film resembling a pellicle. Adjacent natural tooth surfaces did not seem to carry such a thick pellicle coating. Both crowns had considerably less plaque than the neighboring natural tooth surfaces.

In a separate experiment one of the Dicor crowns was treated with a reactive low-molecular weight polysiloxane as a positive control. After curing a transparent, relatively thick polydimethylsiloxane film was obtained. As described

earlier [8], this surface treatment results in very minor plaque accumulation.

Conclusions

A method has been developed to attain a dense PEG grafting on glass. It has been shown that such a surface treatment is an effective way to reduce adsorption of proteins, as illustrated both with a model protein, IgG, and with parotid saliva. However, this surface modification seems not to be a fruitful way to reduce binding of oral bacteria in the presence of saliva and does not seem to markedly influence the amount of plaque accumulation in vivo.

A polysiloxane surface layer on the same glass substrate does not result in a reduction in adsorption of either IgG, or saliva proteins. This strongly hydrophobic surface gives much less binding of oral bacteria in the presence of saliva than does the hydrophilic surface, however. A tentative explanation of the reduced amount of bacteria bound on the polysiloxane surface is that the recognition sites for bacteria binding are lost due to surface induced conformational changes.

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Author's address:

Dr. Krister Holmberg
Institute for Surface Chemistry
P.O. Box 5607
S-114 86 Stockholm
Sweden