

Direct Measurement of Interactions between Tethered Poly(ethylene glycol) Chains and Adsorbed Mucin Layers

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Direct force measurements were used to investigate the interactions between grafted poly(ethylene glycol) (PEG) (M_w 2000) and both adsorbed and soluble bovine submaxillary gland mucin. These measurements show that both soluble and adsorbed mucin adhere weakly to the grafted PEG layers. Soluble mucin mediates a concentration-dependent, bridging attraction between the PEG films. Similarly, PEG adheres to adsorbed mucin layers in both the presence and absence of 0.2 mg/mL mucin solutions. The heteropolymer attraction is pH dependent, with the adhesion being substantially higher at pH 2 than at pH 7.2. The latter suggests that hydrogen-bonding interactions are responsible for binding. The weaker mucin interactions at pH 7.2, however, allow for PEG-coated surfaces to adhere to surface-bound mucin in the presence of soluble mucin. Such characteristics are particularly desirable for coatings for oral drug carriers.

Introduction

A mucous layer covers epithelial surfaces throughout the body including the gastrointestinal tract.¹ Generally, mucus provides protection and lubrication of underlying epithelium. On the other hand, it is also the primary target for oral drug delivery.² The main nonwater components of the mucous layer are mucins, which are long glycoprotein molecules responsible for the gel nature of the mucous layer.

Mucoadhesive drug delivery vehicles have received considerable attention in the last 2 decades.³ They are designed to exploit the attraction between the mucous layer and the polymer carrier of the drug delivery system. The main advantages of mucoadhesive polymer carriers include the localization of the carriers to a specific site within the body and the prolonged time of delivery. These features greatly enhance the bioavailability of drugs, especially for peptide and protein delivery.^{3,4} Obviously, a better understanding of the basis of mucin–polymer interactions in the physiological environment is essential for the rational design of mucoadhesive delivery systems.

Numerous experimental studies have been performed to test the mucoadhesive properties of various polymers.^{5,6} It is generally thought that mucin-attractive polymers are capable of forming a large number of hydrogen bonds with mucin.² This is a reasonable assumption since mucins contain hydroxyl groups in the branched sugar chains

and carboxylic acid and sulfate groups in the terminal segments of branched chains.^{1,7} For this reason, polymers such as poly(acrylic acid) (PAA) are considered good mucoadhesives, and their reported interactions with mucus have been widely cited.^{5,8}

Despite these reports, the interaction between polymers and mucins in the physiological environment requires more careful consideration, both theoretically and experimentally. The excess water has important implications for hydrogen-bonding interactions between polymer chains. Because inter- and intramolecular hydrogen bonds compete with water for H-bonds, H-bonds do not play the primary role in intermolecular association.^{9,10} Poly(ethylene glycol) (PEG), for example, is very hygroscopic, and readily binds water. Hydrogen-bond formation between PEG and another polymer requires breaking H-bonds between PEG and water and between the second polymer and water. This will only occur if there is a net energy decrease upon complexation. For example, PEG and poly(vinyl alcohol) (PVA) form hydrogen bonded complexes in the dry state.^{9,10} However, because of the unfavorable desolvation energy relative to the PEG/PVA interactions, the polymer interactions in water are repulsive. Additionally, the complexation between PEG and poly(methacrylic acid) (PMAA) decreased when as little as 10% of the acid groups in PMAA dissociated.¹¹ Therefore, the simple consideration of chemical composition is insufficient to predict whether polymers would be mucoadhesive in aqueous media.

From an experimental viewpoint, the current methods used to measure mucoadhesion do not provide direct evidence for polymer/mucin attraction. Moreover, there is no universally accepted test method.^{12,13} This is the principal reason for the scarcity of comparisons of results.

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As a result, there is no consensus regarding which polymers are good mucoadhesives or what characteristics yield good mucoadhesion.^{12,13} The various test methods are also based on measurements of macroscopic parameters, which are not directly related to the molecular interactions between the chains. Additionally, they are either method- or sample-dependent. For example, the Wilhelmy plate method has been used to test the mucoadhesion of water-soluble polymers.⁸ In this approach, the adhesion is inferred from the measured tension exerted on a polymer-coated glass slide, which is partially submerged in a mucin solution. However, the contact time turned out to be the principal variable governing adhesion; namely, short incubation times would not allow sufficient interactions between polymers and mucus, but polymers would dissolve at longer contact times.⁵ More importantly, the molecular-level interactions between various polymers and mucin have only been inferred from indirect macroscopic test methods. Such disadvantages will result in poor correlations with *in vivo* behavior.⁴

Even well-accepted "good" mucoadhesives, such as PAA-type polymers, give disappointing *in vivo* results.¹⁴ The inconsistency was explained^{4,14} by the fact that free mucins and other biopolymers are abundant in the gastrointestinal lumen. Thus, mucoadhesive polymers would be rapidly coated by soluble mucin and thereby deactivated before reaching the mucus layer on the epithelium. This explanation is supported by recent studies, which show that mucin layers repel in aqueous media.^{15–17} It has, therefore, been suggested¹⁴ that successful mucoadhesives should specifically bind to the mucous layer but not to free mucin in the lumen. Alternatively, the polymer should exhibit mucoadhesive properties, even after partial coverage by other biopolymers. This problem clearly requires the establishment of molecular design criteria for mucoadhesive materials. However, the determination of the molecular mechanisms and conditions that favor mucoadhesion is still required.

The surface force apparatus (SFA) has been used in numerous investigations to determine the molecular forces and mechanisms responsible for polymer interactions. For example, studies with end-grafted PEG revealed the properties of PEG brushes that are likely responsible for increased circulation times of liposomes in the blood and the protein resistance of grafted PEG films.^{18,19} This study used the surface force apparatus to measure the interaction between grafted PEG chains and adsorbed mucin layers. We used PEG because (i) PEG had been proposed to act as a mucoadhesive,^{20–22} (ii) PEG is a widely used component in drug delivery,^{23,24} and (iii) the characteristics of the PEG/mucin interaction are unknown. The SFA

studies were complemented by surface plasmon resonance (SPR) measurements of mucin adsorption onto grafted PEG films. Together these investigations quantified mucin-PEG adhesion and identified parameters affecting the range and magnitude of the interaction.

Materials and Methods

Materials. Bovine submaxillary gland mucin (BSM) was purchased from Sigma (M-3895, St. Louis, MO). Since fluorescence microscopy showed the presence of aggregates in this material, when dissolved in buffer, mucin solutions were centrifuged at 11 000 rpm for 30 min and then filtered through a 0.2 μm filter prior to use. The final mucin concentration in solution was determined from the measured UV absorbance at 280 nm, using an extinction coefficient $1.18 \times 10^{-6} \text{ M}^{-1}$.

High-purity 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE), and PEG (MW 2000)-conjugated DSPE were purchased in powder form from Avanti Polar Lipids Inc (Alabaster, AL). All inorganic salts were high purity (>99.5%) and were purchased from Aldrich (Milwaukee, WI). All aqueous solutions were prepared with water purified with a Milli-Q UV-Plus water purification system (Millipore, Bedford, MA). Water had a resistivity of 18.2 M Ω cm. HPLC grade methanol and chloroform from Mallinckrodt (St. Louis, MI) were used to prepare lipid solutions. Methoxy-poly(ethylene glycol)-undecanethiol $\text{CH}_3\text{O}(\text{CH}_2-\text{CH}_2\text{O})_{45}(\text{CH}_2)_{11}\text{SH}$ (PEG-thiol) was a generous gift of Professor M. Grunze (Universität Heidelberg, Germany). High-purity gold (Canadian Maple Leaf coin, 99.99%) and silver shot (99.99%, Aldrich, Milwaukee, WI) were used for the evaporation of metal films. Chromium chips (99.997%) for the evaporation of adhesion layers between gold and glass were purchased from Alfa Aesar (Ward Hill, MA).

Mucin was fluorescently labeled with Alexa Fluor 488 from Molecular Probes (Eugene, OR) according to the manufacturer's instructions. The labeling ratio measured by UV spectroscopy was found to be ~ 9 dye molecules/mucin.

Supported Lipid Bilayers Displaying Grafted PEG Chains. Solutions of PEG 2000-lipid (DSPE-PEG 2000) and pure DSPE were prepared in 9:1 (volume ratio) chloroform/methanol mixtures. A solution containing 4.5 mol % DSPE-PEG and 95.5 mol % DSPE was prepared by mixing solutions of the pure compounds in the appropriate proportions. Surface pressure vs area measurements were made with a commercial Langmuir-Blodgett trough (NIMA, type 611), equipped with a standard Wilhelmy microbalance.

The grafted PEG layers (Figure 1) were prepared by Langmuir-Blodgett deposition of the mixed lipid monolayers either onto hydrophobically modified mica or onto an alkanethiol monolayer on a gold film evaporated on glass.^{18,25} The monolayers comprised 4.5 mol % DSPE-PEG 2000 and 95.5 mol % DSPE. After the lipids were spread on the water surface, they were compressed to an average area of 43 \AA^2 per lipid, or 960 \AA^2 per grafted PEG chain. The Flory radius of PEG is 35 \AA . As previously reported, at this grafting density, the neighboring polymer chains overlap weakly, and the steric thickness of the layer is 45 ± 5 \AA .^{18,26} The monolayer was then deposited at a constant surface pressure of 42 mN/m onto a hydrophobic, crystalline monolayer of DPPE. The latter was prepared by Langmuir-Blodgett deposition onto freshly cleaved mica.^{18,19}

Surface Plasmon Resonance (SPR) Measurements. SPR measurements were carried out with a home-built, computer-controlled setup, which was described previously.²⁵ A flow cell with a volume of 0.4 mL, and containing the gold-coated glass slide, was used to measure mucin adsorption *in situ*. This volume was large enough that we could disregard any depletion of the bulk protein solution due to adsorption. The flow rate was 12–13 mL/h.

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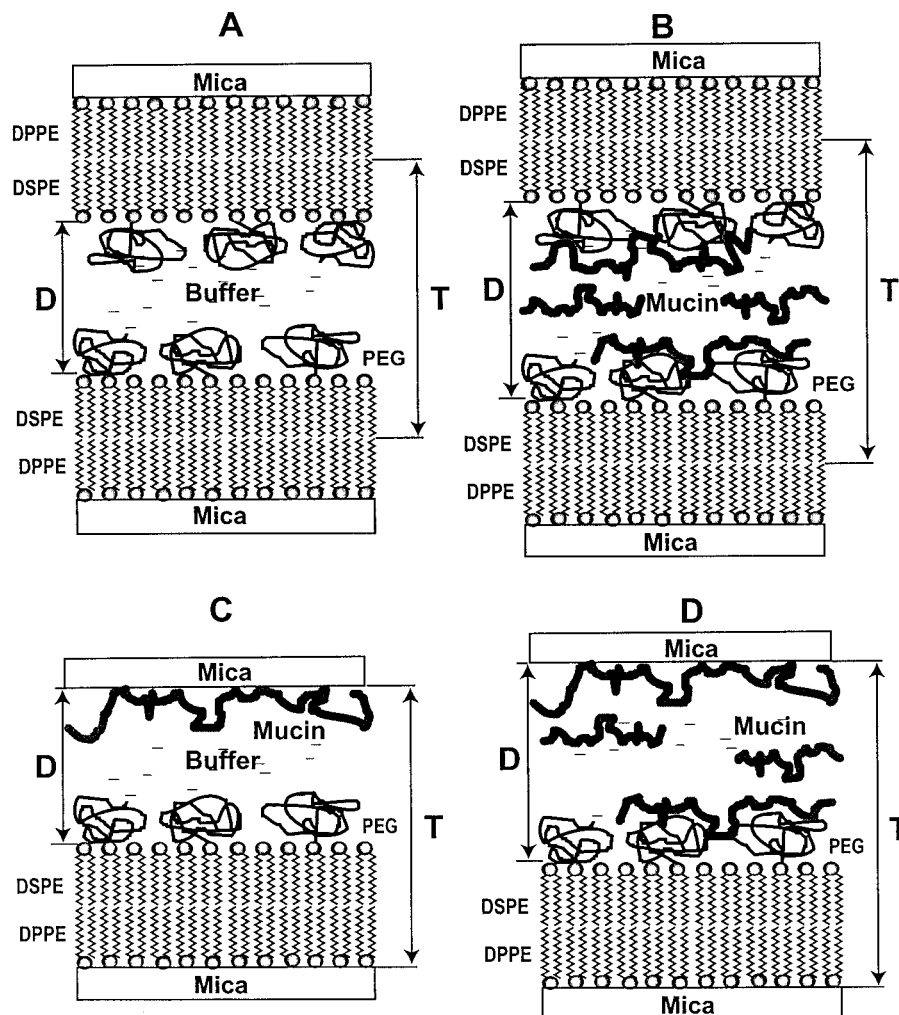


Figure 1. Schematic of the samples used in direct force measurements at pH 7.2. The illustrations show two opposing PEG–lipid coated mica surfaces (A) and bathed in a solution of soluble mucin (B). Figure C shows adsorbed mucin interacting with grafted PEG in the absence of mucin, and figure D shows PEG interacting with adsorbed mucin across a mucin solution.

The samples used for SPR experiments comprised several stacked films. The glass slides were thoroughly cleaned with a mixture of concentrated hydrochloric acid, hydrogen peroxide, and water (1:1:1, volume ratio) at 60 °C. The dried slides were then coated with a 20 Å chromium adhesion layer and a 480–500 Å gold overlayer by the resistive evaporation of these metals in a vacuum. The gold-coated substrates were immersed in a 1 mM solution of 1-octadecanethiol (98%, Aldrich, Milwaukee, WI) in ethanol immediately after the metal evaporation and left for 24 h. The slides were then rinsed with ethanol and blown dry with filtered nitrogen. The advancing water contact angle was measured with a Gaertner goniometer–microscope, and was $110 \pm 3^\circ$. The mixed lipid monolayers of PEG–lipid with DSPE were then deposited on these slides as described above.

For each of the SPR samples, we measured the dependence of the reflected beam intensity I_r on the incident angle Θ before and after adsorption of the overlayers. The obtained I_r vs Θ profiles were fit to theoretical dispersion curves, which were calculated using Fresnel reflectivity coefficients for a multilayer system.²⁵ Provided that the refractive index n of the sample material in the dry state is known, this fitting process allows us to determine a calibration slope k . This slope relates the changes in the resonance angle Θ_r to the changes in the effective optical thickness Δd of the sample layer: $\Delta d = k\Delta\Theta_r$. In addition, for a known specific gravity ρ of the adsorbed material, the changes in the effective optical thickness can be readily converted into the surface density $\Gamma = \rho\Delta d$. In this study, we used $\rho = 1.612$ g/mL and $n = 1.5$ for mucin.^{27,28}

Self-Assembled Monolayers of PEG–Thiol on Gold. The gold-coated substrates were immersed for 48 h in a 0.1 mM solution of PEG 2000–alkanethiol in ethanol immediately after the metal evaporation. The slides were then rinsed with copious amounts of ethanol and water and blown dry with filtered nitrogen. The advancing water contact angle was measured with a Gaertner goniometer–microscope and was $24 \pm 2^\circ$. The grafting density, as determined by ellipsometry, was $100 \text{ \AA}^2/\text{molecule}$.²⁹ Atomic force microscopy (AFM) images of thiol-functionalized and bare gold substrates were measured in air in tapping mode with a Digital Instruments Nanoscope MultiMode scanning probe microscope. Surface areas ($4 \mu\text{m}^2$) that are comparable to the contact area typically probed in force measurements ($10 \mu\text{m}^2$) were scanned. The root mean square roughness was found to be about 15 Å for bare gold substrates as well as for thiol-functionalized ones. There were also occasional 50–150 Å microcrystals.

Fluorescence Imaging of Adsorbed Mucin Layers. Epifluorescence microscopy was used to determine the lateral homogeneity of the adsorbed mucin layers. In these experiments, freshly cleaved mica sheets, either bare or covered with a PEG–lipid bilayer, were placed in a flow-through cell used for fluorescence microscopy. The cell was filled with a mucin solution (0.02 or 0.2 mg/mL) in pH 7.2 buffer (5 mM phosphate, 150 mM NaCl) at room temperature. Mucin was allowed to adsorb for 2 h, after which the cell was flushed with buffer solution.

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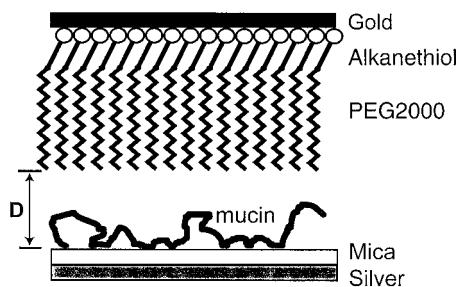


Figure 2. Schematic of the sample architecture used to measure forces between PEG–thiols and adsorbed mucin at pH 2.0.

Fluorescence images were acquired with an Olympus BX-60 microscope equipped with an 80 \times objective (total magnification of the microscope was 1400 \times), a mercury arc lamp source, a set of filters, and a CCD camera (Photometrics, 512 \times 512 pixels, liquid nitrogen cooled). The CCD camera was connected to a computer and controlled via PMIS software (Photometrics).

AFM of Adsorbed Mucin Layers on Mica. Adsorbed layers of mucin on mica were formed by the incubation of freshly cleaved mica surfaces in mucin solutions in pH 7.2 buffer (5 mM phosphate, 150 mM NaCl) at room temperature. The mucin concentrations were 0.02 and 0.2 mg/mL. Solutions were pre-centrifuged at 11 000 rpm for 30 min and then filtered through a surfactant-free 0.2 μ m Durapore membrane (Millipore). The mucin concentration was determined after filtration. Substrates were incubated with mucin for 2 h and then rinsed. AFM images of adsorbed mucin layers on mica, as well as bare freshly cleaved mica, were measured in buffer in the tapping mode with a Digital Instruments Nanoscope MultiMode scanning probe microscope.

Force Measurements. Force measurements were conducted with the Mark III surface force apparatus³⁰ (SurForce, Santa Barbara, CA) in a temperature-controlled room at 25 $^{\circ}$ C. Forces were measured (1) between two supported PEG 2000 bilayers (Figure 1A) immersed in a solution of mucin (Figure 1B) and (2) between a supported PEG 2000–lipid bilayer and an adsorbed layer of mucin on mica in the absence (Figure 1C) or presence (Figure 1D) of exogenous mucin. The chamber of the instrument housing the samples was filled either with 5 mM phosphate buffer containing 150 mM NaNO₃ (pH 7.2) or with a 50 mM HCl/KCl solution containing 100 mM NaNO₃ (pH 2.0). In all surface force measurements, the salt used was NaNO₃ instead of NaCl since the halide ions corrode the silver mirrors of the interferometer. The solution was prefiltered twice through a surfactant-free 0.2 μ m Durapore membrane (Millipore). In some experiments, the SFA chamber was filled with a prefiltered mucin solution in buffer (Figure 1B,D).

The sample configuration used in the force measurements between the PEG-thiol self-assembled monolayer and mucin corresponded to an asymmetrical “two-layer” interferometer (Figure 2). In this configuration, a 400 Å layer of gold was deposited directly on the surface of the silica disk used for force measurements. An alkanethiol monolayer was then self-assembled on top of it, using the procedure described above. In this case, the distances between the surfaces were determined by the multilayer matrix method described elsewhere.^{31,32}

Results

Fluorescence Microscopy. Figure 3 shows fluorescence images of preadsorbed mucin layers prior to (Figure 3A) and after (Figure 3B) centrifugation. With unfiltered mucin, there are a substantial number of aggregates, up to several micrometers in diameter and having mostly dendritic shapes. By contrast, an adsorbed mucin layer prepared from the centrifuged solution exhibited uniform

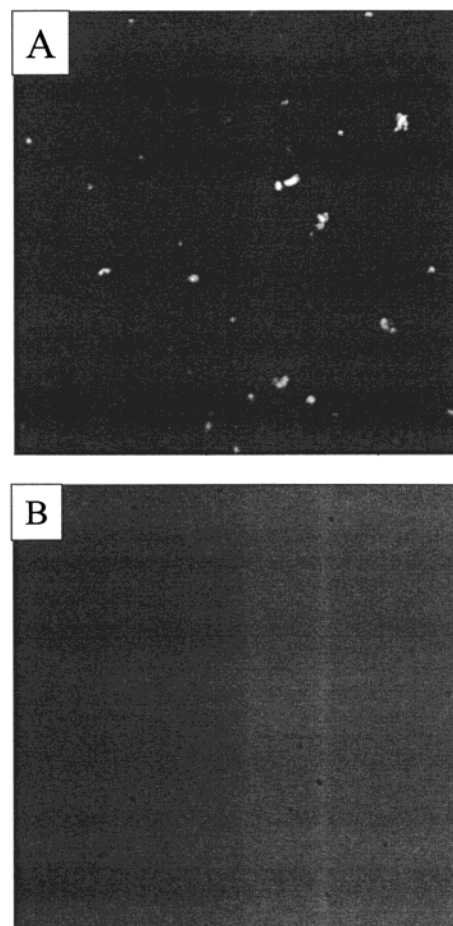


Figure 3. Fluorescent micrographs (1400 \times magnification) of adsorbed mucin layers on mica prepared with mucin solutions prior to (A) and after (B) centrifugation.

fluorescence (Figure 3B), which indicated the removal of aggregates. The fact that the integral fluorescent intensity decreased by only 12% in sample 3B compared to sample 3A suggested that aggregation was not a consequence of the mucin-labeling procedure. The aggregates were probably denatured molecules in the commercial mucin. Therefore, centrifuged mucin solutions were used throughout the rest of this study, and most solutions were filtered.

AFM Imaging of Adsorbed Mucin Layers. The lateral distribution of mucin layers adsorbed on mica was determined with tapping mode AFM. Surface areas of 1–2 μm^2 were scanned. The root mean square roughness and Z_{range} values were determined for (i) bare mica, (ii) mica incubated for 2 h with a 0.02 mg/mL mucin solution, and (iii) mica incubated for 2 h with a 0.2 mg/mL mucin solution (5 mM phosphate, 150 mM NaCl at pH 7.2). Representative scans for bare mica and mucin layers on mica, adsorbed from 0.02 and 0.2 mg/mL solutions, are shown in Figure 4. The surface roughness increased from 2.1 to 7.2 Å after incubating mica in 0.02 mg/mL mucin for 2 h. The Z_{range} of the surface roughness increased even more from 18 to 85 Å . If mica was incubated in a 0.2 mg/mL mucin solution instead, the root mean square roughness, as well as the Z_{range} of surface roughness, of the adsorbed mucin layer was the same as that for bare mica (Table 1). These data suggest that only submonolayers or islands of mucin were formed on the surface after 2 h with 0.02 mg/mL mucin. If a 0.2 mg/mL solution was used instead, a laterally homogeneous adsorbed layer of mucin formed over the same period of time. Perez et al.¹⁶ reported significant mucin desorption from mica upon dilution of the bulk

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Table 1. Range and Magnitude of PEG–Mucin Adhesion

system	medium	mucin layer thickness, ^a Å	jump-out position, Å	F_{adv}/R , mN/m
grafted PEG vs grafted PEG	+0.05 mg/mL mucin, pH 7.2	150 ± 10	200 ± 17	-1.0 ± 0.5
adsorbed mucin vs PEG	-mucin, pH 7.2	700 ± 20	480 ± 20	-0.4 ± 0.2
adsorbed mucin vs PEG	+0.2 mg/mL mucin, pH 7.2	1000 ± 20	690 ± 10	-0.3 ± 0.2
adsorbed mucin vs PEG	+0.05 mg/mL mucin, pH 7.2	850 ± 50	590 ± 30	-0.3 ± 0.1
adsorbed mucin vs PEG	-mucin, pH 2.0	475 ± 6	358 ± 8	-1.4 ± 0.3

^a Determined from the range of the repulsive force.

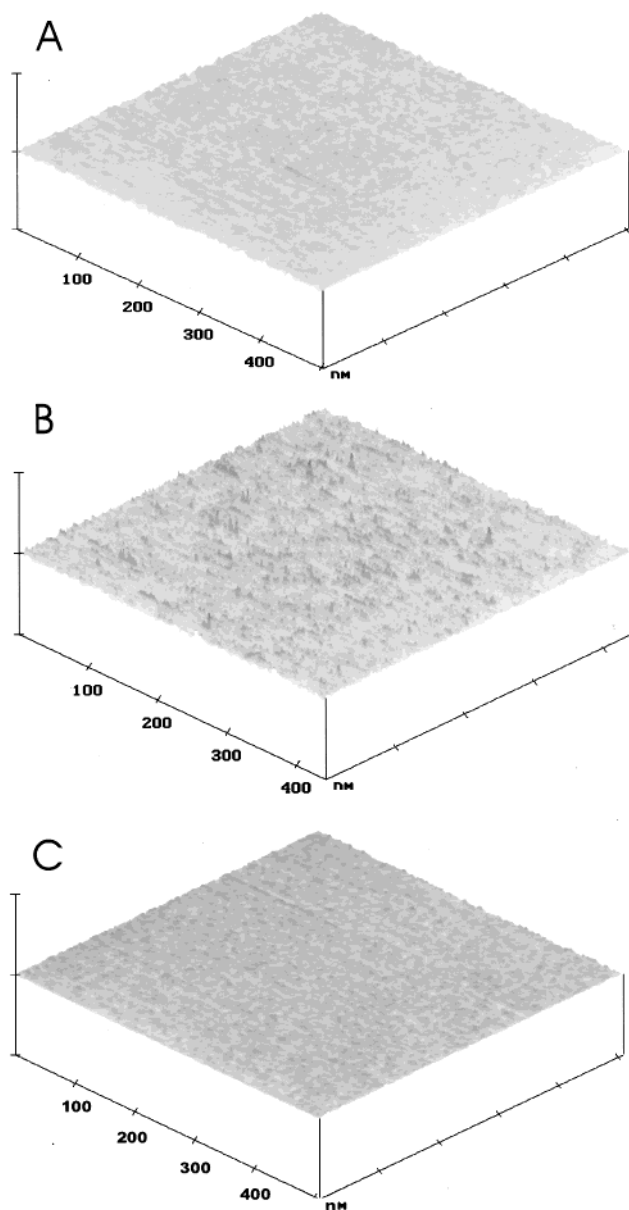


Figure 4. AFM images of bare mica (A) and mucin layers on mica, adsorbed from 0.02 (B) and 0.2 mg/mL (C) solutions. Images were acquired with a Digital Instruments Nanoscope MultiMode scanning probe microscope using tapping mode AFM in a fluid cell. The tick mark on the vertical bar corresponds to 10 nm.

solution. These AFM measurements, however, show that, despite initial desorption, a significant amount of mucin remains adsorbed to mica in 0.15M NaCl (or NaNO₃) in the absence of soluble mucin.

PEG–Mucin Interactions at pH 7.2 Studied by SPR. The mucin adsorption to a lipid monolayer exposing grafted PEG chains was studied in situ using surface plasmon resonance. Figure 5 shows an adsorption time

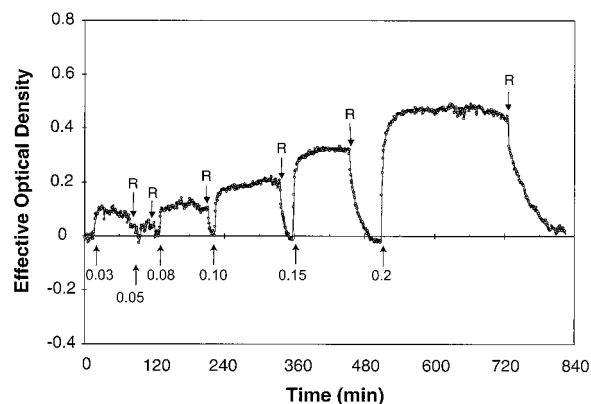


Figure 5. SPR measurements of mucin adsorption and desorption on a supported PEG monolayer. The time course was measured by SPR. Arrows with numbers indicate injection of mucin solution with concentrations from 0.03 to 0.2 mg/mL. The *R*'s indicate the postadsorption rinsing with 10 mL of buffer lacking soluble mucin, and the arrows indicate the time of injection of bulk mucin at the specified concentration. The buffer contained 5 mM phosphate and 150 mM NaCl at pH 7.2 and 25 °C.

course. The adsorption was initiated by injecting the solution with the lowest mucin concentration into the flow cell. After the system equilibrated, the mucin solution was rinsed from the cell with buffer. The same sequence of actions was then repeated with successively increasing mucin concentrations.²⁵ As seen in Figure 5, the adsorption of mucin onto PEG was fully reversible at pH 7.2—that is, upon removal of mucin solution from the cell, all mucin rapidly desorbed from the surface.

On the basis of Langmuir adsorption theory,³³ the maximum surface concentration Γ_{max} and the adsorption equilibrium constant (or binding constant), K , can be expressed as

$$\Gamma = \left(\frac{K[P]}{1 + K[P]} \right) \Gamma_{\text{max}} \quad (1)$$

Here P is the concentration of the bulk mucin solution. The values of K and Γ_{max} can be obtained by plotting the reciprocal surface concentration $1/\Gamma$ against the reciprocal mucin solution concentration $1/[P]$. Linear regression analysis of the data gave the intercept $1/\Gamma_{\text{max}}$ and the slope $1/K\Gamma_{\text{max}}$.²⁵ The parameter Γ_{max} was thus determined to be 1.3 ± 0.1 mg/m², and K was found to be 3 mL/mg. If we assume the average molecular weight of mucin to be 1.6×10^6 Da,²⁸ then the affinity constant $K = (4.9 \pm 0.5) \times 10^6$ M⁻¹.

The Γ_{max} determined in this study was approximately 2 times lower than the surface coverages of 2.8 ± 0.3 mg/m² and 2.25 mg/m² reported for mucin adsorption onto hydrophobic surfaces.^{15,28} We, therefore, measured mucin binding to a hydrophobic, self-assembled 1-octadecanethiol

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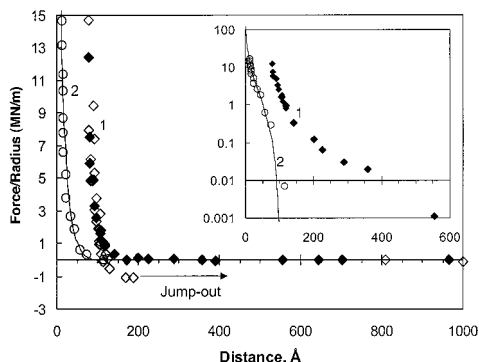


Figure 6. Normalized force–distance curve between identical PEG monolayers in the presence and absence of a 0.05 mg/mL BSM solution. Curve 1 indicates the forces measured across a 0.05 mg/mL BSM solution in 5 mM phosphate buffer (pH 7.2) containing 150 mM NaNO_3 at 25 °C (Figure 1B). The filled diamonds correspond to forces measured during approach, and open diamonds correspond to forces measured upon separation. The open circles show the force profile measured after replacing the soluble mucin solution bathing the samples with pure buffer. The solid line through the data corresponds to the theoretically calculated interaction profile between two monolayers of weakly overlapping PEG2000 chains (Figure 1A). For details of this calculation, see refs 18 and 25. In this experiment, $D = 0$ Å corresponds to contact between the opposed lipid bilayer surfaces (Figure 1B). The inset shows the force curves plotted on a logarithmic scale.

monolayer on gold. In this case, a considerable amount of mucin irreversibly bound to the surface. Γ_{max} was 2.9 ± 0.2 mg/m², in good agreement with previous results,^{15,28} and the binding constant K was 15 mL/mg, or $(2.4 \pm 0.3) \times 10^6$ M⁻¹.

Force Measurements. Definition of $D = 0$. In force measurements between supported PEG-2000 bilayers, $D = 0$ Å is defined as contact between the lipid headgroups beneath the PEG chains (Figure 1A,B). The difference in the distance of closest intersurface approach before and after deposition of the DSPE–PEG 2000 monolayer defines the total thickness T of both outer lipid monolayers plus the polymer headgroups. The distance D between the lipid bilayers is determined by subtracting twice the thickness of the DSPE monolayer from the total thickness T : $D = (T - 2)t_{\text{DSPE}}$, where t_{DSPE} is the thickness of the DSPE monolayer. For these calculations, we used the measured value of $t_{\text{DSPE}} = 28$ Å.³⁴

In measurements between grafted PEG 2000 and adsorbed mucin, we define $D = 0$ Å as contact between the mica surface and the headgroups of the lipid bilayer on the opposite surface (Figures 1C,D). The difference in the distance of closest intersurface approach between two bare mica sheets before and after deposition of the PEG-2000 lipid bilayer on one surface defines the total thickness T of the lipid bilayer together with the PEG brush. The distance D between the lipid bilayer and second mica surface is determined by subtracting the thickness of both the DPPE and DSPE monolayers from total distance T between the mica sheets (Figure 1C,D). For these calculations, we used 27 Å for the DPPE thickness.³⁴

Forces between Grafted PEG Chains in Bulk Mucin Solutions at pH 7.2. The force curves between two PEG 2000 bilayers bathed in a 0.05 mg/mL solution (pH 7.2, 150 mM NaNO_3) are shown in Figure 6 (diamonds). At distances $D < 400$ Å, we measured a repulsive force between the two PEG monolayers. This indicated the presence of adsorbed mucin on the PEG, since the

PEG 2000 monolayer thickness is only ~ 50 Å at the grafting density used in this study.^{18,26,35,36}

The force–distance profiles measured after 2 and 24 h were essentially identical. Therefore, the adsorption equilibrium was reached after 2 h, in agreement with our SPR data (cf. Figure 5). However, as reported previously,^{15,16} during approach, after each decrease in D , the force between the surfaces re-equilibrated slowly. To measure the equilibrium force–distance curves, it was necessary to perform the measurements sufficiently slowly. In these studies, the system relaxed for 1–2 min before further decreasing or increasing the surface separation. Longer waiting intervals had no effect on either the advancing or receding force curves and indicated that these measurements represent the quasi-equilibrium force–distance profiles between mucin and PEG.

The repulsive force increased roughly exponentially from 400 to 100 Å. The decay length is ~ 66 Å (Figure 6), whereas the theoretical Debye length for the double-layer force in 0.15 M 1:1 electrolyte is only 8 Å. The measured long-range force is therefore attributed to steric, rather than strictly electrostatic, interactions. Below 100 Å, the steep increase in the force is attributed to the osmotic and steric repulsion between highly compressed mucin chains and PEG chains. The steep repulsion may also reflect, in part, the lower compressibility of the more dense protein core. The solid line in Figure 6 shows the force curve between the PEG monolayers.

The maximum thickness of the mucin layer, adsorbed from a 0.05 mg/mL mucin solution (5 mM phosphate, 150 mM NaNO_3 at pH 7.2), was determined from the range of the repulsive force. The thickness of the adsorbed layer on each surface was $(400 \pm 20)/2 = 200 \pm 20$ Å. The thickness of a single mucin layer is therefore 200 Å – 50 Å (PEG thickness) = 150 ± 20 Å (Table 1).

There was a noticeable hysteresis between the advancing (loading) and receding (unloading) profiles that was unaffected by slower separation rates (Figure 6). The force decreased with separation to about 200 Å, and then the surfaces jumped out of adhesive contact from 200 ± 17 Å (Table 1). This behavior was reproducible in five successive measurements and was observed at several different regions of the 1 cm² sample surface. The observed jumps apart were also very slow, so that the surfaces came to rest only ~ 120 s after the beginning of the jump apart. If the measurements were not performed sufficiently slowly, the jump-out was easily missed. This is consistent (i) with the previously observed slow relaxation of adsorbed layers of pig gastric mucin and rat gastric mucin on mica¹⁷ and (ii) with the detachment of adsorbed polymer layers on mica.³⁷

Figure 6 (curve 2) shows the forces measured between the surfaces after the bulk mucin solution was exchanged for pure buffer (cf. Figure 1A). The range of the repulsive force decreased substantially to 100 ± 10 Å, and the resulting curve is identical to both the predicted and measured force profile between 4.5 mol % PEG-2000 monolayers.^{18,26,38,39} Additionally, there was no adhesion, and the measured force profiles were completely reversible. These data indicated that mucin fully desorbed from the

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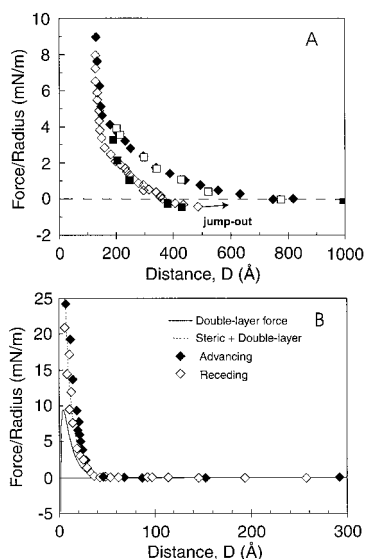


Figure 7. (A) Normalized force between grafted PEG and adsorbed mucin on mica. Filled diamonds and open squares correspond to forces measured during approach. The filled diamonds correspond to the initial measurement. Open diamonds and filled squares correspond to the receding curves. The samples were bathed in 5 mM phosphate and 150 mM NaNO_3 at pH 7.2 and 25 °C. (B) Normalized force profile between grafted PEG and bare mica. The samples were bathed in 5 mM phosphate and 150 mM NaNO_3 at pH 7.2 and 25 °C. The filled and open symbols correspond to the forces measured during approach and separation, respectively. The solid line is the electrostatic double layer force, calculated using the nonlinear Poisson–Boltzmann equation and surface potentials of -75 and -41 mV for mica and the PEG bilayer, respectively. The dotted line corresponds to the superposition of electrostatic and steric forces. In both parts A and B, $D = 0$ Å corresponds to contact between mica and opposed lipid bilayer surface (see Figure 1C).

surfaces. This agrees with our SPR results, which showed that mucin adsorption on PEG layers is reversible at pH 7.2.

Bridging forces will depend on the amount of bulk mucin in solution. To determine whether this applies for mucin–PEG interactions, we measured the force curves between PEG–lipid membranes bathed in 0.02 mg/mL mucin. The profiles were similar to those seen in Figure 6. However, the magnitude of the repulsion was smaller and there was no adhesion. This difference is most likely because, at the low mucin surface concentration (6% of a full monolayer), there are too few bridging interactions to generate any measurable adhesion.⁴⁰

Forces between Grafted PEG and Adsorbed Mucin in Buffer. The normalized force profile between a PEG–bilayer and a mucin layer adsorbed on mica (Figure 1C) is shown in Figure 7A. For these experiments, mucin was adsorbed from a 0.2 mg/mL mucin solution. There was no mucin in the bathing solution, but the AFM images (Figure 4) show that mucin remains on the surface. During the initial approach, the range of the repulsion between the surfaces was 750 ± 20 Å. The long-ranged repulsive force is attributed to the steric repulsion between PEG and the adsorbed mucin layer on mica. Because the PEG thickness is 50 Å, the thickness of the adsorbed mucin layer was ~ 700 Å ($=750 - 50$ Å) (Table 1). The layer was also highly compressible, so that, under a moderate load of 10 mN/m (1.6 mJ/m²), it could be compressed to 120 Å. Given the

structure of mucin,⁴¹ it is likely that the weakly adsorbed polymer forms compressible loops and trains on the mica surface (cf. Figure 1C).

Upon separation, the distance between the surfaces increased gradually to 480 ± 20 Å, where the surfaces jumped out of weak adhesive contact (Table 1). The normalized adhesive force was -0.4 ± 0.2 mN/m, and the adhesion energy was 0.08 mJ/m² (Table 1). This was very reproducible (number of experiments $N > 5$) (Figure 7A), provided that the applied loads did not exceed 18 mN/m. The adhesion energy is weaker than that between PEG films in 0.05 mg/mL mucin, but still high enough to cause the adsorption of PEG-coated particles onto mucin films. The pronounced hysteresis between the loading and unloading force profiles measured at < 18 mN/m (cf. Figure 7A) is qualitatively similar to previous measurements between identical mucin layers adsorbed on mica.^{15,16} Here the materials on the two surfaces are different, and because of this, the measured hysteresis and adhesion are clearly due to mucin–PEG interactions.

Higher applied, normalized loads (> 18 mN/m) caused successive force curves to shift to smaller distances, and this was accompanied by a loss of adhesion. While this could be due to a flattening of the chains onto the mica surface, the more likely explanation is that the weakly adsorbed mucin was squeezed out of the contact region. This reduction of the amount of mucin in the gap would also reduce the magnitude of the PEG–mucin adhesion. Consistent with this interpretation, when the mica was incubated in a more dilute 0.02 mg/mL mucin solution (pH 7.2, 5 mM phosphate, 150 mM NaNO_3) for 2 h prior to measurements, and then rinsed, there was no attraction. Additionally, the range of the repulsive force was reduced to 200 Å (data not shown).

Control measurements of the force profile between the grafted PEG chains and bare mica are shown in Figure 7B. The force profile, which reflects the superposition of electrostatic double layer and steric forces, was repulsive at all separations, and there was no adhesion. The solid line shows the contribution of the electrostatic double-layer force. This was calculated by numerically solving the nonlinear Poisson–Boltzmann equation with surface potentials of -41 and -75 mV for the PEG–lipid and mica surfaces, respectively.^{42–44}

PEG Interactions with Adsorbed Mucin in the Presence of Soluble Mucin. The force profile measured between the PEG bilayer and an adsorbed mucin layer bathed in a 0.2 mg/mL mucin solution (pH 7.2) is shown in Figure 8. In this experiment, the conditions were closer to those in the gastrointestinal lumen where soluble mucin is present at concentrations of up to 2 mg/mL. The range of the intersurface repulsion increased up to 1000 ± 20 Å, compared with the 750 ± 20 Å range of the repulsion measured in pure buffer (Figure 8, curves 1 and 2, Table 1). The PEG and mucin layers still adhered, but the adhesive minimum was farther out at 690 ± 10 Å (Table 1). The normalized adhesive force was -0.3 ± 0.2 mN/m (Table 1), and the adhesion energy was 0.06 ± 0.04 mJ/m². The force curves were practically unchanged after several successive measurements at the same contact region.

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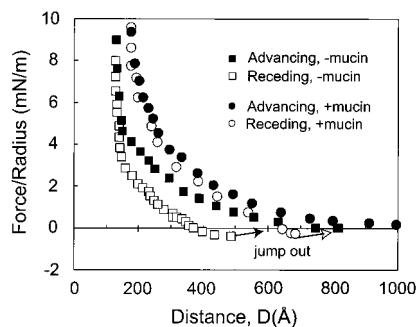


Figure 8. Normalized force–distance profiles between grafted PEG and adsorbed mucin on mica. The bathing solution contained 5 mM phosphate and 150 mM NaNO₃ at pH 7.2, and the temperature was 25 °C. Filled and open squares show the advancing and receding curves, respectively, measured across pure buffer (Figure 1C). Filled and open circles show the forces measured across a 0.2 mg/mL mucin solution (Figure 1D).

In 0.05 mg/mL mucin, the force profiles were qualitatively similar. However, the range of the repulsion was slightly smaller at 850 ± 50 Å (Table 1). The surfaces jumped out of adhesive contact from 593 ± 30 Å, and the adhesive force was -0.3 ± 0.1 mN/m (Table 1).

PEG Interactions with Adsorbed Mucin at pH 2.0.

Additional experiments were performed at pH 2.0, since this pH corresponds to the acidity in the human stomach.⁴⁵ However, because acid catalyzes the hydrolysis of phospholipid headgroups,⁴⁶ the DSPE–PEG used in the first part of the study would be unstable at pH 2.0. Therefore, we used PEG-terminated alkanethiol (PEG 2000–alkanethiol) self-assembled on gold. These monolayers are stable under acidic conditions.⁴⁷

In the SPR measurements, the mucin adsorption was initiated by the injection of the mucin solution into the flow cell. In this experiment, the amount of mucin adsorbed to the PEG–thiol layer was comparable to the amount adsorbed to PEG–lipid bilayer at the same bulk mucin concentration at pH 7.2. In this case, however, the adsorption was faster, and little desorption occurred after flushing the cell with pure buffer at pH 2.0. Subsequent rinsing with deionized water at pH ~ 5.7 resulted in the complete desorption of the mucin.

Force–distance profiles of the interaction between a self-assembled monolayer of PEG–thiol on gold and an adsorbed mucin layer on mica at pH 2.0 in the absence of soluble mucin (Figure 2) are shown in Figure 9. Here D corresponds to the distance between the mica and the surface of the opposed PEG–alkanethiol layer (Figure 2). The mucin layer was adsorbed from a 0.2 mg/mL mucin solution at pH 2.0. It is not possible to measure the forces between two PEG–alkanethiol layers on gold across a mucin solution (cf. Figure 1D) since the interferometer of the SFA requires the thickness of samples between the two reflecting metal surfaces to be on the order of the wavelength of visible light.⁴⁸

During the initial approach, the range of the repulsion was $D < 475 \pm 6$ Å. At separations below ~ 340 Å, the repulsive force increased steeply. The difference between the range of the repulsion at pH 2 relative to that at pH 7 suggests that the mucin chains are less extended on the

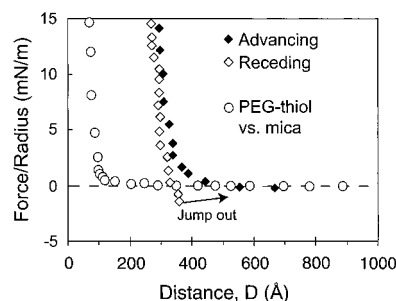


Figure 9. Normalized force–distance curves between a PEG–thiol monolayer and adsorbed mucin at pH 2.0. Samples were bathed in a pH 2.0 solution containing 100 mM NaNO₃. Filled and open diamonds correspond to the advancing and receding force curves, respectively, between the self-assembled PEG–thiol on gold and an adsorbed mucin layer on mica (cf. Figure 2). Open circles show the force profile measured between the PEG–thiol monolayer and bare mica. Here D corresponds to the distance between the mica and the outer surface of the opposite alkanethiol layer (Figure 2).

mica. This is consistent with the reduction in mucin–mica repulsion and with stronger mucin–mica adsorption. Mucin is slightly positively charged at pH 2.0 and would be attracted to the negatively charged mica surface. The layer was also less compressible than at pH 7.2 (cf. Figures 7 and 8), and could be compressed to $D = 190 \pm 6$ Å at 16 mN/m. During separation, the measured adhesion at 358 ± 8 Å was -1.4 ± 0.3 mN/m (Table 1), which is nearly 5-fold larger than the adhesion measured at pH 7.2.

The force–distance curve between the PEG–thiol monolayer and bare mica at pH 2.0 is shown in Figure 8 (open circles). In this case, the force curves were repulsive and reversible. Subtraction of the force–distance profile between the PEG–thiol monolayer and bare mica from the force–distance profile between the PEG–thiol and adsorbed mucin (filled diamonds) gave the 350 ± 10 Å thickness of the unperturbed mucin layer on mica at pH 2.

Discussion

These studies show that PEG adheres weakly to mucin at physiological pH. The hysteresis in the force curves between PEG layers across a mucin solution is similar to the forces observed between dilute, adsorbed mucin layers on mica interacting across a mucin solution.^{15,16} In the latter case, mucin was adsorbed on mica, instead of on grafted PEG, but the adhesion was attributed to mucin–mica bridging forces. In this work, measurements done at different rates of approach and separation indicate that the adhesion is not due to mechanical entanglements. Since (i) PEG monolayers repel,^{18,26} (ii) dense BSM films repel each other,^{15,16} and (iii) mucin adsorbs to the grafted-PEG, we also attribute the attractive force between the two PEG films in 0.05 mg/mL mucin to mucin-mediated bridging between the two PEG layers.^{40,49,50}

The possibility exists that the mucin could also adhere to the bilayers supporting the grafted PEG. This is unlikely for several reasons. First, the membranes are negatively charged and are more likely to repel mucin. Second, although the PEG is not in a dense brush, there is a significant osmotic penalty for penetrating the layer.²⁵ This impedes interpenetration of opposed PEG films and their penetration by proteins.^{18,19,25} Finally, we measured adhesion between mucin and densely grafted PEG–

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alkanethiol monolayers, which both lack the lipid bilayer and are too dense for large molecules to penetrate.

The adhesion due to mucin-mediated bridging was determined from the pull-off force $F_{po} = -1.0 \pm 0.5$ mN/m (Table 1). From the Johnson–Kendall–Roberts (JKR) theory of the adhesion between deformable solids, the adhesion energy per area is related to the pull-off force by $E = F_{po}/1.5\pi R = 0.21$ mJ/m².^{40,51} This value of the adhesion energy is on the order of the van der Waals attraction between two bare lipid bilayers³⁴ and would be sufficient to cause the flocculation of PEG-coated particles.

Typically, bridging forces should exhibit a bell-shaped dependence on the surface coverage of the bridging chains⁵² or, alternatively, on the bulk polymer concentration.⁴⁹ The trend reported here is consistent with this mechanism. Namely, the surfaces did not adhere when the membranes were bathed in a low concentration 0.02 mg/ml mucin solution. Little mucin adsorbs at this concentration, so that there are likely too few mucin–PEG contacts to generate measurable adhesion. The PEG surfaces do, however, adhere in 0.05 mg/mL mucin solutions. Because the amount of adsorbed mucin increased with the bulk concentration, we attribute the onset of adhesion to the formation of a larger number of mucin–PEG bonds. Unfortunately, we could not determine the mucin concentration at which the adhesion begins to decrease with increasing mucin, because the solutions become too viscous to filter the 150 mL volumes needed for these measurements.

The measured steric thickness of the mucin layers adsorbed on mica is in good agreement with the measured 160 Å diameter of porcine gastric mucin adsorbed on mica.⁴¹ It suggests that the chains lie flat on the PEG brush and do not form loops and trains. Indeed, the low force between 200 and 400 Å indicates that mucin attraction to the surface is sufficient to cause the proteoglycan to flatten and even, perhaps, to penetrate into the PEG monolayer. The measured jump-out from 200 Å is consistent with some mucin penetration. Given the large entropy penalty associated with polymer stretching,⁵³ this is at first somewhat surprising given the weak measured adhesion between the two different polymers. However, the bending modulus of mucin is undoubtedly much higher than most polymers on account of the high grafting density of carbohydrates along the protein backbone. Confining these biopolymers on a surface should therefore be energetically less costly.

The mucin rigidity could also explain the smaller steric thickness of the weakly adsorbed mucin on mica at pH 7.2 relative to other weakly adsorbed, freely jointed chains in good solvent.^{50,54,55} The reported thickness of weakly adsorbed polymer layers in good solvent was two to three times the radius of gyration.^{50,54} The radius of gyration of soluble mucin is 1400 Å,^{56,57} but the thickness of the adsorbed film was <1000 Å. However, because of its stiffness, mucin may be unable to form loops on the surface as easily as simple chains. This could result in thinner adsorbed polymer layers.

In dilute mucin solutions (0.02 mg/mL) the maximum normalized repulsive force F/R at $200 < D < 400$ Å (Figure 6) is less than 1 mN/m. According to the Derjaguin approximation,⁴⁰ the normalized force between two crossed cylinders of average radius R is related to the interaction energy per area by $E = F/2\pi R$. Thus, the repulsive energy per area is <0.2 mJ/m². Both the flat, adsorbed configuration and the low adsorbed amount of mucin (~13% of a full monolayer) would result in little mucin–mucin interaction across the gap. The low mucin coverage would also account for the weak interaction at $D < 400$ Å, as the mucin layers interdigitate. The observed steep increase in the repulsive force at $D < 200$ Å is attributed to mucin–PEG repulsion.

The measurements between PEG and adsorbed mucin films confirm that PEG weakly adheres to both soluble and immobilized mucin. Again, the force curves observed in this study are qualitatively similar to those measured between two mucin films on mica in bulk mucin.^{15,16} In the latter case, the origin of the attraction was less certain. By contrast, because PEG does not adhere to bare mica, the adhesion reported in this study can only be due to PEG–mucin binding.

The range of both the repulsion and attraction varied somewhat with the bulk mucin concentration (Figure 8). This behavior is consistent with the mucin adlayer thickening as more chains adsorb to the mica at higher bulk concentrations^{15,17} and/or mucin rearrangements in response to additional adsorption. Consistent with this, the position of the adhesive minimum between PEG and adsorbed mucin also moves out at the higher mucin concentration. However, mucin adsorption onto the PEG also contributes to the thickness changes, and the increased range of the repulsion is similar to the 150 Å thickness of adsorbed mucin on PEG (cf. Figure 6). This makes attributing the adhesion to either mucin–mucin or mucin–PEG interactions more difficult. Nevertheless, given the presence of attraction between mucin and PEG in the absence of bulk mucin (cf. Figure 7A) and the absence of adhesion between dense BSM layers on mica,^{15,17} we attribute the adhesion in Figure 8 to mucin–PEG attraction.

The adhesion between PEG and adsorbed mucin in the presence of soluble mucin is of some practical importance, since mucin both covers the inner epithelial lining and exists in soluble form in the gastrointestinal lumen. Our data suggest that at the mucin concentrations investigated, soluble mucin will only partially coat the PEG layer. This would allow a PEG-coated particle to still bind to the mucin-coated lumen in the presence of soluble proteoglycan. The critical issue, however, is the bulk concentration at which the soluble mucin out-competes immobilized mucin for adsorption sites. This remains to be determined.

Hydrogen bonding between mucin and PEG was proposed as a mechanism of adhesion, and this motivated the development of PEG-based oral drug-delivery devices. However, significant formation of hydrogen bonds between PEG and BSM at pH 7.2 is unlikely, because most of the acidic groups (H-bond donors) of BSM are dissociated. The very weak adhesion measured could, however, be due to the formation of a small number of hydrogen bonds. The adhesion of the PEG monolayer and adsorbed mucin layer at pH 7.2 could also originate from chain “entanglements” and the slow relaxation of mucin during surface separation.^{15,17} However, in contrast to the expected behavior for mechanical entanglements,⁵⁸ the apparent

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adhesion did not increase with the detachment rate. This suggests that the attraction is due to physical chemical interactions between the chains and not to nonequilibrium interactions.

The comparison of mucin-PEG adhesion at pH 7.2 with that at pH 2.0 suggests that H-bonding between the carboxylic acid protons of mucin and the ether oxygen on PEG may in fact mediate polymer adhesion.^{59,60} The mucin adsorption results obtained by SPR indicate that, at pH 2.0, mucin binds more strongly to the PEG monolayer than at pH 7.2. BSM has an isoelectric point of around 3.0. At the lower pH, most of the carboxylic groups on the proteoglycan should be protonated and could hydrogen bond to the ether oxygens. Similar pH-dependent hydrogen bonding to carboxylic acid groups was recently reported by Schneider et al.,^{59,60} and the pH-dependent coacervation of PEG and proteins is attributed to similar H-bonding interactions.⁶¹

In comparing the magnitude of adhesion at pH 7.2 and pH 2.0, one should also keep in mind that the PEG surface densities differed significantly in the two grafted-PEG preparations. The area per DSPE-PEG was 960 Å², and the polymer coils on the surface were only weakly overlapping. With self-assembled PEG-alkanethiol monolayers, the polymer chains are densely packed, with a mean area per polymer of ~100 Å².²⁹ In the latter case, interpenetration and entanglements between PEG and mucin are less likely. This would reduce the possibility of chain entanglements contributing to the adhesion. It would also reduce the adhesion by substantially reducing the extent of chain-chain contacts that can form.

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Given the large differences between the adsorption behavior of mucin on PEG at the two pH values, it may seem surprising that the adhesion differed by only a factor of ~5. There are two possible reasons for this. First, the dense packing in the PEG-alkanethiol monolayer may reduce the extent of polymer-polymer contact and, hence, the adhesion. Second, the 15 Å surface roughness of the self-assembled monolayer on gold is much larger than on mica (<1 Å), and this will decrease the effective contact area between the surfaces. Since the force scales with the contact area, this would similarly reduce the adhesion relative to that between two smooth surfaces.⁴⁰

Conclusions

These measurements demonstrate directly that PEG exhibits mucoadhesive properties that may be directly relevant to oral drug delivery. The data show that PEG weakly adheres to soluble mucin at neutral pH. PEG also adheres to adsorbed mucin films, both in the presence and absence of bulk mucin, and the adhesion depends somewhat on the bulk polymer concentration. The pH dependence of the binding suggests that these polymers adhere via H-bonds between the ether oxygen of the EO segments and hydrogens on the carboxylic acid on the mucin backbone. Further studies with other polymers should clarify the role of H-bonds in mucoadhesion and identify molecular design criteria for creating better mucoadhesives.

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