

Forces between Mica Surfaces Covered with Adsorbed Mucin across Aqueous Solution

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The forces between two mica surfaces covered with an adsorbed layer of protein (mucin BSM) across aqueous solution were measured as a function of distance. Parallel adsorption measurements were performed with radiolabeled mucin. The analysis of the force/distance curves for different mucin surface densities and ionic strengths have shown that (1) in all the studied cases, steric repulsive exponentially varying forces were observed, which increased with the mucin surface density. (2) For a low coverage of the mica surfaces by mucin, bridging effects give rise to large-distance attractive interactions. They disappear at high coverages of the mica surfaces. (3) The mucin macromolecules are more rigid at physiological salt concentrations than at low ionic strengths. © 1987 Academic Press, Inc.

INTRODUCTION

The behavior of proteins at interfaces is of great practical importance in various situations. Proteins can have a stabilizing or destabilizing effect on biocolloids: in a biological membrane, they control many of its functions and when a prosthesis is implanted in a biological medium, the first layer of protein which adsorbs on it determines its subsequent rejection or acceptance by the organism (1, 2). In soils, mucoproteins have important bearings on the aggregation properties of mineral particles (3).

In most of these situations, the adsorbed protein modifies the structure of the interface and consequently the interaction forces between such interfaces. So far, very few publications have been devoted to the measurements of surface forces with adsorbed proteins. Silberberg and Klein (4) reported interaction between two surfaces bearing adsorbed collagen layers.

The present paper deals with the measurements of such forces between two surfaces covered with bovine submaxillary mucin (BSM). The main function of mucin in the

living organism, along with similar mucoproteins found in respiratory, gastrointestinal, and reproductive tracts, is to lubricate epithelial cells and protect them from the external environment by repulsing the bacteria that come over the surface (5). Also, human ocular mucus contains mucin-like high-molecular-weight complexes (6, 7). Different biosurfaces, such as dentures, contact lenses, or intrauterine contraceptive devices, in spite of different functions have one common feature: all are in contact with a mucosal surface. It is, therefore, extremely important in the science and technology of biomaterials to acquire a better understanding of mucin/biomaterial interactions and of the mucin properties at the interfaces.

Mucin Structure

Bovine submaxillary mucin (BSM) is a large, flexible rod-like macromolecule (8), with a molecular weight of 4×10^6 (9). Its isoelectric point is at pH 3. With the oligosaccharide side chains attached to the polypeptide core mucin has the form of a bottle-brush structure (10). Its length is about 800 nm (11) and its radius

of gyration measured by light scattering (11–13) is 140 nm.

Mucin at Interfaces

Adsorption isotherms of BSM have been studied on various substrates including polymers and mica. The main results are that mucin adsorbs indefinitely, without reaching any plateau. One part of the adsorbed layer is reversibly adsorbed while another part of the layer remains irreversibly adsorbed on the surfaces (14, 15).

The stability of thin mucin free films, as reported by Proust *et al.* (16), depends on the manner in which they are formed. When a reasonably long time was allowed for BSM adsorption at two boundary interfaces, the films were unstable and thin at low ionic strength values. They were infinitely stable and thick at physiological NaCl concentrations (16).

Preliminary measurements of the forces between two mica surfaces bearing adsorbed mucin layers (15) revealed large-distance attractive effects, a hysteresis of the force/distance profiles, and steric repulsions at short distances.

The effect of NaCl concentration in the aqueous mucin solutions and of the adsorbed amounts on the nature of different contributions to these forces is the main purpose of the present work. Previous experiments on mica surfaces (14) have shown that the irreversibly and reversibly adsorbed quantities of mucin are linear as a function of mucin concentration in solution. As these quantities also depend (as shown in this paper) on ionic strength, a suitable choice of mucin solution concentration may give the same adsorbed amounts for two different ionic strengths.

MATERIALS AND METHODS

The water used was tridistilled, the second distillation being made from acid potassium permanganate, and had very low bubble persistence. The pH value of the water was typically 5.6.

The NaCl was obtained from Merck Suprapur 99.5% and roasted 6 h at 600°C.

BSM was isolated from fresh salivary glands obtained at a slaughterhouse by a procedure described by Tettamanti and Pigman (17). Purification was achieved by extensive dialysis against tridistilled water. The lyophilized mucin was analyzed to determine the sialic acid content which specifically characterizes glycoproteins. Using the periodate–resorcinol method (18), the mucin was found to contain about 30% sialic acid, a value in good agreement with that reported in Ref. (17). The details of the extraction, purification, and radiolabeling of mucin are given in Ref. (19).

Adsorption Measurement Technique

The *in situ* adsorption at the mica/aqueous solution interface was measured with the ^{14}C labelled mucin. The adsorption was performed in a cell (see Fig. 1), the bottom of which was a window made of a thin mica sheet ($\leq 10\ \mu\text{m}$). The radioactivity coming from the adsorbing mucin was directly measured with the help of a gas flow counter. An allowance was made to correct for the background activity and the adsorption of radioactivity by a mica window. The ^{14}C -labeled mucin was shown to have the same surface properties as the unlabeled one by the constancy of measured adsorption with varying isotopic dilution. The technique is described in detail in Ref. (19).

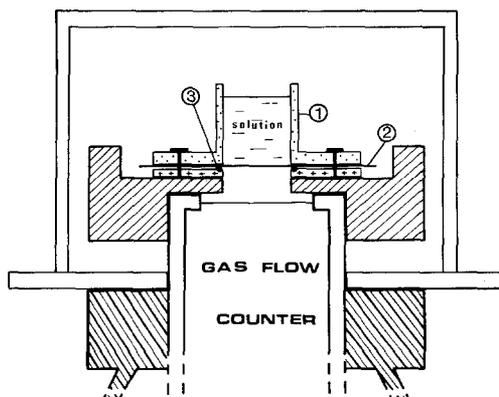


FIG. 1. Adsorption measuring device: (1) glass cell, (2) mica window, (3) "O" ring.

Forces Measurement Technique

The force F between two molecularly smooth curved (of radius $R \approx 2$ cm) mica surfaces as a function of distance D was measured using the method developed by Israelachvili and extensively described in Ref. (20). The main part of the system is a three-stage translation device, composed of a micrometric translation (accuracy $1 \mu\text{m}$) coupled with a differential spring system (accuracy 1 nm) and a piezoelectric tube (accuracy better than 0.1 nm). The distance D between the mica surfaces is measured by multiple-beam interferometry to 0.1 – 0.2 nm , and the refractive index of the intervening medium is measured by the same method. The translations are calibrated in the absence of forces. Then, the surfaces are brought toward each other, and any difference between the actual change in distance and the one obtained from calibration, when multiplied by the stiffness coefficient of the leaf spring (100 N/m), is equal to the interaction force between the two surfaces.

In our apparatus (see Fig. 2), the mica surfaces were immersed in the fluid within a stainless-steel cell (volume $\approx 9 \text{ cm}^3$) which reduced the necessary volume of fluid considerably and thus reduced the risk of contamination.

The apparatus was thermostated at around 20°C in such a way that the temperature drift was always smaller than $10^{-3} \text{ }^\circ\text{C min}^{-1}$. The value F/R is plotted in the graphs as a function of D and is equal to $2\pi E$, where E is the corresponding energy between flat surfaces (21).

The mica surfaces are first immersed in water and left for an hour. Prior to the measurements, a necessary check is made to ensure the absence of organic or particulate contaminations. This is made by bringing the surfaces toward each other so that they can jump into contact from a repeatable distance of 2.5 nm . Then, 10^{-3} M NaCl is added and the force/distance profiles are determined.

To measure force/distance profiles with mucin, a known amount of either mucin alone or mucin with NaCl is added to the cell, de-

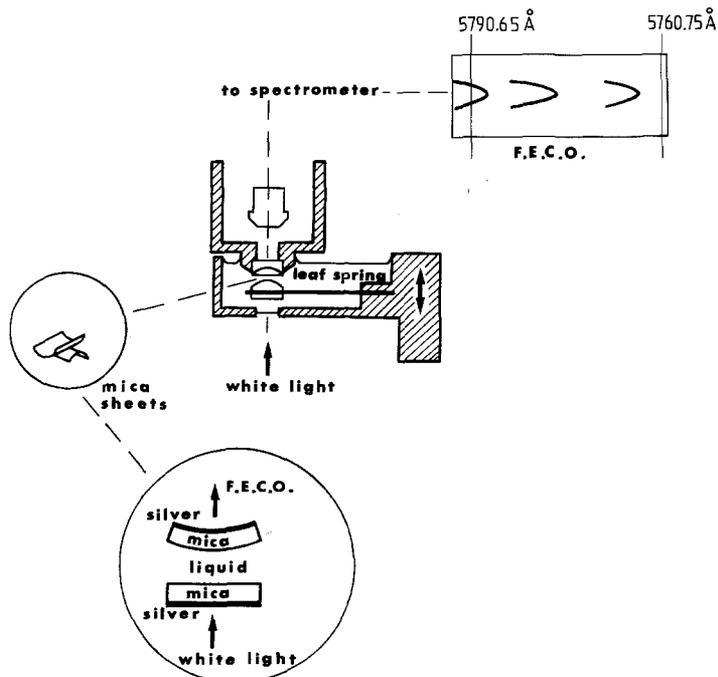


FIG. 2. Surface forces measuring cell.

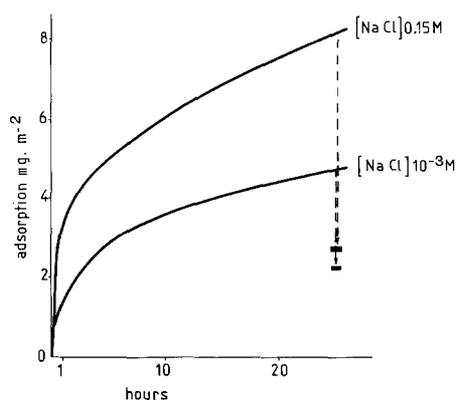


FIG. 3. Adsorption kinetics of mucin 0.1 g/liter at two different NaCl concentrations. Dashed lines indicate mucin desorption.

pending on the studied case. Three hours are allowed for adsorption before each experiment is performed.

In our experiments, the mica surfaces were brought from 1200 nm to the smallest possible distance with *monotonic* displacement. These working conditions required the use only of the differential spring system (20). Then, the surfaces were taken apart monotonously up to 1200 nm, and surface forces were measured during this movement. This cycle was repeated twice.

RESULTS

Adsorption of Mucin at the Mica/Solution Interface

Adsorption measurements were performed at 0.1 g/liter mucin solution concentration with two different ionic strengths: 10^{-3} and 0.15 M NaCl (see Fig. 3). The results show

that the adsorption of mucin in NaCl 0.15 M is twice as large as that in NaCl 10^{-3} M. Two situations where the mucin adsorption is the same are 0.1 g/liter mucin + NaCl 0.15 M and 0.2 g/liter mucin + NaCl 10^{-3} M. The desorption measurements show that in 0.15 M NaCl, mucin is more reversibly adsorbed than in 10^{-3} M NaCl. Similarly, the adsorption at 0.05 g/liter mucin + 0.15 M NaCl solution concentration was found to be equal to that at 0.1 g/liter mucin + 10^{-3} M NaCl. The adsorbed amounts of mucin after 3 h are given in Table I. These solution concentrations were also used for the surface force measurements.

Forces between Two Mica/Aqueous Interfaces Coated with Adsorbed Mucin

The force/distance measurements showed that no significant difference could be observed between three successive cycles for each of the studied cases. The results were qualitatively reproducible. Some scattering, however, especially at large separations where small forces varied slowly with distance, was observed. We had thus used statistical averages to determine large-distance interactions.

A relaxation toward an equilibrium distance always occurred when displacing one of the surfaces at distances below 170 nm.

As it has already been mentioned above, four cases (A, B, C, and D) were studied.

(A) *Mucin 0.1 g/liter + NaCl 10^{-3} M.* In this case, three regimes of forces could be distinguished according to distance:

At large distances (between 500 and 170 nm), weak attractive forces (Fig. 5) are noticed.

TABLE I

Surface Density of Total Adsorbed Mucin

	0.1 g/liter mucin + 10^{-3} M NaCl	0.2 g/liter mucin + 10^{-3} M NaCl	0.1 g/liter mucin + 0.15 M NaCl	0.05 g/liter mucin + 0.15 M NaCl
δ_{mucin} at 3 h (mg/m ²)	2.4 ^a	4.3 ^a	4.5	2.3

^a From Ref. (14).

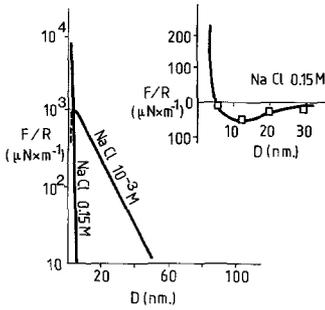


FIG. 4. Forces between two mica surfaces across pure NaCl solution.

They vary slowly with distance and are difficult to characterize. As they are measured with the differential spring system, the uncertainty is large and statistical averages must be determined in order to show their existence.

For the distances D below (170 nm), repulsion sets in with an exponential regime and the decay length is equal to 41 nm (see Fig. 5). One can compare them with the forces between mica surfaces across pure NaCl 10^{-3} M solution (represented in Fig. 4). These are exponentially repulsive with a decay length of 9.5 nm, close to the theoretical Debye length. We can deduce therefore that the repulsive exponential interactions of mica with an adsorbed mucin layer cannot be explained by the DLVO theory.

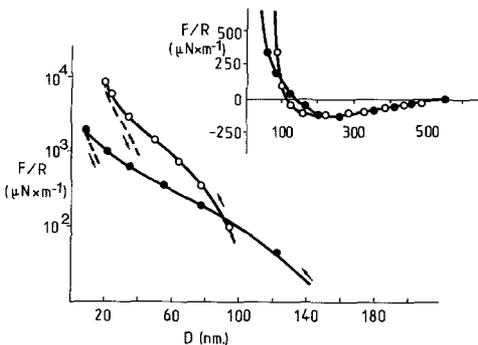


FIG. 5. Forces between two mica surfaces covered with adsorbed mucin at 3 h. (●) Mucin 0.1 g/liter + NaCl 10^{-3} M (case A), (○) mucin 0.05 g/liter + NaCl 0.15 M (case D). Dashed lines: forces measured in the separation movement.

Below $D \approx 40$ nm, the repulsion becomes steeper and the forces are not reversible. When the surfaces are separated the receding curve is not superimposable on the advancing one and some adhesion can be noticed.

When the solution is replaced by a pure 10^{-3} M NaCl solution (rinsing), the measured forces are the same as those during the advancing movement before rinsing. Then, the surfaces are separated and a very strong adhesion is observed. The measured forces become negative, down to about -2000 N/m. The hysteresis is much larger than before rinsing.

(B) Mucin 0.2 g/liter + NaCl 10^{-3} M. When bulk mucin concentration is doubled, the surface density of mucin is also doubled. In this case (Fig. 6), no large-distance attraction was detected and the exponential repulsive forces starting from 205 nm were larger than those in case A. The decay length is equal to 43 nm. When the repulsions become too large, at 8 nm, the surfaces cannot be brought closer. The curves are not reversible and the hysteresis is less pronounced than that in the case A.

The replacement of the mucin solution by a pure electrolyte does not noticeably modify the measured forces, neither upon bringing the surfaces together, nor upon separating them.

(C) Mucin 0.1 g/liter + NaCl 0.15 M. The surface density of mucin in this case is the same as that in case (B). However, the measured forces are considerably different from those in case (B) (see Fig. 6). No large-distance

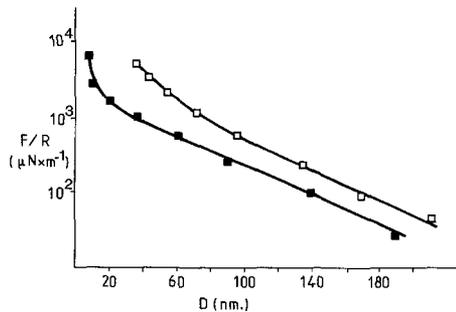


FIG. 6. Forces between two mica surfaces covered with adsorbed mucin at 3 h. (□) Mucin 0.2 g/liter + NaCl 10^{-3} M (case B), (■) mucin 0.1 g/liter + NaCl 0.15 M (case C).

attraction is observed and the forces are exponentially repulsive (decay length, 41 nm), starting from 230 nm. They are perfectly reversible, more reproducible than at low ionic strength, and much higher than those in case B. We cannot bring the surfaces closer than 34 nm. These forces can be compared with the force/distance profiles in pure 0.15 M NaCl solution (Fig. 4), where a secondary minimum is followed by a steep repulsion. It is even more obvious here than in case (A) or (B) that the forces measured when mica is covered by an adsorbed mucin layer cannot be explained in terms of the DLVO theory.

Upon replacing the mucin solution by pure NaCl 0.15 M (rinsing), a strong desorption is noticeable from the force/distance profiles shown in Fig. 7. After rinsing, the forces are reduced and the surfaces can be brought to distances as close as 12 nm. The forces are no longer reversible as shown in Fig. 7.

(D) *Mucin 0.05 g/liter + NaCl 0.15 M.* At this concentration, the adsorption of mucin is equal to that in case (A). The forces start by large-distance attractive interactions from 500 nm, followed by a repulsion from about 110 nm (see Fig. 5). This repulsion is much stronger than that in case (A). Upon separating the surfaces, the measured forces are different from those obtained with decreasing D .

Refractive index measurements. The refractive indices of the medium between the mica surfaces were measured in cases (B) and (C) (see Fig. 8). In case (B), they are equal to

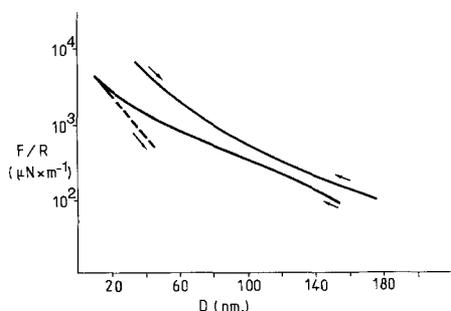


FIG. 7. Effect of rinsing with NaCl 0.15 M. Upper curve (case C), before rinsing; lower curve (case C), after rinsing.

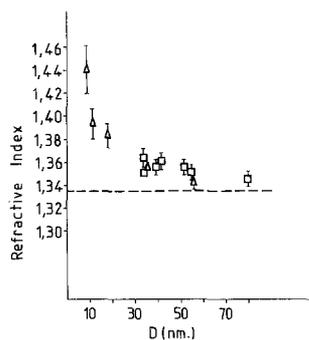


FIG. 8. Refracting indices versus distance. (Δ) Case B, (\square) case C.

those in case (C) at the same mica/mica distances, which confirms that the adsorptions are equal. With mucin and NaCl 0.15 M (case C), the refractive indices are given for case (C) down to separations of 34 nm which is the minimum accessible distance in regard to the intensity of the repulsion. The corresponding maximum measured refractive index is 1.36.

DISCUSSION

In a previous paper on the same subject (15), where only one case was studied (mucin 0.05 g/liter + NaCl 10^{-3} M), we had observed in the force/distance profiles three particular behaviors:

- large-distance attractive interactions;
- hysteresis and adhesion;
- exponential repulsive steric interactions.

In the present work, these three effects have been observed. It is possible now to determine the conditions under which each of them may occur or not and to explain them. The behavior of the studied systems is summarized in the Table II.

Attraction, Hysteresis, and Adhesion

Large-distance attraction was the main effect, pointed out in our previous paper (15), on forces between mucin-coated mica surfaces. There, only one mucin surface concentration

TABLE II
Attractive and Adhesive Effects in Different Cases

	$10^{-3} M$ NaCl		$10^{-3} M$ NaCl Forces data from Ref. (15)	$0.15 M$ NaCl	
	A	B		C	D
δ_{mucin} (mg/m ²)	2.4	4.3	2.0	4.5	2.3
Large-distance attraction	+		+		+
Hysteresis	++	+	++		+

($\delta_{\text{mucin}} = 2 \text{ mg/m}^2$) was investigated. Here, this attraction regime is observed in cases (A) and (D), and it is absent from cases (B) and (C). It thus seems obvious that this effect is not related to ionic strength but depends essentially on the surface concentration of the mucin layer.

With adsorbed macromolecules such as mucin, two mechanisms can produce large-distance attractive effects between two walls:

attraction between adsorbed macromolecules;

bridging of the two walls by one macromolecule attached simultaneously on both surfaces.

The first process may be supported by a well-established property of the self-association of mucin in solution. However, this process must increase with adsorption and is thus ruled out. Bridging would be limited by the degree of coverage of mica surfaces by macromolecules. Increasing surface density would limit bridging, and at high coverages, no bridging is possible as the mica sites are not accessible to mucin molecules adsorbed on the opposite wall. On the contrary, at low coverages, enough adsorption sites may remain on one mica surface to bind mucin that is adsorbed on the other wall. We thus conclude that this attractive regime is due to bridging.

Hysteresis of the force/distance profiles had been observed in our previous paper (15). Here strong hysteresis and some adhesion are observed in case (A). Small hystereses are ob-

served in cases (B) and (D) while no hysteresis at all is obtained in case (C) where the forces are perfectly reversible.

The possible origins for a hysteresis of the force/distance profile may be:

modification of the adsorbed layers by compression during force measurements;

bridging of the mica surfaces by mucin; entanglement between mucin chains.

The first possibility can be checked by performing a second cycle of force measurements. As no significant change in the force/distance profiles was obtained relative to the first cycle, we can rule out this explanation.

As we have seen above, bridging occurs in cases (A) and (D). The origin of hysteresis can thus be explained by an increase in the number of bridges at small distances. However, the occurrence of entanglement effects should not be excluded. In case (C), neither bridging nor chain entanglement may occur. Finally, in case (B), in spite of the fact that the surface density of mucin is equivalent to that in case (C), small hysteresis is observed. As this situation cannot be attributed to the presence of bridges, we must attribute it to chain entanglements. This difference in behavior may be explained if we assume that the mucin chain mobility is restricted in NaCl 0.15 M.

Repulsive Regimes

All the repulsive regimes are characterized by an exponential domain which in some cases is limited by the attractive regime at large distances. At very small distances, the repulsion steepens in all cases.

The repulsive profiles illustrated in Figs. 5 and 6 show two distinct regions. At relatively large distances, an exponential domain is observed. Comparison of Fig. 4 with Figs. 5 and 6 clearly shows that the DLVO theory cannot be applied to interpret the shape of the repulsive forces. The only relevant explanation of

the observed phenomenon would therefore be the existence of steric repulsive forces which originate from the decrease in the possible number of configurations allowed for the adsorbed macromolecules at these distances. At smaller distances, the repulsive profiles change and a steeper part of the curve can easily be distinguished. While steric repulsions are still present in this region, they are likely to be complemented by the existence of intra- and intermolecular segment/segment forces which enhance the repulsive effect. Indeed, it can easily be imagined that when the distances become very small, the mucin chain movements are completely blocked and static interactions between mucin segments may occur.

The comparison of case A with case B shows (cf. Figs. 5 and 6), as expected, that an increase in surface coverage increases the repulsive forces. However, as can be seen by comparing case (B) with case (C) and case (A) with case (D), the quantity of adsorbed mucin is not the only relevant parameter determining the forces. The increase in repulsive forces with NaCl concentration must come from the stiff increase in mucin macromolecules. This confirms the above-mentioned assumption that the presence of NaCl has an important effect on chain entanglement.

Replacement of Mucin Solution by Pure Solvent

When the mica surfaces are rinsed with mucin free electrolytes, hysteresis increases in all studied cases. In case (C), where it does not initially exist, hysteresis can be observed after rinsing.

Only in case (C) in the repulsive domain may a strong mucin desorption be observed directly. This is attributed to the fact that in 0.15 M NaCl, the reversible adsorption is much more pronounced than in 10^{-3} M. The forces in this domain are also higher.

General Discussion

How far do the adsorbed mucin macromolecules span from the surface? This ques-

tion is related to many problems such as bio-lubrication of epithelial cells and stability of precorneal and prelens tear films. In all these situations, it is important to know the actual thickness of the aqueous mucus layer protecting epithelial tissues. Many authors evaluate this thickness from the total quantity of the dried adsorbed mucoprotein (23–25). From these experiments the mucus layer is believed to have a thickness of 20–40 nm. However, the relevant parameter is the thickness of the aqueous mucus layer as it is directly linked to the protective role of this layer. The protection effect is essentially obtained by repulsing all the bacteriae that come over the surface (26), and the range of repulsion is directly characterized by our method of measurement.

Let us first notice that the mica/aqueous solution interface is negatively charged (20) and so is mucin at this pH. This implies that the adsorbed mucin molecules are anchored to mica by very few points, and that they are unlikely to be very perturbed and denatured.

The extension of adsorbed mucin from the surfaces may be evaluated from the distance at which the interactions become observable in the process of bringing the surfaces together.

When the interactions start by an attraction regime, this distance is 500 nm. When they start by a repulsion, this distance is equal to 220 nm. It may be surprising that a weak mucin adsorption can lead to a larger interaction distance than a stronger one. However, the attractions are due to bridging and repulsions are due to steric effects. As it has been shown above, the bridging observed in the present work is a one-molecule process (one molecule adsorbing simultaneously on both surfaces). The steric interaction may be either a one- or a two-molecule process. We have shown above that in cases where the interaction starts by a repulsion regime, it is a two-molecule process. If l_c is the characteristic spanning of a non-confined adsorbed mucin macromolecule, the steric repulsion is expected to start from a dis-

tance smaller than $2 \times l_c$ (27). This allows an estimate of the value of l_c : $l_c \simeq 110$ nm, which is close to the mucin radius of gyration (11–13).

The attractive interactions by bridging are more complex. Clark and Lal (28) have discussed the possibility of a polymer chain bridging two surfaces, with two varying parameters: the surface distance and the monomer/surface interaction $\Delta\epsilon$. Their main conclusions are:

The bridging probability increases with increasing $\Delta\epsilon$, it reaches a maximum, and it decreases when $\Delta\epsilon$ is so strong that it favors a flat configuration of the chains.

The probability distribution of bridge length is shifted toward large distances when $\Delta\epsilon$ decreases.

At large distances, the likelihood of bridge formation due to a tail attaching to the second surface will be greater than that due to the transformation of a loop into a bridge.

In our case, we observe attractive interactions due to bridging, at distances much larger than l_c . This is the evidence that bridges come from tail attachment, and thus, the hypothesis of a weak mica/mucin interaction is confirmed. Far from being excluded, the possibility of shorter bridges may not be appreciated as its effect may be mixed by the steric repulsions.

Mucin is well known for its self-associative properties (12). We have measured no force that can be attributed to that, probably because it is too weak when compared to the accuracy of the technique.

CONCLUSION

This study enabled us to gain an insight into the behavior of one protein at many interfaces. It can bridge two surfaces when adsorption is low and create attractive interactions. When confined, it can oppose a steric force. Under physiological conditions, as opposed to low ionic strength conditions, quantities of mucin

greater than those at low ionic strength can be adsorbed and the reversibility of adsorption is greater. In this situation mucin chains are less mobile and the macromolecules are less compressible.

Mucin has a lubricating and protecting role. The present work has shown that if a low quantity of mucin is adsorbed, it can then play a negative role. The ideal case is when the mucin layer is incompressible and has a reversible behavior in regard to adsorption and response to compression. This was observed for physiological salt concentration and high adsorption. In this case, the extension from the surface of the protective and lubricating layer made of mucin molecules and bound water is about 110 nm. This thickness is similar to that found for the stability of wetting films made of mucin solution on contact lens materials (16) and to the minimum-thickness lacrymal films on cornea (29).

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