

BOVINE SUBMAXILLARY MUCIN (BSM) ADSORPTION AT SOLID/LIQUID INTERFACES AND SURFACE FORCES

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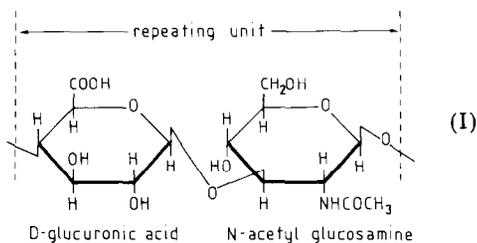
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ABSTRACT

Bovine submaxillary mucin (BSM) was obtained from salivary glands through successive precipitations and dissolutions. It was labelled by acetylation with [$1-^{14}\text{C}$]acetic anhydride. Direct and continuous measurement of the total (reversible and irreversible) adsorption has been taken on muscovite mica, polyethylene, oxidized polyethylene, silicone and poly(vinyl pyrrolidone) grafted silicone films. Force measurements between mucin layers adsorbed on two mica surfaces have been made in the distance range 0–600 nm. Adsorption/desorption and force distance measurements allow us to distinguish between reversibly and irreversibly adsorbed protein molecules. The results also show that chemical modification of polymer surfaces enhances mucin adsorption.

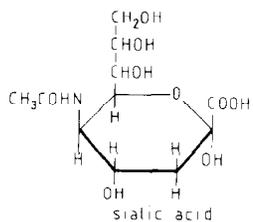
INTRODUCTION

Glycoproteins represent a group of proteins where carbohydrates (hydroxy-aldehydes or hydroxyketones) are linked to the main polypeptide chain. The carbohydrates, also known as sugars, may be monosaccharides (simple sugars), disaccharides or polysaccharides. The polysaccharide moieties of most glycoproteins are very complex and contain more than one type of unit. One of the most important units found in polysaccharides of glycoproteins is hyaluronic acid (I) which consists of alternating residues of *N*-acetylglucosamine and glucuronic acid.



This polysaccharide is of great biological importance and is found in the skin, in the umbilical cord, in the humour of the eye and in the synovial fluid. It forms aqueous solutions of high viscosity.

Another class of glycoproteins is the sialic acid-containing (II) group of glycoproteins (mucoproteins).



(II)

N-acetylneuraminic acid (NANA)

The oligosaccharides of the mucoproteins, mainly sialyl-*N*-acetylglucosamine, are linked to the peptide through glycosidic bonds between *N*-acetylglucosamine and the hydroxyl group of serine or threonine [1].

Mucins of a variety of species have been shown to be predominantly sialic acid-containing glycoproteins, especially in the mucous secretions of submaxillary glands. Their function, along with similar mucoproteins found in the respiratory, gastrointestinal and reproductive tracts, is to lubricate epithelial cells and protect them from the external environment [2]. Recent studies of the macromolecular components of human ocular mucus show the presence of mucin-like high molecular-weight complexes [3, 4].

The role of mucous glycoproteins as a macromolecular surfactant is of extreme importance in the science and technology of biomaterials. Such different biosurfaces as dentures, contact lenses or intrauterine contraceptive devices, in spite of different functions, have one common feature: all are placed on a mucosal surface. In order to obtain a better understanding of mucin—biomaterial interactions, they should be studied in more detail.

The aim of this paper is to describe the interactions of bovine submaxillary mucin (BSM) with two specific examples of solid surfaces: polymers and mica. Extraction, purification, ¹⁴C-labelling and in situ adsorption measurements of BSM on these surfaces, as well as the measurement of forces in BSM solution between two mica surfaces according to the Israelachvili technique [5], have been performed.

EXPERIMENTAL

Bovine submaxillary mucin (BSM): isolation, purification and labelling

Bovine submaxillary mucin (BSM) consists of a long protein chain with numerous disaccharide and oligosaccharide side chains. Threonine, serine, proline, glycine and alanine are the major amino acid residues. The carbohydrate content of BSM consists of *N*-acetylneuraminic acid, galactose, fucose, *N*-

acetylglucosamine and *N*-acetylgalactosamine [6]. The BSM macromolecule was reported to have a molecular weight of about 4×10^6 [7]. Light-scattering measurements show that at low ionic strength the molecule is a rigid rod and when the ionic strength is increased, the molecule becomes a stiff, compact coil [8]. It has also been suggested [8] that the stiffness of BSM molecules is largely maintained by the repulsive electrostatic forces of the sialic side chains.

Bovine submaxillary mucin was isolated from fresh salivary glands obtained at a slaughterhouse by a procedure described in detail in Ref. [9]. This procedure, containing five distinct steps, follows the same pattern as that of Tettamanti and Pigman [6]. The total sialic acid content of the obtained lyophilized mucin was about 30%, which is in good agreement with the data reported for a mixture of minor and major mucins [6].

^{14}C -labelling of lyophilized mucin was achieved by acetylation with [$1\text{-}^{14}\text{C}$]-acetic anhydride and is described in Ref. [9]. The method is based on that used by Hoagland [10] for acetylation of β -casein. The solutions of acetylated mucin were found to have the same surface tension values as the solutions of the non-acetylated mucin, indicating that the surface tension properties of the mucin were not modified during its acetylation [9].

Polymers and mica

Adsorption of BSM was studied on the following surfaces: polyethylene, oxidized polyethylene, silicone, poly(vinyl pyrrolidone) grafted silicone and mica.

Polyethylene was low-density Cryovac film of thickness $19 \mu\text{m}$ (Grace, France). Silicone was Rhône-Poulenc poly(dimethyl siloxane) film of thickness $50 \mu\text{m}$.

Surface properties of polymers were modified either by oxidation in $\text{KClO}_3/\text{H}_2\text{SO}_4$ mixture for polyethylene, according to the method described in Ref. [11], or by grafting with poly(vinyl pyrrolidone) onto silicone samples. In the latter case, the silicone films were irradiated with a mercury vapour lamp in the presence of air and then immersed in air-free (purged by nitrogen bubbling) *N*-vinyl pyrrolidone during 30 min at 95°C . To separate the non-grafted homopolymer, the sample was washed several times in boiling water and then dried. The average degree of grafting was 3–5%.

Mica samples used for adsorption were freshly cleaved under a laminar flow cabinet; their average thickness was $10 \mu\text{m}$.

Adsorption measurement techniques

Two distinct techniques were used to measure BSM in situ adsorption on studied surfaces. They are illustrated in Fig. 1. The densities of polymers are lower than that of water and the polymers float when placed on top of BSM solutions. Circularly-punched polymer samples are gently placed at the sur-

face of a BSM solution, care being taken to ensure that no air bubbles (Fig. 1a) are trapped between polymer and solution. For mica surfaces a specially-constructed circular container was used (Fig. 1b). The flat, ground part of the glass cell was covered with molten paraffin. Three fixing screws and a Viton "O" ring ensured a tight seal between glass and plastic parts of the container with a mica window inserted between them. The radioactivity-counting device (gas-flow chamber) placed at a fixed position below a sample measured the radioactivity and displayed it on a recorder as a function of time. Counting corrections are necessary to obtain the net adsorption values: allowance was made for the absorption of β -radiation by a polymer or a mica surface and the radioactivity originating from the bulk of the BSM solutions was subtracted. A calibration graph is also needed, to convert the adsorption values from counts min^{-1} into μg of BSM cm^{-2} . Details of the method may be found in Ref. [9].

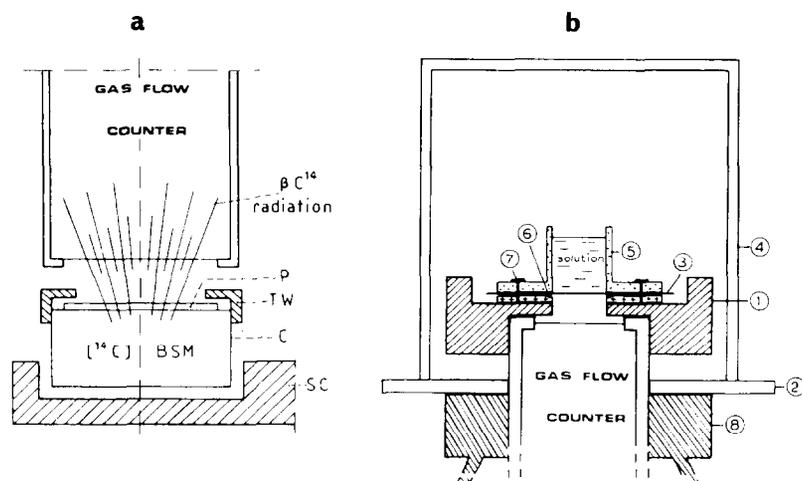


Fig. 1. Adsorption measuring devices: (a) for adsorption on polymers; (b) for adsorption on mica. Legend: (P), floating polymer film; (TW), teflon window; (C), glass container; (SC), support; (1), (2), (8), supports ensuring reproducibility of geometry; (3) mica window; (4), cover; (5), glass cell; (6), "O" ring; (7), cell assembling screws.

The necessary test was made to ascertain that the surface concentrations obtained in this way were independent of the ratio of labelled to unlabelled protein, indicating that no preferential adsorption occurred on the surface (Fig. 2).

Desorption experiments were performed at the end of each adsorption cycle. Polymer samples were placed on buffer substrates which did not contain BSM and in experiments with mica the BSM solution was replaced by a buffer solution.

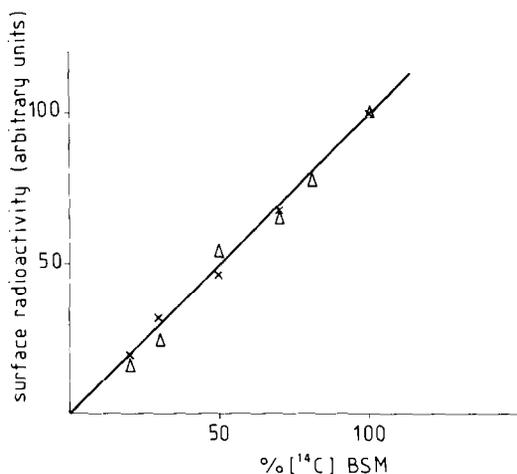


Fig. 2. Test showing the equivalence of adsorption of labelled and unlabelled BSM. Total BSM concentration of the labelled/unlabelled mixture in solution 0.1 mg ml^{-1} ; 10^{-3} M NaCl; adsorption time = 5h. (X) Adsorption on mica; (Δ) adsorption on polyethylene.

Force measurement technique

In order to measure the force vs distance between two curved mica sheets immersed in a protein solution, a reproduction of the system designed by Israelachvili [5] was used. It is made of a calibrated three-stage translation mechanism capable of displacing one mica sheet relative to the other within $\pm 0.1\text{--}0.2 \text{ nm}$ accuracy. The real distance between the mica surfaces is measured with the same accuracy by multiple-beam interferometry. The force

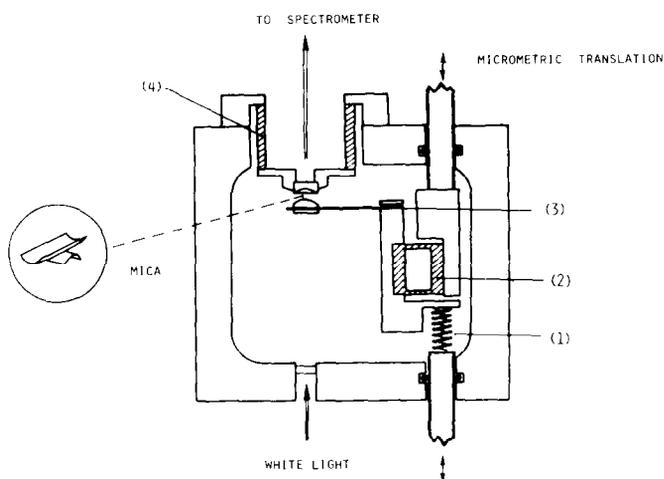


Fig. 3. Schematic drawing of apparatus for surface force measurements: (1), helical spring; (2) stiff double cantilever spring; (3), spring; (4) piezoelectric tube.

between the surfaces is obtained optically by measuring the deflection of the leaf spring. The system is thermostatted (Fig. 3).

The mica sheets mounted in the system are first immersed in NaCl solution and forces are measured. Then they are kept several μm apart, the protein solution is injected into the measuring cell by means of a syringe, the liquid is mixed with a magnetic stirrer and the system is left at this separation for a given time interval, during which the protein adsorption takes place. At the end of this time interval the forces are measured again.

RESULTS AND DISCUSSION

Adsorption measurements

A typical example of the rate of BSM adsorption on different surfaces is illustrated in Fig. 4. Adsorption during the initial time interval was rapid; a slower and more gradual increase was then observed. It can be noticed that on all surfaces studied, the amounts of BSM adsorbed continue to increase with time. In the previous paper [9] we have shown that no plateau value is attained up to a 60-h adsorption time.

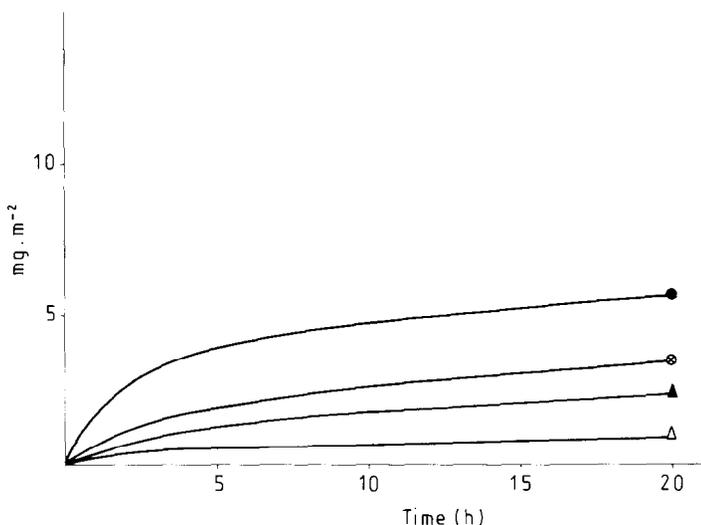


Fig. 4. Kinetics of BSM adsorption on various solid substrates: (Δ), polyethylene; (\blacktriangle), oxidized polyethylene; (\circ), silicone; (\times), mica; (\bullet), PVP grafted silicone; BSM concentration = 0.05 mg ml^{-1} ; 0.15 M NaCl ; pH 7.2.

When the adsorption curves were compared with the curve of concentration dependence of variation of BSM surface tension with time (the case in which the transport of protein molecules is diffusion controlled), it was concluded that all surfaces exhibit different barriers (steric or electrostatic) for

adsorption of mucin molecules arriving at the solid surface [12]. Such types of barriers, slowing the adsorption of protein towards adsorbing surfaces, have also been observed by others [13].

Thick BSM layers are found on all surfaces with increasing bulk protein concentration (Fig. 5). The data in Fig. 5 clearly show that chemical modification of polymer surfaces (grafting or oxidation) enhances BSM adsorption. Except for polyethylene, all other surfaces adsorb more than the amount corresponding to the saturation value at the BSM solution/air interface (2.5 mg m^{-2}) [9]. Continuous increase of the amount of adsorbed BSM is observed as the bulk protein concentration increases.

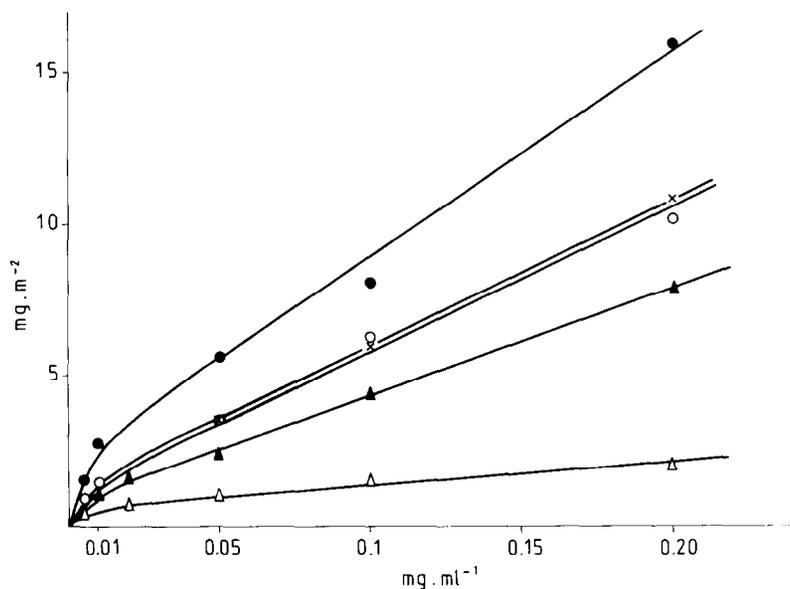


Fig. 5. Adsorption vs BSM bulk concentration. Symbols as in Fig. 4. 0.15 M NaCl ; pH 7.2; adsorption time = 20 h.

The desorption experiments illustrated in Fig. 6 show that the loosely bound BSM fraction is significant on mica and chemically modified polymers and almost negligible on untreated polyethylene. The amount of loosely-bound BSM increases with the protein bulk concentration.

Forces between two mica surfaces immersed in mucin solution

The effect of $5 \times 10^{-2} \text{ mg ml}^{-1}$ BSM solution in 10^{-3} M NaCl on the forces between mica surfaces is shown in Fig. 7(a). It can be seen that the measured forces in the presence of adsorbed BSM layers are not reversible, while in pure aqueous NaCl solution, they are reversible. Figure 7 illustrates the occurrence of hysteresis when, after bringing together two mica surfaces (advancing

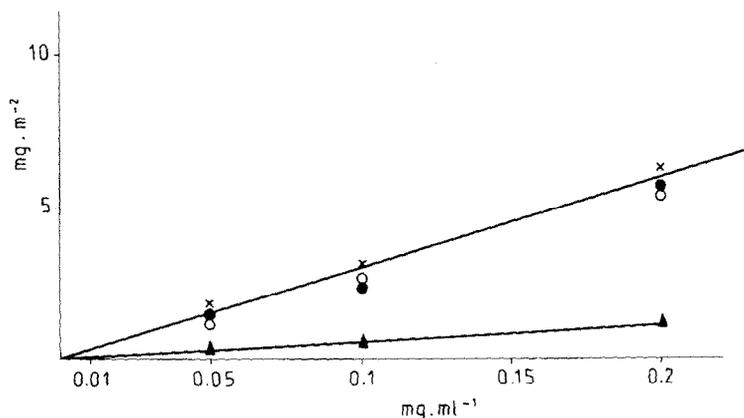


Fig. 6. Loosely-bound BSM vs protein concentration. Symbols and conditions as in Fig. 5. Loosely-bound BSM function on untreated polyethylene is zero (not shown).

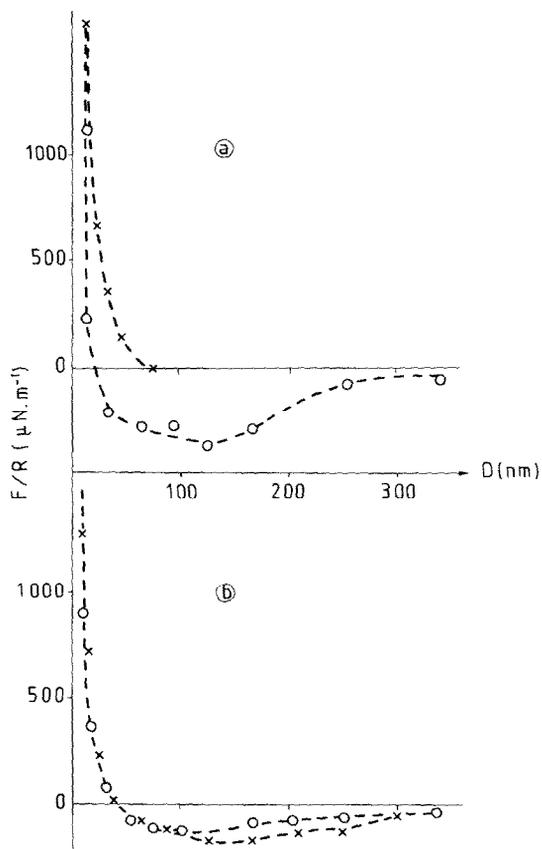


Fig. 7. Force—distance curves measured in $5 \times 10^{-2} \text{ mg ml}^{-1}$ BSM solution; 10^{-3} M NaCl ; adsorption time = 20 h. (a) First advancing (x), first receding (o); (b) second advancing (x), second receding (o). R = radius of cylindrical mica surface.

movement), they are separated (receding movement). Figure 7 also shows that only repulsive forces arise during the first advancing movement, whilst on separation, attractive forces are also observed (their extension is beyond 300 nm).

When a second cycle of bringing together and separating mica surfaces is performed (Fig. 7b) the behaviour of BSM molecules is different from that during the first cycle (Fig. 7a). Here, during the advancing step of the cycle, initial attractive forces are present and then at the end of the advancing movement repulsion is observed. The hysteresis is less pronounced than during the first cycle. The third and successive cycles, not shown in Fig. 7 exhibit only a negligible hysteresis.

The attractive portion of the force/distance curves in the second and third cycles may be attributed to a bridging effect between two adsorbed BSM layers. This bridging effect is visible below 250 nm.

The portions corresponding to the repulsive forces are plotted on a logarithmic scale vs distance and are illustrated in Fig. 8. The difference between the two curves, corresponding to the first and the second advancing movement, may be attributed to the stripping of the loosely bound mucin layers.

The dimensions and shape of mucin molecules are subjects of controversy [8, 14, 15]. Bettelheim and Dey [8] have said that the mucin molecule is a rigid rod of about 800 nm at low ionic strength, while Gottschalk and MacKenzie [14] have assumed that its shape is that of a random coil, which has some rigidity. Recently Shogren et al. [15] came to the conclusion that a polydisperse system of spheres or coils could, in light scattering measurements, give the same results as for rod-shaped molecules.

Whatever the BSM molecule structure is, one can anticipate that BSM mole-

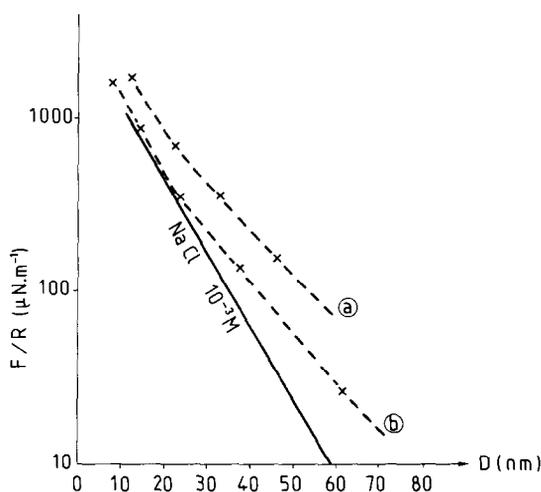


Fig. 8. Log F/R vs distance for first (a) and second (b) advancing movement of curves represented in Fig. 7.

cules lie in a flat orientation at the mica surface. Then, on approach of the BSM-covered surfaces, bridging may occur at small distances, whereas, on separating from the surfaces, the interacting BSM molecules may change their orientation and conformation so that they extend in the solution over a large distance before the bridges are destroyed.

CONCLUSIONS

(1) In situ adsorption/desorption measurements and force measurements at the interface between a solid and an aqueous BSM solution show the presence of a loosely-bound mucin layer (in addition to irreversibly-bound BSM) on all studied surfaces. Such loosely-held protein layers account for the behaviour of mucin during the advancing and receding movements of mica surfaces in force measurements, and may also be relevant to exchange processes with other constituents of biological liquids in contact with solid surfaces.

(2) Surface-chemical modification of polymer films by oxidation or grafting enhances mucin adsorption, showing the important effect of superficial hydrophilic groups upon the adsorption process.

(3) Force/distance measurements in mucin solutions give additional information about the nature of adsorbed BSM layers on mica surfaces. The adsorbed molecules (total adsorption) lie flat on the mica surfaces. Bridging occurs when two mucin-covered surfaces are brought into contact.

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