Spectroscopic Ellipsometry of Mucin Layers on an Amphiphilic Diblock Copolymer Surface

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ABSTRACT

Both visible and infrared (IR) spectroscopic ellipsometry have been employed to study the structure of thin layers of bovine submaxillary mucin (BSM) adsorbed on poly(acrylic acid-block-methyl methacrylate) (PAA-*b*-PMMA) copolymer and poly(methyl methacrylate) surfaces at three pH values (3, 7 and 10). The adsorbed mucin layer on the copolymer surface had the greatest thickness (17 nm) when adsorbed from a mucin solution at a pH of 3. For the first time, IR ellipsometry was used to identify adhesive interactions and conformational changes in mucin/polymer double layers. After applying the regularized method of deconvolution in the analysis, the formation of hydrogen bonds between the carboxyl groups of the BSM and PAA-*b*-PMMA copolymer in double layers has been found. The IR ellipsometry data, in agreement with the visible ellipsometry analysis, indicate the pH dependence of adhesion of mucin to the copolymer surface. There is an increase in the amount of hydrogen-bonded carboxyl groups in mucin deposited at a pH of 3. There is no evidence that the mucin's amide groups participate in this bonding. At the lower pH, the IR ellipsometry spectra after deconvolution reveal an increase in the proportion of β -sheets in the BSM upon adsorption on the copolymer surface, indicating a more unfolded, aggregated structure. The IR ellipsometry data also indicated some changes in the conformational states of the side groups in the copolymer induced by entanglements and bonding interactions with the mucin macromolecules. Deconvolution provides an unprecedented level of information from the IR ellipsometry spectra and yields important insights.

Index Headings: IR ellipsometry; IR spectroscopy; Deconvolution; Mucoadhesion; Hydrogen bonding; Mucin

INTRODUCTION

Mucins are natural amphiphilic biomolecules that have potential applications in biotechnology.¹⁻⁴ It has been shown recently^{5,6} that mucin layers adsorbed on polymer surfaces reduce bacterial adhesion. Hence, creating robust mucin coatings and exploring their structure and properties is an important step in the development of improved coatings.

The mucin macromolecule is considered to be a block copolymer made up of a long, linear peptide backbone (~20-50%) with regions of densely-packed linear or branched carbohydrate side chains (~50-80%) and “bare” regions with no or few carbohydrate side chains.¹⁻³ Molecular weights range from 0.5 to 40×10^6 Daltons, depending on the source.^{1,2} The carbohydrate branched chains of mucin have a large charge density mainly because of sialic acid and sulfate residues. Mucin has an overall negative charge in neutral solutions, which diminishes as the pH decreases.³ It is worth noting that the composition, structure, conformation and interfacial properties of mucins differ somewhat depending on their origin.^{1,2}

Mucin adheres to hydrophobic surfaces via its unglycosylated, hydrophobic protein blocks, while glycosylated hydrophilic blocks tend to stretch out away from the surface.⁵ Hence, adsorbed mucin layers impart hydrophilicity to an otherwise hydrophobic surface. It has been reported earlier that mucin coatings on hydrophobic polymeric materials, such as poly(methyl methacrylate) (PMMA) and polystyrene, can suppress the adhesion of some bacterial species due to the reduction of the hydrophobicity of their surface.^{5,6} However in these previous works,^{5,6} there was no attempt to control the adsorbed mucin layer thickness or to explore the structure of mucin layers adsorbed onto polymer surfaces.

Our approach to creating the mucin coatings has been inspired by previous studies of mucoadhesion, a term which refers to the attachment of materials to a mucus layer.²⁻⁴ It has been well documented that there is strong adhesion between mucin macromolecules and poly(acrylic acid) (PAA) in aqueous solutions.^{3,7-10} We therefore propose to use an amphiphilic diblock copolymer of PAA and PMMA (PAA-*b*-PMMA) with the aim of attaching mucin to the polymer surface through mucoadhesive interactions. Whereas previous studies of bacterial reduction relied on mucin adsorption through hydrophobic interactions^{5,6} the present approach relies on hydrogen bonding, as will be demonstrated herein.

The purpose of this work is to explore the structure of bovine submaxillary mucin (BSM) layers adsorbed on PAA-*b*-PMMA copolymer surfaces and to identify the molecular mechanisms of the adhesion and conformational changes in the BSM/PAA-*b*-PMMA double-layer system using spectroscopic ellipsometry. In this work we also determine the effect of pH on the thickness of BSM layers adsorbed on PAA-*b*-PMMA copolymer surfaces, as compared

to more hydrophobic PMMA.

The exact mechanisms of mucoadhesion are under continuing investigation.²⁻⁴ In the literature, at least three mechanisms for mucin/PAA adhesion in aqueous solutions have been proposed. One mechanism involves electrostatic interaction and hydrogen bonding mainly between the carboxyl and sulphate groups in the mucin's carbohydrate side chains and the carboxyl groups in poly(acrylic acid).^{3,7-9} In aqueous solutions, the hydrophobic protein backbone of mucin is largely protected by the carbohydrate side chains, which are exposed to the water due to their hydrophilic nature.³ A second possible mechanism, proposed by Patel et al.,¹⁰ is that amide groups of the mucin participate in hydrogen bonding with the carboxyl groups of PAA. Furthermore, interpenetration of mucin into polymers and the creation of molecular entanglements provide a physical adhesion mechanism.^{3,4,7-9} This third mechanism is dependent on the swelling behaviour of the mucin and the polymer, and it is time-dependent.

Various spectroscopic methods, in particular attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy, FTIR and nuclear magnetic resonance spectroscopy, dynamic light scattering, together with atomic force microscopy have been successfully applied to study the complex structure of mucin and molecular mechanisms of its adhesion.⁸⁻¹³ Considerable attention has been given to understanding the adsorption behavior of mucin and its important functional properties.¹⁴⁻¹⁷ However, the molecular structure of mucin layers adsorbed onto polymer surfaces has not been sufficiently explored so far. Little is also known about the conformational changes of mucin that might occur, as is the case for proteins, upon adsorption onto various polymer surfaces.¹⁶⁻¹⁸

Over the past decade, IR ellipsometry has been demonstrated to be very sensitive technique for structural analysis of thin films and multi-layers.¹⁹⁻²¹ Ellipsometry in the infrared range shows also considerable promise as a tool for exploring the molecular structure of thin biological layers, and hence is well suited to studies of mucoadhesion in multi-layered systems. This non-invasive technique can study thin multi-layers with much greater sensitivity than can be obtained from transmission spectroscopy,²² but with greater penetration depths than afforded by internal reflection spectroscopy yielding information on thickness.¹⁹ It has been applied with good success to studies of interfacial chemistry²³ and bonding,^{24,25} to polymer orientation,²⁶ to interdiffusion in polymer bilayers^{27,28} and polymer brushes.²⁹ There are a few examples of applications of IR ellipsometry to the study of biological molecules in thin films and at interfaces,^{19,30-32} but – until now - the technique has not been applied to detailed studies of biomolecular interfacial bonding and conformation.

In our work we apply the IR ellipsometry technique to identify structural and

conformational changes in BSM/PAA-*b*-PMMA double layers caused by mucin adhesion to a copolymer surface. Whereas there is previous study of mucin/PAA adhesion in aqueous solutions,^{2-4,7-10} we are not aware of any reported investigations of mucin adsorption on an amphiphilic copolymer, such as PAA-*b*-PMMA. We also use spectroscopic ellipsometry in the visible range to examine the thicknesses of adsorbed mucin layers on the PAA-*b*-PMMA copolymer, as compared to PMMA surface. The application of regularized method of deconvolution^{33,34} to the IR ellipsometry spectra allows us to reveal the fine structure of the complex bands and greatly increases the amount of useful information obtained via the IR ellipsometry analysis.

MATERIALS AND METHODS

Materials. PAA-*b*-PMMA was used as received from Polymer Source, Inc. (Montreal, Canada). The molecular weights of the blocks were 2.8×10^4 g/mole for the PAA block and 1.0×10^4 g/mole for the PMMA block. The molecular weight polydispersity was $M_w/M_n = 1.14$. Poly(methyl methacrylate) was purchased from Polymer Laboratories, UK ($M_w = 8.0 \times 10^4$ g/mole). Bovine submaxillary mucin, type 1-S as-lyophilized powder with approximately 12% bound sialic acid (Sigma Inc.), was used as received.

The experiments used two different types of substrates: (1) silicon and (2) gold. Single-crystal (100) wafers were used as-received for all visible ellipsometry experiments. A typical thickness of the native SiO₂ layer was determined to be 2.5 nm. Gold substrates, used for all infrared ellipsometry analysis, were prepared by the thermal evaporation of gold onto cleaned glass slides to create 55 nm films. Gold and silicon substrates were used because of their high IR and visible light reflectivity, respectively.

Thin Film Preparation. Copolymer films (ca. 150 nm thick) were created by spin-casting PAA-*b*-PMMA 3 wt.% solutions in tetrahydrofuran onto the gold substrates using a photoresist spin-coater (Cammex Precima Ltd., Colchester, UK) at 2000 rpm for 6 s. Thinner copolymer films (18-20 nm) were spin-cast onto the silicon substrates from 0.5 wt.% solutions. PMMA films were spin-cast from toluene solutions. All types of polymer films were heated in a vacuum at 90°C for 1 h to remove remanent solvent and to allow the polymer conformation to relax.

In the IR ellipsometry analysis, single films (copolymer or mucin) were first examined to identify the characteristic bands. Bilayers consisting of a mucin film on a copolymer film on a gold substrate were also examined. Mucin films were deposited from 1 mg/ml solutions of BSM in de-ionized (DI) and filtered water. Approximately 150 μ l of the solution was spread

across an area of 3 cm x 4 cm, and the film was left to dry in a desiccator containing silica gel for 24 h. The resulting mucin film thicknesses ranged between 400 and 500 nm. Mucin solutions were made at one of two pH values (3 and 7) obtained by the addition of a 0.1 M HCl solution or a 1 M NaOH solution. BSM/PAA-*b*-PMMA bilayers were created by successive deposition of the copolymer followed by the mucin films on gold substrates by the procedures just described.

Very thin mucin layers were also created by adsorption from mucin solutions onto one of three different surfaces: PMMA; PAA-*b*-PMMA; and silicon (for comparison). The substrates were soaked in 1mg/ml BSM solution for 24 h while shaking. The pH values of the BSM solutions were adjusted to 3, 7 or 10. The substrates were then removed and rinsed three times with DI water to remove any unattached mucin from the surface. The films were left to dry in a desiccator at ~ 0% RH. These adsorbed mucin films were then analysed with visible ellipsometry technique. The polymers and mucin are both glassy, and neither is subject to crystallization. They can be considered to be isotropic.

Spectroscopic Ellipsometry. The thicknesses and refractive indices of the films (spin-cast and adsorbed layers) were determined using a variable-angle spectroscopic ellipsometer (J.A. Woollam Co., Inc., Lincoln, NE, USA) in air. The angle-of-incidence ranged between 64 and 75° depending on the substrate and the particular system, and the wavelength ranged across the visible region from 400 to 800 nm. The central area of the film, where it was most smooth and uniform, was probed. Spectra were analysed using commercial software (WVASE 32, J.A. Woollam Co., Inc.). All films were stored at in a desiccator prior to their analysis in order to ensure reproducibility of the thickness measurements. (The copolymer thickness was found to increase with the relative humidity, but the initial thickness was recovered when placed in a low humidity atmosphere.)

Infrared ellipsometry spectra were obtained in air using a Fourier transform IR ellipsometer (Model GESP5-FTIR, SOPRA Sa., Bois-Colombes, France) at an angle-of-incidence of 65°. The spectra were obtained with a spectral resolution of 4 cm⁻¹ over a range from 4000 to 650 cm⁻¹. A typical measurement used eight scans for each spectrum, and 32 spectra were averaged to yield a higher signal-to-noise ratio.

The Ψ and Δ parameters obtained from the IR ellipsometry spectra were then converted to real and imaginary optical density values using these relations:

$$\text{Re } D = \ln(\tan \Psi_o / \tan \Psi_f)$$

and

$$\text{Im } D = i(\Delta_o - \Delta_f)$$

where the subscript o and f refer to the original surface and to the final surface after the deposition of a surface layer. Only the $\text{Re } D$ data will be presented here because, as shown earlier,²⁷ for isotropic organic films on metallic substrates over a limited range of thicknesses, the $\tan \Psi$ IR ellipsometry spectra are similar in shape to ordinary absorption spectra.

Infrared Spectroscopy. IR spectra were also obtained from the mucin in bulk form rather than in a thin film. KBr and mucin were separately crushed to create fine powders. KBr powder was dried in a fan oven at 40 °C, and the mucin was dried at room temperature in a desiccator containing silica gel for 24 h. The two powders were mixed, and then sufficient pressure was applied to make a solid pellet. FTIR spectrometer (Perkin Elmer system 2000 FTIR, Waltham, Ma., USA) was used to obtain the absorption spectra at a resolution of 2 cm^{-1} and averaging 128 scans.

Regularized Method of Deconvolution. The theory of the regularized method of deconvolution has been described in detail elsewhere.^{33,34} The deconvolution method was applied to the measured $\text{Re } D$ ellipsometry data. The deconvolution parameters - the Lorentzian contour half-width (W_L) and the passband value of the deconvolution filter (L) for the ellipsometry spectra - were set for the various spectral regions as follows: $W_L = 16.0\text{-}17.0$ cm^{-1} (1800-1700, 1600-1000 cm^{-1}), $W_L = 20\text{-}22$ cm^{-1} (1700-1600 cm^{-1}), and $L = 0.12$ cm. For the IR absorption spectrum in the 1700-1600 cm^{-1} range, W_L was set 26 cm^{-1} and L was 0.1 cm. These values of the deconvolution parameters were chosen to provide the highest resolution enhancement in the experimental spectra at the given signal-to-noise ratio. The reliability of the results of deconvolution was confirmed by coincidence of the deconvolved spectra of several samples of the same compounds.

RESULTS AND DISCUSSION

Visible Ellipsometry of Mucin Layers Adsorbed on Polymer Surfaces. Spectroscopic ellipsometry in visible range was used to examine the thicknesses of adsorbed mucin layers on both the PAA-*b*-PMMA copolymer and PMMA surfaces on Si substrates. While PMMA is relatively hydrophobic, the PAA-*b*-PMMA copolymer is amphiphilic^{35,36} owing to the presence of a hydrophilic poly(acrylic acid) block.

Figure 1 shows, as an example, the ellipsometry spectra obtained from mucin layers adsorbed at a pH of 3 and 10 onto a PAA-*b*-PMMA film in comparison to the original copolymer film. There are obvious differences in the spectra that are attributed to the

adsorption of mucin, and an effect of pH is also readily apparent in the spectra. (In all cases, the samples had been dried in a desiccator prior to the measurement to ensure a fair comparison.)

Analysis of the ellipsometry data has been performed with the use of an effective medium approximation (EMA).³⁷ This model allows the calculation of the refractive index, n , of a composite of one phase dispersed in another. In our case, the mucin coatings were described as a composite of dense mucin and air voids. The refractive index therefore provides an indication of the void content (or density). The refractive index of dense (void-free) mucin was obtained from an independent measurement on a thick, dry layer (ca. 200 nm). The dependence of n on the wavelength, λ , was described using the Cauchy equation, $n = A + B/\lambda^2$, where A was found experimentally to be 1.61 and B found to be $0.001 \mu\text{m}^2$.

The ellipsometry spectra were modeled using a standard slab model^{24,28,30} under the assumption of the isotropy of the optical properties of the polymer and mucin films. The thicknesses and refractive indices of the silicon's native oxide layer and the polymer films were obtained in preliminary measurements. After the mucin adsorption, the model consisted of a mucin layer of unknown thickness, the polymer film, the native oxide layer, and silicon. Note that the application of a similar multilayer optical model for the evaluation of ellipsometry spectra of protein layers adsorbed on polymer surfaces, as shown earlier,^{38,39} provides sufficiently reliable characterization and good resolution.

The quantitative results of data modeling (Table I) reveals differences in thickness in the very thin layers of biomolecules. Table I indicates that the thickness of adsorbed mucin layers on the more hydrophobic PMMA surface is in the range expected for a molecular monolayer coating (about 4 nm), and which is consistent with the data reported by others.⁶ The thickness of BSM layers on PMMA does not vary with pH. (The reported thickness values are an average for the layer.)

At all pH values, however, the thickness of BSM layers adsorbed on PAA-*b*-PMMA is greater than found on PMMA. Furthermore, unlike the case for PMMA, the average layer thickness on the PAA-*b*-PMMA is strongly dependent on pH. At a pH of 3, the mucin thickness is more than double that at higher pH values. There is not any significant difference in the BSM layers thickness when adsorbed at pH values of 7 and 10.

The values obtained for the void fractions rely on the assumption that a thick mucin layer is fully dense, but in any case, the data in Table I clearly indicate trends in the layer densities. The analysis of the ellipsometry data shows that the percentage of voids in the BSM coating on PAA-*b*-PMMA is the smallest at acidic pH, and slightly increases under neutral conditions

(Table I). From this analysis, it can be concluded that the most complete mucin coverage on the PAA-*b*-PMMA surface is obtained at a pH of 3.

On the basis of previous studies of mucin adhesion on PAA,^{3,7,8} we suspect that the observed increase in the thickness of adsorbed BSM layers on the copolymer surface at a pH of 3 can be explained by an increase in the proportion of protonated carboxyl (COOH) groups of mucin and PAA able to form hydrogen bonds. However, details of the particular hydrogen bonding mechanism, and whether the mucin's amide groups are contributing, cannot be obtained from these results alone, which prompted IR ellipsometry analysis.

Proteins are known to undergo conformational changes and re-orientation upon adsorption on various surfaces.³⁸⁻⁴³ These changes are influenced by the hydrophobicity of the surface, charge, protein concentration, pH and various other chemical and physical factors. In particular, it was revealed^{42,43} that the protein aggregation at solid interfaces correlates with an increase in the content of β -sheets in the secondary structure. The extent of conformational changes of some proteins was found⁴¹⁻⁴³ to be dependent on the amount of adsorption. However, unlike the situation for proteins, there are scarce reports in the literature^{17,18} about the secondary structure of the protein backbone of BSM adsorbed on polymer surfaces. However, IR ellipsometry is shown hereafter to determine the conformational changes caused by the adsorption of BSM on PAA-*b*-PMMA surface.

In investigating the adhesion and conformational changes of mucin on an amphiphilic diblock copolymer surface we analysed $\tan\Psi$ IR ellipsometry spectra of BSM/PAA-*b*-PMMA double layers. The ellipsometry spectra of mucin layers and copolymer films on gold were used as a reference to reveal characteristic changes in the spectra of bilayers due to adhesion.

IR Ellipsometry Spectra of Mucin Layers and Copolymer Films. The IR ellipsometry spectra of mucin layers on gold (Fig. 2a) are consistent with what is known about the structure of mucin. The spectra are characterized by the presence of Amide I (1657 cm^{-1}), Amide II (1540 cm^{-1}), and Amide III (1240 cm^{-1}) bands and by several components in the $1200\text{-}1000\text{ cm}^{-1}$ range, which mainly originate from the carbohydrate side chains of mucin.^{10,44-46}

The pH of the mucin solution is found to affect the spectra of the layers that are deposited from them. Some characteristic differences are observed between the spectra of mucin layers deposited at a pH of 3 and 7 (Fig. 2a). Specifically, increasing the pH value from 3 to 7 leads to a decrease in the intensity of the carbonyl band ($\nu(\text{C=O})$) near 1720 cm^{-1} , which is attributed to the COOH groups of the mucin's sialic acid.⁴⁶ Together with this, an increase in the absorption near 1556 and 1400 cm^{-1} corresponding, respectively, to the antisymmetric $\nu_a(\text{COO}^-)$ and

symmetric $\nu_s(\text{COO}^-)$ stretching vibrations of carboxylate groups is observed with increasing pH.

The original ellipsometry spectrum of the PAA-*b*-PMMA copolymer film shows the presence of a characteristic carbonyl band at 1732 cm^{-1} , CH_2 deformation at 1450 cm^{-1} , and the broad absorption bands in the $1300 - 1150\text{ cm}^{-1}$ range, mainly related to the CO and CC stretching vibrations of the copolymer's side groups (Fig. 2b).⁴⁷⁻⁴⁹

SPECTROSCOPIC EVIDENCE FOR HYDROGEN BOND FORMATION IN THE BSM/PAA-*b*-PMMA BILAYER

Carbonyl Stretching Region. Bilayers of mucin deposited onto copolymer films on gold were used to gain insight into the bonding mechanism between the two substances and conformational changes induced by their interactions. It is particularly useful to compare the spectra from the bilayer to the “sum spectra” obtained through the mathematical combination of the relevant spectra of the individual layers. Because of the relatively weak contribution of the $\nu(\text{C=O})$ band of carboxyl groups of mucin near 1720 cm^{-1} in comparison to the band of the copolymer, the sum spectra in the carbonyl stretching range are virtually identical to each other at the two pH values (Figs. 2c and 3a). In the original experimental spectra of mucin/copolymer bilayers, a low-frequency shift and a change in the shape of the $\nu(\text{C=O})$ band of the copolymer is observed when comparing to the sum spectra (Fig. 3a).

Further important information is provided by the deconvolution of the spectra (Fig. 3b). It is revealed that the asymmetric carbonyl band of the copolymer consists of two components with different intensities. The component at 1736 cm^{-1} is attributed to the $\nu(\text{C=O})$ stretching vibrations of “free” (i.e. non-hydrogen-bonded) ester and carboxyl groups in the PMMA and PAA units of the copolymer, respectively. On the other hand, the component at 1712 cm^{-1} is known to be a characteristic of the hydrogen-bonded COOH groups in PAA.^{47,48} There are marked differences in the deconvolved spectra of the bilayers in comparison to the sum of the spectra of the single films. In particular, there is a shift towards lower frequencies and a broadening of the band corresponding to the hydrogen-bonded C=O groups of the copolymer at 1712 cm^{-1} (Fig. 3b). This fact is indicative of the formation of hydrogen bonds between the copolymer and mucin macromolecules.⁴⁸ It is worth noting that the band of the “free” C=O groups is also shifted in the deconvolved spectra of bilayers. This result could reflect changes in the chemical environment around the carbonyl groups of the copolymer because of entanglements with mucin chains.

Mucins in aqueous solution are known to have a “bottle-brush” structure with hydrophilic

carbohydrate branch chains exposed at the outermost surface.³ Therefore carboxyl groups in the mucin side chains are considered to be the most probable sites for hydrogen bonding upon mucoadhesion. Furthermore, it has been shown elsewhere that the surface rearrangement of amphiphilic block copolymers also occurs when in contact with water.^{35,36} Hydrophilic blocks tend to locate at the surface, whereas hydrophobic segments are buried under the surface. It follows that upon exposure of the mucin solution, the surface of PAA-*b*-PMMA copolymer film will be enriched with COOH groups of the hydrophilic PAA block. Hence, the formation of hydrogen bonds is favored in the double-layer system via the COOH groups of both the mucin and the copolymer. Figure 4 shows the proposed types of hydrogen bonding.

Some differences between the deconvolved spectra of the bilayers at pH values of 3 and 7 can be seen in Fig. 3b, namely, the contribution of hydrogen-bonded C=O groups to the complex $\nu(\text{C=O})$ band contour is lower at a pH of 7 in comparison to a pH of 3. This result suggests the participation of terminal COOH groups of sialic acid in hydrogen-bonding interactions during mucoadhesion. It worth noting that the complex structure of the $\nu(\text{C=O})$ band in the spectra of bilayers prevents reliable quantitative estimate of the extent of hydrogen bonding of mucin and copolymer macromolecules at different pH values.

It is well-known^{3,8} that at a pH of 7 most of the carboxyl groups of sialic acid (with a pKa of 2.6) are ionized and consequently are not available for hydrogen bonding. Likewise, the surface properties of amphiphilic copolymer films have been shown to be greatly influenced by pH.^{35,36} At pH values higher than the pKa of 4 to 5,9 deprotonation of the COOH groups of PAA blocks at the interface occurs, which leads to a electrostatic repulsion between negatively-charged PAA and mucin groups. Due to the repulsion of uncoiled polymer chains, the density of chain packing decreases, which can result in reducing interdiffusion and molecular entanglements.⁸ In accord with this concept, a more pronounced low-frequency shift at a pH of 7 for the band of “free” C=O groups at 1736 cm^{-1} (Fig. 3b) can be attributed to the greater changes in the structure of the copolymer chain packing because of the strong repulsion of negatively-charged, uncoiled PAA blocks.

Thus, the IR ellipsometry results confirm that the considerable increase in the thickness of the adsorbed mucin layers at pH 3, as compared to pH 7, revealed by visible ellipsometry, can be explained by a larger extent of hydrogen bonding between mucin and copolymer macromolecules. Interestingly, a similar tendency for the adsorption of a protein (bovine serum albumin) on a PAA brush layer to decrease at pH values higher than 6 has been revealed elsewhere.⁵⁰

It is worth noting that, according to visible ellipsomery data, the average thickness of

mucin layer adsorbed on the copolymer surface at pH 7 and 10, when the carboxyl groups are ionized, is two times larger than that on PMMA. This observation can be attributed to the fact that introducing PAA blocks increases interdiffusion at the mucin/copolymer interface and the extent of molecular chain entanglements,^{8,9} which results in more mucin adsorption on PAA-*b*-PMMA copolymer surfaces, as compared to PMMA.

1350-1000 cm⁻¹ Region. In the original IR ellipsometry spectrum of copolymer in the 1300-1150 cm⁻¹ range, as already mentioned, a broad contour with maxima at about 1256 and 1192 cm⁻¹ is observed (Fig. 2b). The vibrational bands in this region can be mainly assigned to the combined CO and CC stretching vibrations in the copolymer's ester and carboxyl side groups.⁴⁷⁻⁴⁹ These bands are known to be very sensitive both to conformational changes in polymer macromolecules and to hydrogen bonding.^{47,48}

With the application of the deconvolution method, the fine structure of the complex copolymer bands in 1300-1150 cm⁻¹ range has been revealed (Fig. 5b). The existence of four additional new components has been established (1292, 1271, 1167, and 1157 cm⁻¹). Multiplicity of the bands in this range can be related to different conformers of side groups in copolymer macromolecules, as has been reported earlier for PMMA.⁴⁹ According to the literature for PMMA,⁴⁹ in the 1300-1220 cm⁻¹ range, the absorption bands mainly arise from antisymmetric $\nu_a(\text{C-C-O})$ stretching vibrations of ester groups in the *trans*- and *cis*- conformations, whereas in the 1220-1150 cm⁻¹ range, the bands are associated with $\nu_a(\text{C-O-C})$ vibrational modes of different rotational isomers of ester groups of the PMMA block. Moreover, CO stretching vibrations coupled with OH in-plane bending of carboxyl groups of PAA segments contribute greatly to the absorption in the given intervals.^{47,48}

In Fig. 5 it is seen that most of the bands of the copolymer in the 1350-1150 cm⁻¹ range appear in the sum spectra. Comparing the experimental and the sum spectra, pronounced differences are observed in the 1300-1150 cm⁻¹ range in the deconvolved spectra (Fig. 5c) that were less apparent in the original spectra (Fig. 2c). Thus, a redistribution of the relative intensities of the bands near 1271/1250 cm⁻¹ and 1194/1159 cm⁻¹ together with a decrease of the 1292 cm⁻¹ component are all observed in the experimental spectra but not in the sum spectra. In accordance with the assignments of the bands in this range, the observed spectral differences could be indicative of changes in hydrogen bonding of carboxyl groups in PAA segments caused by mucoadhesion. Furthermore, some changes in the conformational states of side groups of the copolymer induced by entanglements and interactions with the mucin macromolecules could have occurred. That is, an increase in the relative intensity of the band near 1271 cm⁻¹ can be related to a larger content of *trans* conformations of ester groups in the

PMMA block. A similar tendency for conformational changes of the side-chain ester groups has been previously reported for mixing in thin polymer double layers.²⁷

In the 1150-1000 cm^{-1} region, the bands originating from the carbohydrate residues of mucin contribute significantly to the sum spectra (Fig. 5a,c). The spectral alterations in the given range are consistent with those observed in the above regions and indicate the formation of hydrogen bonds between carboxyl groups of mucin and copolymer macromolecules.

Effects of pH are also observed in the spectral changes in the 1350-1000 cm^{-1} range. Some differences between the spectra of bilayers at the two pH values are found, because of vibrations involving COOH groups of PAA and mucin. This observation confirms the conclusions drawn above regarding the formation of hydrogen bonding in the bilayer via COOH groups of copolymer and mucin (Fig. 4).

As mentioned in the Introduction, according to IR spectroscopy data from mucin/PAA mixtures,¹⁰ hydrogen bonds can be formed between carboxyl groups of PAA and amide groups of mucin. However, our analysis here does not support this mechanism. If the mucin's amide groups were solely contributing to the hydrogen bonding, then changes in the bands related to the vibrations of COOH groups of mucin would not have been observed.

SECONDARY STRUCTURE OF MUCIN LAYERS ON GOLD AND COPOLYMER SURFACES

Amide I Region. The amide I band is mainly the result of stretching vibrations of hydrogen-bonded C=O groups in the protein backbone of mucin with some contribution from the stretching vibrations of CN groups.^{10,18} The band is well-known to be very sensitive to the conformational changes in the secondary structure of protein molecules and to the degree of hydrogen bonding.^{18,51-53} Study of the amide I band therefore provides important insights to any conformational changes and bonding that might occur during mucoadhesion.

The protein backbone of mucin is arranged into several distinct regions.¹ Glycosylated peptide blocks are rich in serine, threonine and proline, whereas unbranched peptide blocks or regions with little glycosylation have a high content of cysteine. The domains that are rich in cysteine are believed to be globular.^{1,2}

The mucin macromolecules in solution are shown to have a mainly random-coil structure at $\text{pH} \geq 4$ and anisotropic extended conformation at $\text{pH} < 4$.¹¹ It has been found elsewhere that the secondary structure of BSM in the solid state differs from that in solution.¹⁸ In the original absorption IR spectrum of the mucin powder (Fig. 6a, curve 1) the amide I band is observed near 1650 cm^{-1} . With the use of the deconvolution method, a number of individual components

of the given band have been revealed (Fig. 6a, curve 2). According to the literature,^{43,51-53} the observed components could reflect the presence of β -turns (1661 cm^{-1}), α -helix (1649 cm^{-1}), random coil (1641 cm^{-1}) and β -sheets (1694 and 1632 cm^{-1}) in the secondary structure of the protein backbone. The given secondary structure elements are consistent with the complex amino acid composition of polypeptide chains of mucin.¹⁻³

Comparison of the results of deconvolution of the amide I band in the dry bulk powder and in the layers (Fig. 6) shows that upon mucin adsorption, significant conformational changes of the protein backbone occur. The amide I band observed in the original ellipsometry spectra of the mucin layers on gold (Fig. 6b, curve 1) is shifted to 1657 cm^{-1} , as compared to the absorption IR spectrum of the mucin powder, which indicate some changes in the secondary structure of the protein backbone of mucin upon adsorption on the gold surface. For the BSM on gold, the results of deconvolution suggest predominantly an α -helix secondary structure of the protein backbone (1657 cm^{-1}) with some noticeable contribution of β -sheets (1632 cm^{-1}) and β -turns (broadly around 1670 cm^{-1}).^{43,51-53} Some differences observed in the 1675 - 1660 cm^{-1} range for the amide I band of the mucin layers on gold could reflect a larger contribution of β -turns in the secondary structure at a pH of 7 compared to the pH of 3 (Fig. 6b, curve 1).

The fine structure of the amide I band of mucin layers on gold, revealed as a result of deconvolution, is compared to that of mucin on the copolymer surface in Fig. 6b. In the deconvolved spectra of the mucin on the copolymer surface at the different pH values (Fig. 6b, curve 2), alterations in the relative intensities of the individual components of complex amide I band, as compared to the mucin layers on gold, are revealed. The band associated with β -sheets (around 1630 cm^{-1}) is stronger for the bilayers compared to the mucin layers on the more hydrophobic gold surface. Furthermore, the maximum of the main component corresponding to α -helix secondary structure is shifted towards lower frequencies in the case of the bilayers. This observation indicates that the adhesion of BSM on the PAA-*b*-PMMA is accompanied by changes in the secondary structure of the protein backbone. Results of deconvolution indicate an increase of the proportion of β -sheets together with a decrease in the amount of β -turns. This suggests a more unfolded rigid structure of the protein backbone of mucin macromolecules after their adhesion on the copolymer surface. It has been shown previously⁴⁰⁻⁴² that the increase in the amount of β -structures can be indicative of the aggregation of proteins at the interface and of the formation of a stronger network. In Fig. 6b it is seen that the proportion of β -sheets is slightly higher at a pH of 3 as compared to a pH of 7. It follows that the larger amount of the BSM adsorbed on the copolymer surface at a pH 3, revealed by visible

ellipsometry, correlates with the higher content of β -sheets.

It is worth noting that an increase in the content of β -sheets in secondary structure has also been reported earlier for BSM irreversibly adsorbed on hydrogel surfaces.¹⁸ In other works on protein adsorption⁴¹⁻⁴³ it was found that a greater amount of adsorbed protein macromolecules leads to greater changes in their secondary structure. Our results reveal a similar correlation between the conformational changes of the protein backbone of mucin molecules and the amount of mucin adsorbed onto the copolymer surface.

Amide II Region. The amide II band (Fig. 2a,7a) is mainly related to an in-plane NH bending coupled with C-N and C-C stretching vibrations.^{18,51-53} It has been shown¹⁸ that upon changes in conformation and hydrogen bonding of the protein backbone of mucin, one can observe a change in the halfwidth of this band and a shift in the direction opposite to that observed for the amide I band.

In Fig. 7a it is seen that Amide II band of the mucin layers (1540 cm^{-1}) is overlapped with the pH-sensitive component near 1556 cm^{-1} related to the antisymmetric $\nu_a(\text{COO}^-)$ stretching vibrations of the carboxylate groups.

Fig. 7c reveals significant differences between the deconvolved ellipsometry spectra of bilayers at the two different pH values. The sum spectra, on the other hand, do not show any difference with pH, indicating that changes are brought on by the bilayer formation. In the ellipsometry spectrum of the bilayer at a pH of 7, a considerable broadening of the spectral bands near 1556 and 1400 cm^{-1} occur, which might indicate changes in the electronic environment of the carboxylate groups because of the repulsion of like-charged chains of mucin and PAA. However, Fig. 7c shows that a significant broadening of the spectral contour in the Amide II region ($1580\text{-}1525\text{ cm}^{-1}$) is also observed at a pH of 3, when most of the COOH groups are protonated. This allows us to draw the conclusion that the spectral alterations revealed in the Amide II region probably reflect changes in the conformations and hydrogen bonding of the protein backbone of mucin upon adhesion.

CONCLUSIONS

Using spectroscopic ellipsometry we have determined the thickness of mucin layers adsorbed onto PAA-*b*-PMMA copolymer and PMMA surfaces under acidic and neutral pH conditions. The average thickness of the mucin layers on the copolymer surface – but not on PMMA - was determined to be pH dependent. The mucin thickness is more than two times greater when deposited at a pH of 3 compared to neutral conditions. The most complete mucin coating was obtained on the PAA-*b*-PMMA copolymer surface at this pH, and this finding can be exploited when preparing coatings to reduce bacterial adhesion.

With the use of IR ellipsometry and the regularized method of deconvolution, spectroscopic evidence for the formation of hydrogen bonds between the copolymer and mucin macromolecules in the BSM/copolymer double-layer system on gold has been found. The IR ellipsometry data, in agreement with the visible ellipsometry analysis, reveal the pH dependence of adhesion of mucin to the copolymer surface. There is an increase in the amount of hydrogen-bonded carboxyl groups in mucin deposited at a pH of 3. There is no evidence that the amide groups of the mucin participate in hydrogen bonding with the COOH groups of PAA, although this mechanism has been proposed in the literature.

Using the regularized method of deconvolution, it has been found that the process of adhesion of mucin on the PAA-*b*-PMMA surface, in comparison to the more hydrophobic gold surface, is accompanied by changes in the secondary structure of the protein backbone. The proportion of β -sheets therewith increases, which indicates a more unfolded, aggregated structure of the mucin macromolecules after adhesion. The conformational changes in the protein backbone of mucin are found to be pH dependent. A slightly higher proportion of β -sheets in the secondary structure of the protein backbone at a pH of 3 has been revealed, which correlates with the greater amount of mucin adsorbed on the copolymer surface at this pH. The deconvolution results of IR ellipsometry spectra allow us to conclude that some changes in the conformational states of the side groups of the copolymer, caused by entanglements and interactions with the mucin macromolecules, occur upon adhesion.

This work is the first detailed use of IR ellipsometry to determine the conformational structure of biomacromolecules and adhesive interactions in double layers. We have shown that spectroscopic ellipsometry is an effective tool for studies of bioadhesion in multi-layered systems. The use of the deconvolution method enables a more reliable identification of the structural changes and molecular interactions due to the adhesion of biomolecules to polymer surfaces.

ACKNOWLEDGMENTS

We are grateful to the Ministry of Higher Education of Saudi Arabia for a Ph.D scholarship for I.A.B., who performed all the experiments reported here, and to the Royal Society for a travel grant to N.A.N. to visit the University of Surrey. We acknowledge assistance from Dr. G. Cavalli (University of Surrey) in the IR spectroscopy experiments.

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585

FIGURE CAPTIONS

Fig. 1. Ellipsometry spectra, obtained at an angle-of-incidence of 72° , for PAA-*b*-PMMA films on Si substrates before and after the adsorption of bovine submaxillary mucin at pH values of 3 and 10, as indicated.

Fig. 2. Re *D* spectra obtained from IR ellipsometry in the $1800\text{-}1000\text{ cm}^{-1}$ region for (a) the mucin layers on Au at a pH of 3 (solid line) and 7 (dashed line), (b) the PAA-*b*-PMMA copolymer film on Au, and (c) the mucin/PAA-*b*-PMMA bilayers likewise at two pH values. Curve 1 shows the experimental data, whereas curve 2 represents the sum of the spectra of the mucin layer and PAA-*b*-PMMA copolymer film. The vertical lines show the division of the spectra into four regions that will be considered individually. Arrows indicate trends in the intensity changes of pH-sensitive bands in the mucin spectra.

Fig. 3. Re *D* spectra in the carbonyl stretching region of the mucin/PAA-*b*-PMMA bilayers on Au at a pH of 3 (solid line) and 7 (dashed line). The original spectra are in (a), and the results of their deconvolution are in (b). In both (a) and (b), curve 1 shows the experimental spectra and curve 2 represents the sum of the spectra of the mucin layer and the PAA-*b*-PMMA copolymer film.

Fig. 4. Proposed types of hydrogen bonding between the carboxyl groups of PAA-*b*-PMMA copolymer and sialic acid residues of the mucin, illustrating (a) cyclic dimers and (b) face-on fashion bonding.

Fig. 5. Deconvolved Re *D* spectra in the $1350\text{-}1000\text{ cm}^{-1}$ region of (a) the mucin layers on Au at a pH of 3 (solid line) and 7 (dashed line), (b) the PAA-*b*-PMMA block copolymer film and (c) the mucin/PAA-*b*-PMMA bilayers also at two different pH values. Curve 1 shows the experimental data, whereas curve 2 represents the sum of the spectra of the mucin layer and the PAA-*b*-PMMA copolymer film. Arrows indicate trends in the intensity changes of the bands in the bilayer spectra caused by adhesion.

Fig. 6. A comparison of spectra obtained in the Amide I region. (a) IR absorption spectrum obtained from a dry mucin powder, comparing (1) the original spectra and (2) the result of its deconvolution. (b) Deconvolved Re *D* spectra obtained from (1) a mucin layer on Au and from (2) mucin/PAA-*b*-PMMA bilayers, all at a pH of 3 and 7, as indicated.

Fig. 7. Deconvolved Re *D* spectra in the Amide II and carboxylate stretching region ($1600\text{-}1350\text{ cm}^{-1}$) of (a) the mucin layers on Au at a pH of 3 (solid line) and 7 (dashed line), (b) the PAA-*b*-PMMA copolymer film, and (c) the mucin/PAA-*b*-PMMA bilayers also at two

619 different pH values. Curve 1 shows the experimental data, whereas curve 2 represents the sum
620 of the spectra of the mucin layer and the PAA-*b*-PMMA copolymer film.
621

622 **Table I. Adsorbed Mucin Layers' Average**
623 **Thicknesses and Void Percentages Estimated**
624 **from Visible Ellipsometry Data Using the**
625 **Effective Medium Approximation.**

Polymer	pH	Mucin layer thickness (nm)	Voids (vol. %)
PMMA	10	4.1 ±1.0	17.3±1.6
	7	3.9±0.8	18.4±2.0
	3	4.5±1.2	22.0±2.6
PAA- <i>b</i> -PMMA	10	7.6±1.3	15.1±1.5
	7	8.1±1.0	13.5±1.5
	3	17.2±1.5	9.0±1.0

626

Figure 1

Figure 2

Figure 3

(a)

(b)

Figure 4

Figure 5

Figure 6

Figure 7