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Annual Progress Report

CHEMICAL INTERACTIONS BETWEEN PROTEIN MOLECULES AND
POLYMER MEMBRANE MATERIALS

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INTRODUCTION

During the past year, we have experienced several unanticipated problems that have caused unexpected delays in the research. These have included (i) an unexpectedly long delay for the new student to learn how to use the Surface Force Apparatus (SFA), (ii) difficulty in automating the measurement taking process, and (iii) inordinate delays in communicating with ex-student Frederic Pincet, who is now at Ecole Normale Supérieure, Paris, France, on our two manuscripts. With respect to operating the SFA, our new doctoral student, Mr. Jeffrey Koehler, joined the project after our previous student, Mr. Frederic Pincet left for France. This unfortunately resulted in an unexpectedly long delay in returning to full operation with the SFA. Mr. Koehler made a special trip to Eastman Kodak Company to learn how to operate the SFA from Dr. Ravi Sharma and Dr. Jonathan Wood. During the past few months he has made reproducible and publishable measurements with the SFA (see later). During the past two years we found it extremely time consuming to take the normalized force-distance data manually and decided to hire an a company (Advanced Idea Mechanics, Troy, NY) to automate our data taking system so that the measurements would flow directly from the SFA to our 386sx personal computer for direct analysis. Unfortunately, after 12 weeks of down-time, the company was unable to do the job. We then hired Mr. Les String, an electronics technician from General Electric Co. Corporate R & D Center, Schenectady, NY, who was able to automate the system. However, it took him 8 weeks to do so! Evidently, the signals from the micrometer read-out and the multimeter were incompatible with that required by the computer, so that a special interface needed to be built. Mr. F. Pincet worked on the project from December 1990 to February, 1992 and then returned to France to complete his doctorate in Physics. He had to study for doctoral candidacy exams and could not assist in writing the manuscripts into this year. This caused a delay in preparing the manuscripts.

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In spite of these difficulties, we have successfully solved all of them. The SFA is now fully operable and data collection is automatic. Also, with Mr. Pincet's assistance we have prepared the two draft manuscripts that will be submitted for publication shortly. Copies of these drafts are attached in the Appendix.

During the past several months, Dr. Jonathan Wood, formerly of Imperial College, London, UK, and recently of Kodak Research and Development Laboratories, Rochester NY joined the project. With his extensive background in using the SFA, he assisted in upgrading our system and helped get the experiments underway. Thus, we have directly measured the intermolecular forces between two layers of hen egg lysozyme on mica using the SFA. We have also begun a complementary study using circular dichroism to determine the changes in protein structure during protein adsorption onto 20 nm modified polystyrene beads and 250 nm silica particles. de Gennes' scaling theory has proved useful in predicting the adsorption of denatured proteins onto mica.

In summary, this report period began with difficulty but has ended with success. We look forward to continuing this study and plan (see our proposal) to use some innovative methods to prepare stable hydrophobic and hydrophilic surfaces for use in the Surface Forces Apparatus (SFA). This will include photochemical modification and low temperature plasma treatment of poly(arylsulfone) films and membranes. A Langmuir-Blodgett method for depositing a hydrophobic layer of condensed octadecyltrimethoxysilane (OTS) onto the mica surface will also be used. *Hydrophilic* monomers such as 2-hydroxyethyl methacrylate (HEMA) and glycidyl methacrylate (GlyMA) will be attached to pretreated polymeric surfaces and tested for their propensity to adsorb proteins in the SFA using cross-mica cylinders. Using the OTS as a base, interfacial polymerization will be conducted on top of its hydrophobic surface to form polyamide films. A variety of *hydrophobic* silane monomers will be attached to poly(arylsulfone) after exposure to low temperature helium/water plasma. A second diagnostic tool, circular dichroism, will be used to determine the influence of surface polarity, as measured by receding contact angle measurements, on the molecular structure of lysozyme during adsorption onto small particles. We also plan to test the scaling theory of de Gennes et al and the discrete lattice model of Scheutjens and Fleer (SF) in predicting the behavior of protein adsorption.

RESULTS

(a) Summary of Previous Research Progress

The main results obtained during the previous period of this study were (1) the adsorption process of human serum albumin (HSA) behaved differently at the pI of the protein as compared with other pH values, (2) the cellulose acetate (CA) films, prepared by dipping, were smooth and no polymeric chains extended into the

solution from the membrane, (3) CA membranes tended to stabilize the denatured state of HSA and ribonuclease A (RNase A) at low pH, (4) HSA is more easily denatured than RNase A in the presence of a CA film emphasizing their relative stabilities, and (5) hydrophobic interactions played an important role between proteins and dimethyldioctadecylammonium bromide (DDOA) at the protein pI. In fact, previous work has shown that proteins were susceptible to denaturation at low pH values [3], and that RNase A was more conformationally stable than HSA during adsorption, i.e., HSA was denatured more easily at interfaces and was more sensitive to pH changes than RNase A [4]. It is also at the pI that more intense fouling occurs with hydrophobic membranes. Thus, these measurements have confirmed, at the molecular level, what has been known for membrane processing of macromolecular solutions and have emphasized the important part played by the membrane in the denaturation process [5].

We have also compared the behavior of natures and denatured adsorbed HSA on mica [2]. Using the surface force apparatus and the scaling theory of De Gennes [6,7], we showed that unfolded (denatured) HSA molecules behave essentially as a linear polymer while adsorbed onto molecularly smooth mica in 10^{-2} M KCl solution at pH 2 in the presence of 10 M urea. This is the first confirmation on a molecular scale that unfolded (and adsorbed) protein molecules can indeed behave as non-interacting linear polymer molecules and that the interactions between the adsorbed molecules, the adsorbent and the solvent determine their molecular state.

Besides surfactants (DDOA), we and others have not previously obtained direct measurements between proteins and *hydrophobic* polymeric surfaces that typify many commercial synthetic membranes. Using low temperature plasma surface modification techniques together with LB and OTS, we plan to prepare such hydrophobic surfaces on mica so that molecular interactions with a model protein, lysozyme, can be obtained in the SFA.

(b) Research Progress During this Report Period

(i) Choice of model protein

Most of our earlier work was with the protein ribonuclease A (RNase A), mainly because it is a very stable protein and is known not to easily denature upon adsorption. This was an important criteria with our first surface forces apparatus measurements. However, we have since decided to choose a "softer" protein such as hen egg lysozyme (EC 3.2.1.17, M.W. approx. 14,400) that more closely represents foulants commonly observed with pressure-driven membrane processes in food, biotechnology and waste water treatment. The reasons for choosing lysozyme (Lyz) as the model protein are: (i) it is a well-studied and characterized protein of relatively small size suitable for membrane permeation experiments, (ii) enzyme activity via the substrate *Micrococcus lysodeikticus* is easily assayed for to determine its structural

stability, and (iii) it is a relatively flexible protein with an intermediate value of the adiabatic compressibility at 25°C of $\beta_s = 4.67 \times 10^{-12} \text{ cm}^2/\text{dyne}$ [between very hard cytochrome C, $\beta_s = 0.066 \times 10^{-12} \text{ cm}^2/\text{dyne}$, relatively hard RNase A of $\beta_s = 1.12 \times 10^{-12} \text{ cm}^2/\text{dyne}$, and very soft bovine serum albumin, $\beta_s = 10.5 \times 10^{-12} \text{ cm}^2/\text{dyne}$] [8,9].

(ii) Protein-protein and protein-mica interactions

Results obtained during this project period, have allowed us to obtain the intermolecular forces between adsorbed lysozyme layers as a function of time and at different pH values. The normalized force-distance curves for Lyz-Lyz interactions at pH 3.2 and 4.73 for compression are presented in Figs. 1 & 2, respectively. The dashed and solid lines are for the classical DLVO theory, which appears to describe the data fairly well with reasonable fitting parameters (i.e. surface potential 50 mV, and $[C] = 6 \times 10^{-4} \text{ M}$ at pH 3.2 and 30 mV $[C] = 4 \times 10^{-4} \text{ M}$ at pH 4.73, respectively). Upon decompression, adhesion forces are observed and decrease roughly with the square root of adsorption time, suggesting a diffusion-driven process (Fig. 3).

(iii) Protein-polymer and protein-surfactant interactions

Direct measurements between selected proteins such as RNase A and HSA and two different synthetic surfaces were obtained [1]. The surfaces include a widely used synthetic membrane material, hydrophilic cellulose acetate (CA), and a hydrophobic dimethyldioctadecylammonium bromide (DDOA) surfactant. Attempts to measure the interactions between proteins and hydrophobic polymeric membranes such as those made from polysulfone were unsuccessful due to the difficulty of attaching molecularly smooth hydrophobic films to mica. In our renewal proposal, we offer a solution to this problem by rendering the mica surface non-polar through treatment with a water plasma and exposure to either chlorotrimethyl silane (CTS) or a preformed polymerized Langmuir-Blodgett (LB) layer of octadecyltrimethoxysilane (OTS). With CTS treatment of mica, we have noticed an increase in the sessile contact angle for a water drop from about 31.8 to 50.8 degrees suggesting an increase in hydrophobicity of the surface. The OTS film will interact with the hydroxyl groups formed from exposure to the water plasma on the mica. On annealing, the OTS film will be anchored to the mica surface. The aligned apolar octadecyl chains will extend away from the surface rendering the mica hydrophobic. Contact angle (θ) measurements of surface modification of glass and mica using a preformed and condensed polymerized LB layer of OTS are shown in Table 1. The most significant result is that even after 2 h exposure to chloroform, the LB-OTS layer is stable and remains hydrophobic. This indicates that the LB-OTS film is attached to the solid substrates. Two possible reasons could explain the drop in θ between the glass and mica surfaces. It is possible that the mica is not completely covered with the LB-OTS layer, although this is unlikely since the transfer ratio from the LB apparatus was close to 1.0 for both surfaces. A more

likely explanation is that the LB-OTS layer is anchored more tightly to the glass than the mica as it has more hydroxyl groups on its surface. Thus, the layer could be less stable and more rough with the possibility of lateral diffusion on the mica. This could result in a lower contact angle for mica than for glass. Possible reasons for the drop in θ on exposure to chloroform are that (i) chloroform may penetrate or sorb into the film, and (ii) it may dissolve that part of the film that is not well anchored to the substrate.

(iv) Protein structure during adsorption by circular dichroism (CD)

Circular dichroism (CD) has been used extensively to measure the changes in secondary structure of proteins in aqueous solution under different conditions. The method has also been applied to protein adsorption to microscopic particles in aqueous solution, which is of interest in this study. An early approach to this type of CD measurement was to study the conformational changes in proteins before and after adsorption to polymer surfaces [10]. The readings, however, were not actually taken while the protein was adsorbed to the surface. The proteins were first desorbed and then the CD measurements were obtained. This obviously does not take into account any changes in configuration due to the desorption process.

This problem was then remedied by using microscopic particles as an adsorption medium. The particles were small enough, approximately 20-250 nm, so that there was not a significant scattering in the light intensity for the measurements. In these experiments, both silica [8,11] and polystyrene [9] particles were used along with various proteins such as ribonuclease A, myoglobin, peroxidase, hemoglobin, bovine serum albumin, cytochrome C, ovalbumin, and lysozyme. The results on silica particles showed that for soft proteins (as measured by adiabatic compressibility, β_s , at 25°C), the secondary structure changed a great deal upon adsorption, whereas, for hard proteins, the secondary structure barely changed. Upon desorption of the proteins from the surface using morpholine, some of the lost secondary structure was regained [8,11]. The dependence of adsorption concentration [9] and pH [9,11] were also probed. Similar results were obtained for the protein adsorption on the polystyrene particles [9].

We have studied the change of secondary structure of Lyz and HSA upon adsorption to silica (c.a. 20 nm diameter, 250 nm long chains) and 21 nm diameter polystyrene beads modified to exhibit either sulfate or carboxylate functional groups on the surface. The experiments were performed in pure water and in mildly buffered solutions. Using CD, we observed a higher loss of secondary structure upon adsorption of the HSA than with the Lyz. This is shown in Fig. 4 where only small differences in the spectra are seen for Lyz in free solution versus in the adsorbed state. For HSA, however, the α -helix predominant region (190-205 nm) and the β -sheet predominant region (205-230 nm) show relatively large differences when

comparing these two states. For future work, we plan to modify the surfaces of the particles using chemical techniques and then to redo the CD measurements to determine the effect of surface polarity on structural changes of the model Lyz protein. These results will nicely complement our SFA measurements.

(v) *Theoretical*

We have successfully used the discrete lattice model of Scheutjens and Fleer (SF) for predicting the adsorption behavior of ribonuclease A interaction with mica and with itself during constrained equilibrium as a function of time (1). In addition, our recent work has used the scaling theory of de Gennes to show that denatured adsorbed proteins behave like polymers (2).

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Table 1 Contact angle measurements of surface modification using a preformed polymerized Langmuir-Blodgett (LB) layer of octadecyltrimethoxysilane (OTS).

Material	Contact angle, Θ	
	Without exposure to CHCl_3	With 2 h exposure to CHCl_3
a) <u>Sessile drop method</u>^{a)}		
OTS on glass slide	103 ± 3	90 ± 4
OTS on cleaved mica	95 ± 3	86 ± 3
b) <u>Capture bubble method</u>^{b)}		
OTS on glass slide	104 ± 3	90 ± 4
OTS on cleaved mica	97 ± 3	88 ± 3

a) The contact angle is observed for a small water droplet in air.

b) The receding contact angle is observed for a small inverted air bubble in water.

Fig. 1 Normalized force versus distance of separation for mica surfaces immersed in a 50 mg/l lysozyme solution at 21.1°C and pH 3.2. The results for 5 compressions after different incubation times after adding the protein solution show: (i) Invariant behavior with time. (ii) A long-range electrostatic interaction that appears to follow classical DLVO theory (dashed line). The predicted electrolyte concentration of $6 \times 10^{-4} \text{ M}$ is very close to that obtained by measurement from the pH ($6.3 \times 10^{-4} \text{ M}$). (iii) At small separations, there is a strong repulsive steric interaction giving a minimum surface separation of 6 nm. This maybe interpreted as a side-on adsorption of the lysozyme molecules ($3 \times 3 \times 4.5 \text{ nm}$). The adhesion forces decrease with coverage and time.

Fig. 2 Normalized force versus distance of separation for mica surfaces immersed in a 50 mg/l lysozyme solution at 21.1°C and pH 4.73. The results for 4 compressions after different incubation times after adding the protein solution show: (i) invariant behavior with time, (ii) a long-range electrostatic interaction that appears to follow classical DLVO theory (dashed line). The predicted electrolyte concentration of 4×10^{-4} M is of the same order as that obtained by measurement from the pH (1.86×10^{-5} M) and the salt added with the Lyz solution (7×10^{-5} M), (iii) at small separations, there is a strong repulsive steric interaction giving a minimum surface separation of 6 nm. This may be interpreted as a side-on adsorption of the lysozyme molecules ($3 \times 3 \times 4.5$ nm).

Fig. 3 Adhesion forces on decompression for the two sets of experiments from Figs.1 & 2 shown in (a) and (b), respectively, as a function of the square root of time.

Fig. 4 Normalized circular dichroism spectra of (a) lysozyme (Lyz) and (b) human serum albumin (HSA) in free solution and adsorbed onto 20 nm silica particles at 1.33 mg/ml. 0.01 M acetate at pH 4.0 and 0.01 M phosphate at pH 7.0 was used for the Lyz and the HSA solutions, respectively.

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