Influence of Salt and pH on the Adsorption of Fibrinogen and Lysozyme to Self-Assembled Monolayers Using a Surface Plasmon Resonance Sensor

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Abstract: *This work examines protein adsorption on surfaces in an attempt to provide a fundamental understanding of the molecular-level mechanism for protein interactions with biomaterials. Carboxylic acid, amine, and hydroxyl terminated self-assembled monolayers (SAMs) are used to model negatively, positively, and hydrophilic/neutrally charged surfaces, respectively. The adsorption of fibrinogen and lysozyme on these SAM surfaces under different ionic strengths and pH values is studied using a surface plasmon resonance (SPR) sensor. It is shown that variations in ionic strength have little effect on the adsorbed amount of fibrinogen and lysozyme on these SAMs at a fixed pH value. Analysis of the effect of pH on protein adsorption at a fixed ionic strength shows that charge interactions are a significant component of protein adsorption. These studies provide insights into the role of hydrogen bonding and electrostatic interactions in protein adsorption.*

1. INTRODUCTION

The development of surfaces able to resist non-specific protein binding is a vital element in the advancement of biomedical devices. Non-specific protein binding to implanted biomedical devices induces the foreign body response. The foreign body response ultimately leads to degradation in device performance [8]. Protein adsorption is frequently used to assess the nonfouling properties of surfaces and to improve our understanding of the molecular level mechanisms in nonfouling surfaces. There are currently four molecular characteristics that are generally associated with adsorption resistant surfaces: surfaces are hydrophilic; they do not include hydrogen bond donors; they include hydrogen bond acceptors and surface charge is neutral [4].

This work aims to provide a fundamental understanding of molecular-level mechanisms for protein interactions. Carboxylic acid (COOH), amine (NH_2), and hydroxyl (OH) terminated self-assembled monolayers (SAMs) are used to model hydrophilic negatively charged, positively charged, and neutral surfaces, respectively. Fibrinogen and lysozyme surface coverage is calculated at varying ionic strengths and pH values through a surface plasmon resonance (SPR) sensor. Varying ionic strength should create a charge screening effect as the ionic solution concentration increases. When pH values change and surpass surface and protein isoelectric values changes in charge interactions will be observed.

2. MATERIALS AND METHODS

2.1 Materials

11-mercapto-1-undecanol [$HS(CH_2)_{11}OH$], 97% from Sigma Aldrich (St. Louis, MO), 11-amino-1-undecanetiol hydrochloride [$HS(CH_2)_{11}$ $NH_3^+Cl^-$] from Dojindo Molecular Technologies, Inc. (Gaithersburg, MD), 11-mercaptoundecanoic acid [$HS(CH_2)_{11}COOH$] from ProChimia (Sopot, Poland), were purchased and used as received. 0, 125, 225, 475, 725, and 975 mM phosphate buffered saline solutions (PBS, pH 7.4) were prepared and used in ionic strength variation experiments. In pH variation experiments, monobasic and dibasic salts from Sigma Aldrich (St. Louis, MO) were used to prepare 0.150 mM PBS solutions at 4.40, 5.54, 7.40, 8.36 and 9.13 pH values. Lysozyme (L6878) and fibrinogen (fraction 1 from bovine plasma) were purchased from Sigma Chemical Co. (St. Louis, MO). Ethanol (100% proof) was purchased from Sigma Aldrich (St. Louis, MO) and used as received.

2.2 Self-Assembled Monolayer (SAM) Preparation

Through e-beam evaporation in a vacuum, glass chips are coated with a 2 nm chromium film to promote the adhesion of a subsequent 55nm gold

film which serves to activate surface plasmons. Self-assembled monolayers are then formed on these gold-coated substrates. SPR chips were rinsed in ethanol and dried under a N_2 stream and placed in an UV Ozone cleaner for 30 min. The chips were then removed from the cleaner, rinsed with de-ionized water, and rinsed with ethanol. Hydroxyl terminated SAMs formed by immersing cleaned gold-coated chips in a 0.5mM ethanolic solution of $[HS(CH_2)_{11}OH]$ overnight at room temperature. Carboxylic acid and amine terminated SAMs were prepared by following previously reported procedures [6].

2.3 Protein Preparation

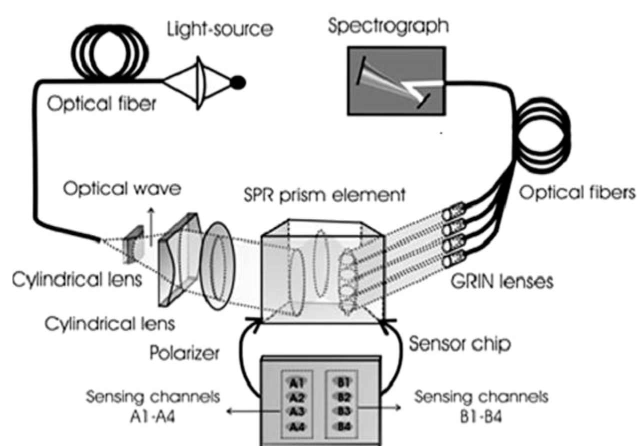
Protein stock solutions were prepared by dissolving 11.0 mg of powdered fibrinogen or lysozyme in their corresponding 1.10 ml PBS solution in a microtube and freezing. For each experiment, the solutions were thawed and the necessary amounts were placed into other microtubes to be diluted with PBS. After experiencing difficulties (protein aggregation, possibly due to temperature changes) with fibrinogen, protein stock solutions were prepared, aliquoted in microtubes, and frozen.

2.4 Surface Plasmon Resonance (SPR) sensor

A custom-built SPR sensor, whose components are illustrated in Figure 1a, was used to measure protein adsorption. The sensor's primary components are a light source, prism element, microflow system (composed of a multichannel peristaltic pump and flow cell), and a spectrophotometer [4].

The SPR sensor light source passes a polychromatic light beam through an optical prism that has been coupled to a gold-coated substrate with immersion oil. Surface plasmons are excited at the metal-dielectric interface. The light reflected then produces two angles of incidence (Figure 1b). A spectrophotometer is used to monitor wavelength changes for each of the eight sensing channels [2].

A baseline is established by flowing PBS for 5 min at a rate of 0.05 ml/min. 1.0 mg/ml fibrinogen or lysozyme in their corresponding PBS solutions are then flowed at a rate of 0.05 ml/min for 20 min. Proteins binding will then change the SPR signal. The protein adsorption changes the local refractive index and therefore the resonant energy of the surface plasmon [3]. This shift in refractive index is detected by observing the wavelength of light which couples to the surface plasmon by measuring a dip in the wavelength-spectrum of the reflected light. Finally, the surface is washed with PBS for 5-10 min at the same flow rate. Refractive index shifts are coupled to resonant wavelength a shift, which were to quantify the amount of bound protein [2].



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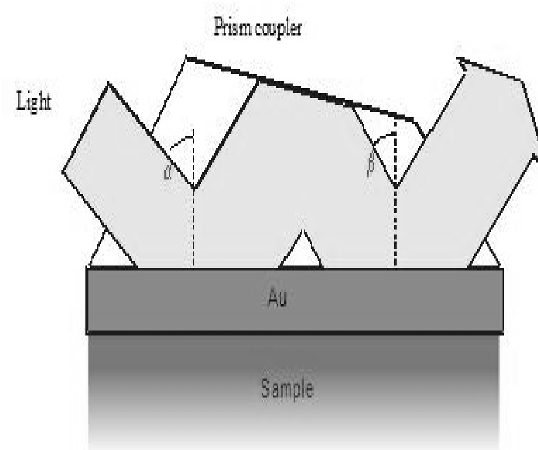

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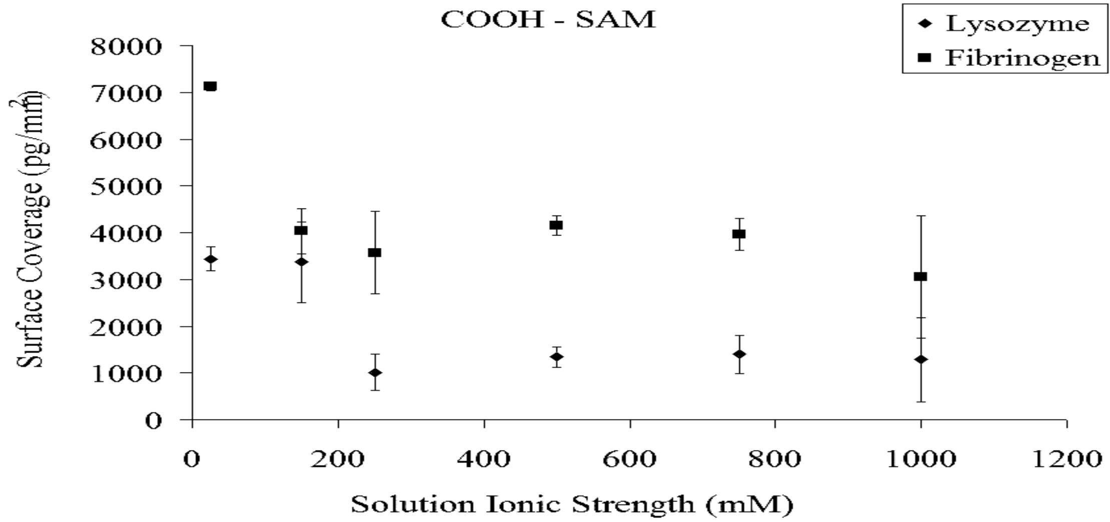
Figure 1: *(a) Schematic of an eight-channel SPR sensor; (b) wavelength division of a polychromatic light source reflected onto a gold surface at two different angles of incidence [For additional information see references 1 and 4. Image used with permission].*

2.5 Calibration of SPR sensor

The eight-channel SPR sensor uses wavemultiplexing to create two sensor regions. In order to compare results from both sensing locations, a calibration constant is required to account for the different excitation energy levels. The bulk and surface refractive index sensitivities are used to determine the calibration ration between the high and low energy surface plasmons. A calibration ratio, previously determined, of 1.92 is applied to shifts arising from low wavelengths [5].



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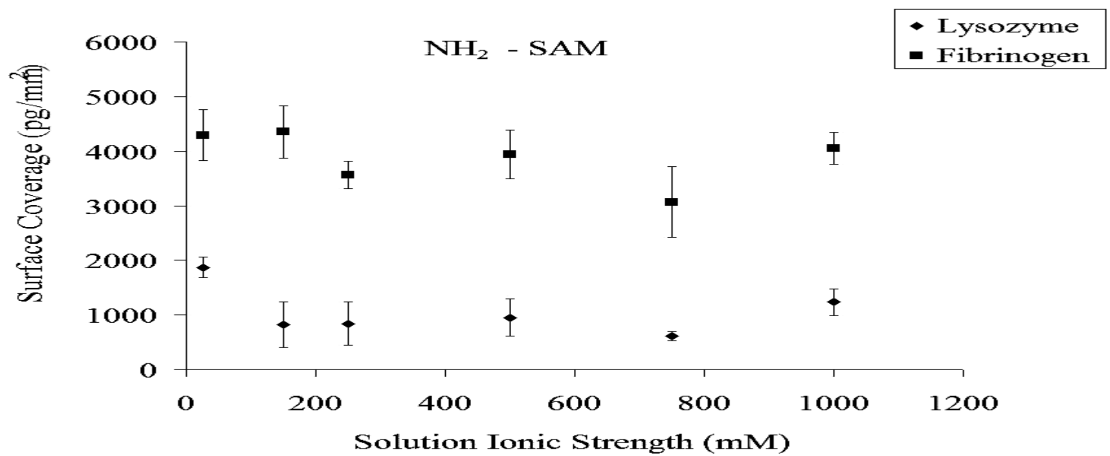

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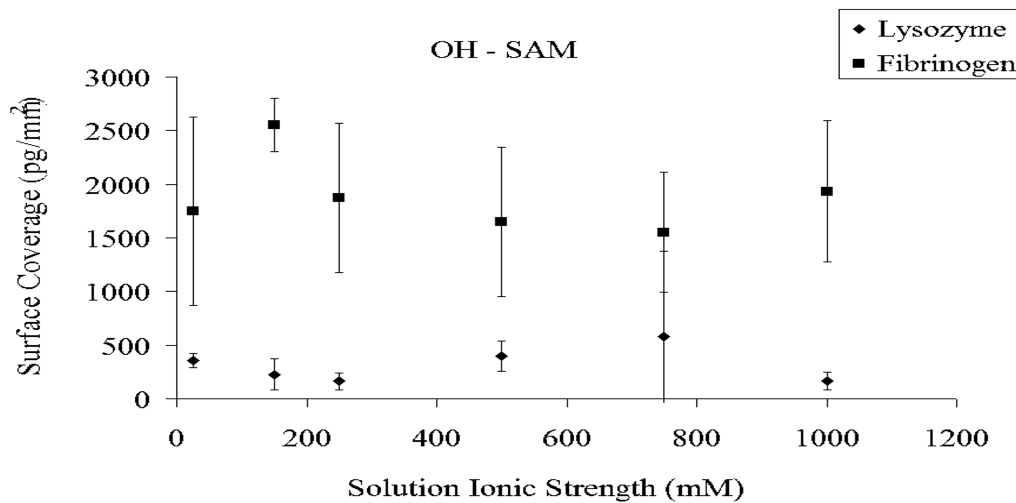

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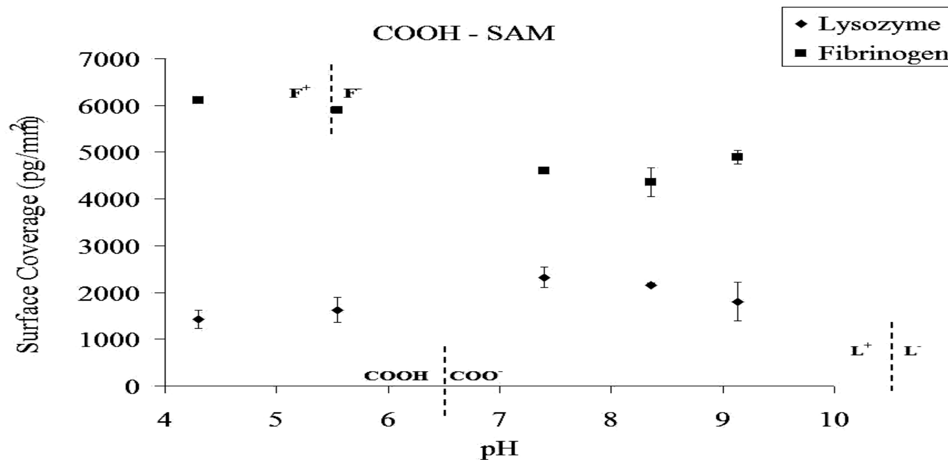
Figure 2: *Lysozyme and fibrinogen surface coverage for (a) COOH (b) NH_2 and (c) OH terminated SAMs under varying ionic strength, pH 7.4*

3. RESULTS AND DISCUSSION

The aim of this study was to expand our understanding of protein adsorption mechanisms by studying adsorption changes under varying ionic strength and varying pH. The study was divided into two parts; in the first part, ionic strength was varied and in the second pH was varied. Figure 2 illustrates lysozyme and fibrinogen adsorption, presented as mass surface coverage, to negatively (COOH SAM), positively (NH_2), and neutrally (OH) charged surfaces as the ionic strength (NaCl concentration) in PBS increases at a fixed pH of 7.4. Because Na^+ and Cl^- molecules are smaller than fibrinogen, they were expected to reach the



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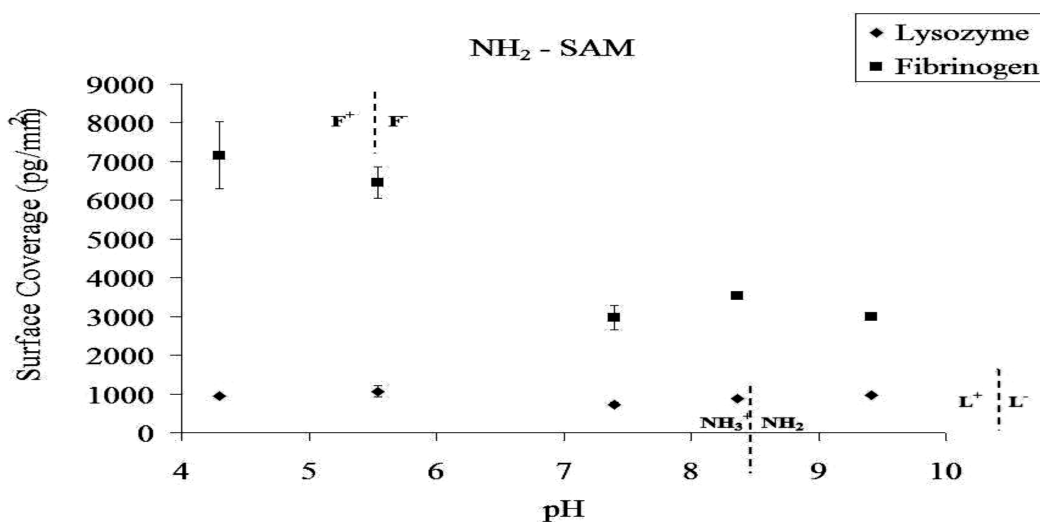

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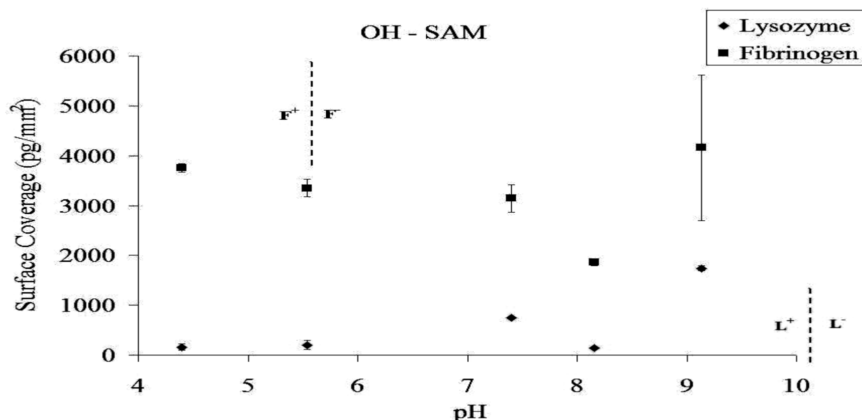

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Figure 3: *Lysozyme and fibrinogen surface coverage for (a) COOH (b) NH_2 and (c) OH terminated SAMs under varying pH. Isoelectric point for lysozyme (L) and fibrinogen is represented by vertical dashed line.*

SAM surface faster; consequently screening surface charge and reducing protein adsorption. It appears, however, that within the range studied, ionic strength did not increase or decrease protein surface coverage. In Figure 2a, surface coverage of lysozyme and fibrinogen remains at a constant value of approximately 1200 pg/mm^2 after ionic strength reaches 200nM. Fibrinogen surface coverage appears to be unaffected by increasing ionic strength after an ionic strength of 200nm. Lysozyme and fibrinogen surface coverage on amine terminated SAMs remains relatively constant as solution ionic strength is increased (Figure 2b). Hydroxide terminated SAMs served as controls for these experiments. These results indicate that within the range studied ionic strength did significantly screen charge to impact protein adsorption.

The second portion of this research studies the effects of pH variation on protein adsorption on COOH, NH_2, and OH terminated SAMs. PBS solution pH ranged from 4.40 – 9.13. This pH range brought forth changes in protein

fibrinogen (IEP=4.4) charge when it surpassed its isoelectric point. Lysozyme (IEP=10.7) was studied as a positively charged protein throughout this study since pH in this study did not exceed 9.13. Carboxylic acid terminated SAMs (pI=6.5) were studied as negatively charged when pH was greater than its isoelectric point and neutrally charged when pH was less than its isoelectric point. Amine terminated SAMs (pI=8.5) were studied as neutrally charged when pH was greater than its isoelectric point and positively charged when pH was less than its isoelectric point.

A commonly accepted thought is that surfaces resistant to protein adsorption do not include hydrogen bond donors [4]. In our study, amine and hydroxyl terminated SAMs were considered to be hydrogen bond donors. Figure 3 illustrates these surfaces ability to be relatively resistant to lysozyme adsorption. Carboxylic acid terminated SAMs exhibited a decrease in fibrinogen adsorption after pH 6.5 when the both fibrinogen and the surface are negatively charged. However, lysozyme protein adsorption increased after pH 6.5. On amine terminated SAMs, lysozyme adsorption remains relatively constant. Fibrinogen adsorption decreases after fibrinogen's isoelectric point is reached. The hydroxyl terminated SAM served as a control.

Overall, results from pH variation experiments support previous studies that also suggest charge interactions are significant component in non-specific protein adsorption [1]. However, because non-zero protein adsorption occurs despite charge screening and pH change, it is believed that hydrogen bonding is still a contributing factor in the formation of a non-fouling surface [7]. This supports studies claiming that surface hydration enables resistance to nonspecific protein adsorption [7]. Therefore charge interactions and chain hydration are critical in determining the ability of a surface to resist non-specific protein binding. Further studies are recommended to determine the specific mechanisms behind these trends.

4. CONCLUSIONS

This work focused on understanding protein and surface interactions through changes in ionic strength and pH. When ionic strength increased, at a constant pH of 7.4, no notable difference in protein adsorption resulted. However, as pH changed and isoelectric points were crossed, a visible difference in fibrinogen protein adsorption occurred on the carboxylic and amine terminated SAMs. Charge appears to be an important factor in the development of non-fouling surfaces. Because, non-zero protein adsorption was experienced despite charge screening and pH variation, this study further suggests that surface hydration resists non-specific protein adsorption.

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