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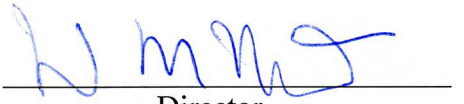

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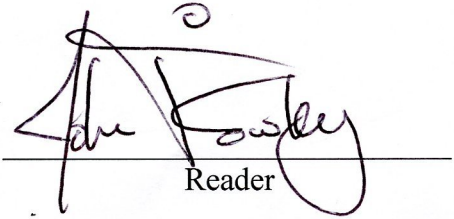

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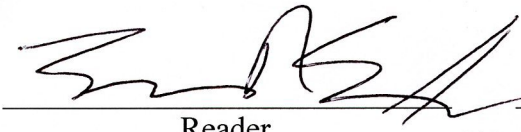

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ADSORPTION OF HUMAN HEMOGLOBIN ONTO CHITIN AND CELLULOSE SURFACES

Honors Thesis for Carroll College

Department of Chemistry, Virginia Polytechnic Institute and State University

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ABSTRACT

When a chitosan (chitin derivative) acetate buffer system is combined with a hemoglobin (Hb) acetate buffer system, three distributions of hydrodynamic radii are observed: Chitosan/H_2O, Hb/H_2O, and a third unexpected distribution – Chitosan/Hb/H_2O complex. This suggests that chitosan and Hb interact aggregate. Neither human serum albumin nor fibrinogen interact or form complexes with either chitin or cellulose. Therefore, chitosan and possibly chitin may be selective to which proteins it binds to. The adsorption of hemoglobin onto chitin and cellulose has yet to be quantified, and this work aims to investigate and quantify a possible affinity of hemoglobin to chitin, cellulose or both. In the present work, hemoglobin solutions were prepared in 50 mM, pH = 7.4 phosphate buffer at concentrations of 0.0125 – 0.5 mg·ml^{-1} and adsorption onto gold, chitin, and cellulose surfaces was studied with surface plasmon resonance (SPR) spectroscopy. First, the hydroxyl groups of chitin and cellulose were masked with trimethylsilyl groups to improve solubility in common organic solvents. Solutions with concentrations of 0.05 mg·ml^{-1} were prepared, in chloroform for chitin and toluene for cellulose for the purpose of preparing spincoated films. Polymer films were spincoated onto gold SPR surfaces, and the hydroxyl groups were regenerated using 10% aq. hydrochloric acid vapor. Hemoglobin solutions were passed across gold, chitin, and cellulose surfaces and resonant angle changes from SPR were converted into surface concentrations (Γ_{SPR}). In all systems, hemoglobin failed to cover the entire surface, therefore equilibrium Γ_{SPR} were less than a monolayer. Hemoglobin adsorbed onto gold surfaces with $\Gamma_{SPR} = 1.2$ mg·m^{-2} and reached equilibrium within 70 min, independent of hemoglobin concentration. Hemoglobin adsorbed faster onto gold than

chitin, however Γ_{SPR} for adsorption onto chitin exceeded gold at high solution concentrations. For cellulose surfaces, hemoglobin adsorption led to smaller Γ_{SPR} at all concentrations tested relative to chitin. Adsorption rates for hemoglobin onto cellulose were substantially smaller than those observed with gold and chitin. These studies suggest stronger interactions between hemoglobin and chitin than hemoglobin and cellulose which are consistent with strong interactions seen between hemoglobin and chitosan derivatives used in drug delivery formulations.

INTRODUCTION

As medicine evolves, research into the safe delivery of drugs to target organs or tissues continues. Chitin and cellulose are common biopolymers,[1,2,3] with potential for drug delivery. An example is chitosan, a cationic derivative of chitin, which remains in negatively charged mucosal membranes.[4] Chitin is found naturally as crystalline microfibrils that associate with several macromolecules to form different composites.[5] Chitinases, hydrolytic enzymes, fight pathogens in plants, regulate growth in fungi, and provide nourishment and parasitism in bacteria.[6,7,8] These enzymes are used synthetically in pharmaceutical, biotechnological, and agricultural applications.[8] Cellulose, ubiquitous in plant and bacterial cell walls and the core of the pulp and paper industry, has a structure and supramolecular organization that is similar to chitin. Trimethylsilylcellulose (TMS-Cellulose) gives rise to very thin and smooth regenerated cellulose films.[3] Kittle et al. used the same approach to obtain smooth chitin films suitable for analysis by SPR from trimethylsilylchitin (TMS-Chitin).[3] Wang et al. created homogeneous, ultrathin chitin films and optimized adsorption conditions for the enzymes and the substrates involved.[1,3]

Hemoglobin is a protein found in all vertebrates. Understanding how hemoglobin interacts with cellulose and chitin is an important factor for the use of cellulose, chitin, and their derivatives for tissue engineering, wound healing and drug delivery. In the present study, polymerization and adsorption kinetics of hemoglobin onto smooth chitin and cellulose surfaces were monitored by means of SPR.[2]

EXPERIMENTAL

Materials. Sodium phosphate dibasic heptahydrate and sodium phosphate monobasic monohydrate were purchased from Sigma-Aldrich. Hydrochloric acid (HCl) was purchased from Fischer Scientific and diluted with ultrapure water to 10% (v/v). Ammonium hydroxide was purchased from Spectrum Science, 30% aqueous hydrogen peroxide was purchased from EM Science, and nitrogen gas was purchased from Airgas. Human hemoglobin was purchased from Sigma-Aldrich. Microcrystalline cellulose (Avicel, PH-101, Fluka) was converted to TMS-Cellulose (degree of substitution (DS) = 2.9) as previously reported.[9] α–Chitin from shrimp shells (> 95% acetylated) was purchased from Sigma-Aldrich, and converted to TMS-Chitin (DS = 2.0) as previously reported.[3,10] All chemicals were used without further purification. The instruments used at Virginia Tech and their model numbers are as follows: UV/Ozone Procleaner (BioForce #4332), spincoater (Gast #0588), SPR (Reichert SR7000 #0031126-02), ultrapure (type 1) water purification system (Millipore #F3NA00568).

Preparation of Thin Films for Surface Plasmon Resonance (SPR). Square SPR gold sensors (20 x 20 mm) were loaded into a UV/Ozone Procleaner for twenty minutes then set face-up in a solution of 1:1:5 (v/v/v) hydrogen peroxide: ammonium hydroxide:

ultrapure water and heated to boiling. After the sensors were in the solution for 40 min, a watch glass was used to cover the container and each sensor was rinsed with ultrapure water (immediately after being taken out of solution) and dried under nitrogen gas. Solutions of TMS-Cellulose (dissolved in toluene) and TMS-Chitin (dissolved in chloroform) at 0.01 $mg \cdot ml^{-1}$ concentrations were spincoated onto sensors at 2000 RPM for sixty seconds. The sensors were then exposed to 10% aq. HCl solution (by volume) vapor for 5 min.

Adsorption of Human Hemoglobin onto Chitin and Cellulose Surfaces. A 10 $mg \cdot ml^{-1}$ hemoglobin solution in phosphate buffer (50 mM, pH=7.4) was used as a stock solution to prepare all other adsorption experiment solutions. Adsorption of hemoglobin from each solution onto chitin, cellulose and gold was studied by SPR. Water was passed through SPR to clean the tubing, then phosphate buffer was run through the flow cell (~12 min), before the instrument was zeroed. Next, the buffer was flowed through the cell for another 5 min to achieve a stable baseline, followed by the hemoglobin solution (~65 min). Finally, buffer (~10 min) was again flowed over the sample. All trials using the SPR were run at 20°C.

RESULTS AND DISCUSSION

Adsorption of Human Hemoglobin onto Chitin and Cellulose Surfaces. Figure 1 provides representative SPR angle changes as a function of time for hemoglobin adsorption onto gold, chitin, and cellulose. The figure illustrates how hemoglobin had the fastest adsorption rates for gold surfaces. The rates for hemoglobin adsorption onto cellulose were much slower. Even though the rates of hemoglobin adsorption onto a

chitin thin film were slower than plain gold, the same amount of hemoglobin adsorbed onto chitin and gold at equilibrium. The initial baseline was set for the first ~700 s with buffer, and buffer was reintroduced at ~4000 s. The fact that resonant angle changes ($\Delta\theta$) did not change after the reintroduction of buffer indicated hemoglobin adsorption was not readily reversible for all three surfaces.

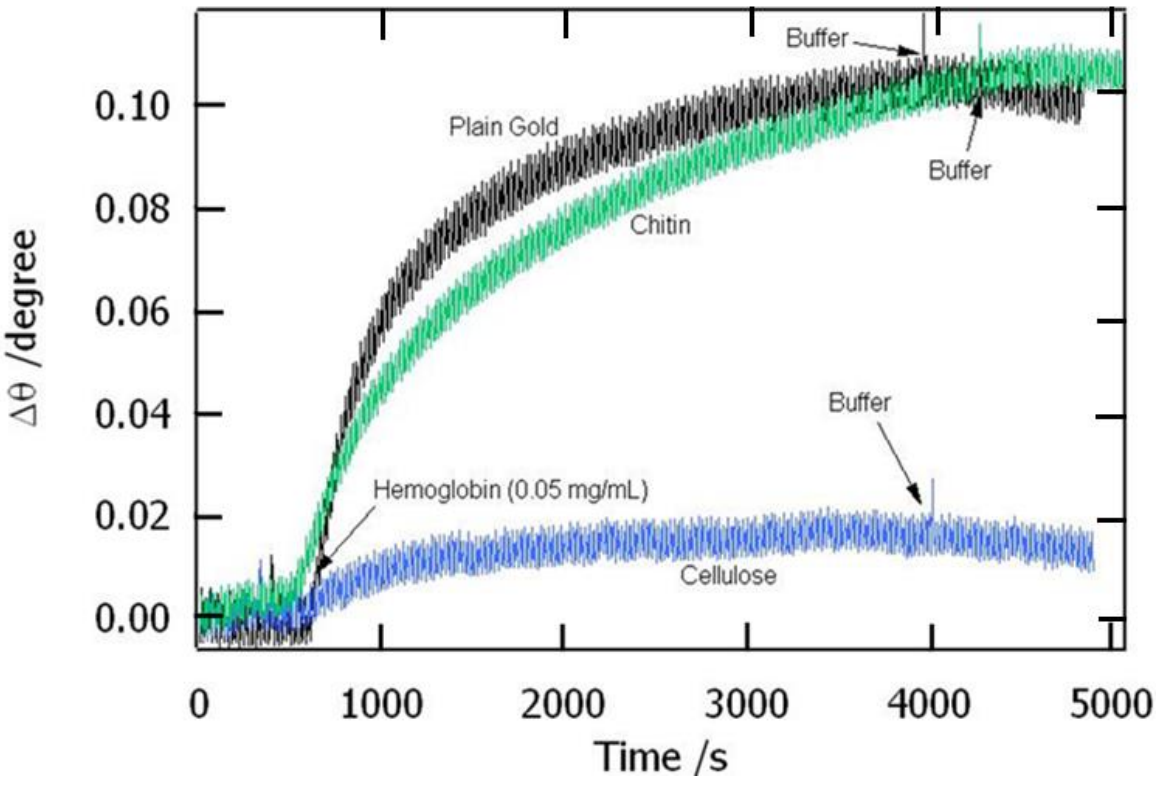

Figure 1. Representative data for hemoglobin adsorption onto a gold, chitin, and cellulose substrates from solutions with concentrations of 0.05 $mg \cdot ml^{-1}$ in pH=7.4 phosphate buffer. Labeled arrows indicate where solutions were switched.

Figure 2 shows adsorption results where Δθ from Figure 1 were translated into surface concentrations (Γ_{SPR}) through the equation of De Feijter et al.[11]

$$\Delta\theta_a = \Delta\theta_{sp} - c\left(\frac{d\theta_{sp}}{dc}\right) = \Delta\theta_{sp} - c\left(\frac{d\theta_{sp}}{dn}\frac{dn}{dc}\right)$$

$$\Gamma_{SPR} = \frac{L(n_a - n_s)}{dn/dc} = \frac{\Delta\theta_a}{d\theta/dL}\frac{(n_f - n_s)}{dn/dc}$$

where $\Delta\theta_a$ represents the resonant angle change due to protein adsorption obtained by subtracting the contribution of the bulk refractive index change in the dielectric medium from the experimental $\Delta\theta_{sp}$, c is the bulk protein solution concentration, $d\theta_{sp}/dn$ represents an instrument specific calibration constant obtained with ethylene glycol solutions (61.5°), dn/dc models the protein solution refractive index increment (with 0.189 mL·g^{-1} assumed for hemoglobin),[12] n_f is the substrate's refractive index (1.45),[13,14] n_s is refractive index of the 50 mM phosphate buffer (1.345),[15] and $d\theta/dL$ represents changes in the resonant angle associated with adsorbed layer thickness obtained from Fresnel calculations (0.042 deg·nm^{-1}).[14] The solid lines in Figure 2 are best fits of chitin and cellulose data obtained from the Freundlich isotherm, which is given as:[16] $\Gamma = K_F c^a$ where K_F represents adsorbent capacity, a is the adsorption affinity constant, and c is the bulk adsorbate concentration. The red trend line is shown as flat because hemoglobin adsorption onto gold appeared to be independent of concentration, and it is was not determined what the minimum concentration necessary was for the adsorption to be zero. Therefore, it should be noted that the actual equilibrium concentration is not 1.25 mg·m^{-2} at 0 mg·ml^{-1}.

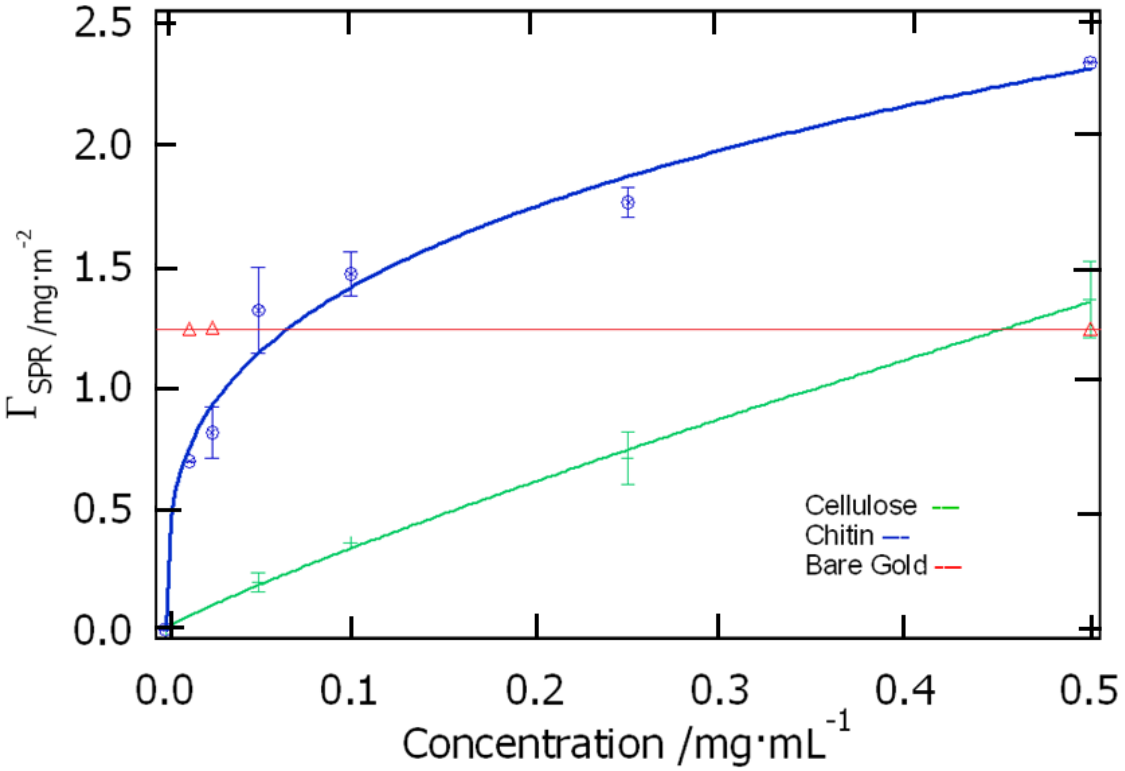

Figure 2. Adsorption isotherms for hemoglobin adsorption onto different substrates as a function of bulk solution concentration.

Adsorption of human serum albumin (HSA), bovine serum albumin (BSA) and human fibrinogen (HFA) onto chitin or cellulose have been reported, but not hemoglobin.[3,4,17,18,19] The end goal was to monitor how chitin and cellulose surfaces interact with human hemoglobin. Previous research has concluded polysaccharide (hydrophilic) barriers hinder protein adsorption due to the active barrier formed from the hydration layer.[20] Therefore, the data in Figure 2 suggest erythrocytes would adsorb less strongly onto cellulose than chitin, with lower values for K_F and a for cellulose and higher values for chitin. Furthermore, as the concentration of hemoglobin in the solution increases, equilibrium was established more quickly. Also, at solution concentrations greater than those tested here, similar amounts of hemoglobin may adsorb on chitin, and

the minimum hemoglobin solution concentration needed to saturate chitin is expected to be lower than cellulose on the basis of Figure 2. One possible explanation for stronger hemoglobin adsorption onto chitin relative to cellulose comes from work by Liuhua et al,[21] who proposed the existence of a complex between chitosan, a chitin derivative, and hemoglobin. Similar complex formation between chitin and hemoglobin could give rise to the behavior seen in Figure 2.

CONCLUSIONS

Analysis of SPR data for the adsorption of human hemoglobin onto chitin, cellulose and gold surfaces showed reduced adsorption onto cellulose, whereas adsorption onto chitin was actually greater than adsorption onto gold except for the lowest concentrations. Therefore, hemoglobin has a greater affinity for chitin than cellulose. Stronger hemoglobin adsorption onto chitin may indicate preferential interactions between chitin and hemoglobin similar to those reported for hemoglobin and chitosan.[21] This behavior is different from studies of human serum albumin and fibrinogen which showed both cellulose and chitin substantially reduced protein adsorption relative to their adsorption onto gold.[19]

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