Using a Stirred Cell to Evaluate Structural Changes in Proteins Adsorbed on Particles

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Introduction

Monitoring the adsorption of proteins onto polymer particles is challenging. It cannot be done by the common techniques used to analyze adsorption on flat surfaces, such as surface plasmon resonance (SPR), *in situ* ellipsometry, or attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy. For particulate surfaces, the established method is to measure the adsorption isotherm, typically obtained by preparing a series of test tubes containing protein solutions and suspended particles, analyzing the protein concentration in the supernatant, and inferring the amount adsorbed. This method involves significant experimental errors, arising largely from the indirect nature of the measurement. To overcome these errors, Cornelius et al. (1992) and Conder and Hayek (2000) used a stirred cell to obtain more accurate isotherms. They injected a small volume of protein solution into the top of a stirred cell (pulse injection), and measured the protein concentration in the effluent, a process analogous to loop injection in analytical chromatography. They obtained more reliable and consistent isotherms than previously.

In this study, we build the work of Conder and Hayek (2000), demonstrating that a stirred cell can be used not only to obtain adsorption isotherms for the proteins on particles, but also to detect structural changes in the adsorbed layer. We have used a stirred cell geometry to obtain a series of breakthrough curves. A breakthrough curve is the change in the concentration of the adsorbate (for example, protein) in the effluent as a function of effluent volume (Kim et al., 1993). Ideally, a breakthrough curve is linear, but mass transfer resistance makes the curve deviate from its ideal form. Film resistance (here, extra-particle film resistance to mass transfer) gives the curve symmetric sigmoidal form, shown in Figure 1a, where C and C_0 are the protein concentrations in the effluent and injected solutions, respectively. For both ideal and symmetric sigmoidal curves, frontal analysis can be performed, as shown in the shaded area in Figure 1a, by evaluating $V_{0.5}$, the eluted volume at which the eluting protein concentration is half the input concentration (Kyprianou and Yon, 1982).

The breakthrough curve can be asymmetric, however, as shown in Figure 1b. This asymmetry arises from a combination of factors related to the efficiency of the sorption process. The most common factor is molecular sieve resistance in densely packed porous particles, such as those used in chromatographic columns (Patwardhan and Ataai, 1997, Mullick et al., 1998; Thömmes, 1999). Changes in the structure of the adsorbed layer can also result in asymmetry. For example, many types of proteins reorient or undergo conformational changes, either upon adsorption or after the surface is fully covered. The molecules may change orientation from side-on to end-on (albumin; Brash and Horbett, 1995), associate with each other (lysozyme; Studebaker et al., 1971), or dissociate into subunits (hemoglobin; Höök et al., 1998). Structural changes in globular proteins may include a loss of α-helix, and gain in β-sheet and random coil content (Baker et al., 2004; Norde and Giacomelli, 2000), which can lead to an increase in the adsorbed layer thickness (Miller et al., 2000). Accordingly, there is a change in the amount adsorbed after $V_{0.5}$, resulting in an asymmetric breakthrough curve.

In the experiments presented here, the substrate consisted of a 1.5 volume percent suspension of nonporous latex particles mixed at high rotation speed (400 rpm). In this system, asymmetry cannot arise from molecular sieve resistance; it should arise only from structural changes in the adsorbed protein layer. We have focused on quantifying structural changes in three adsorbed pro-

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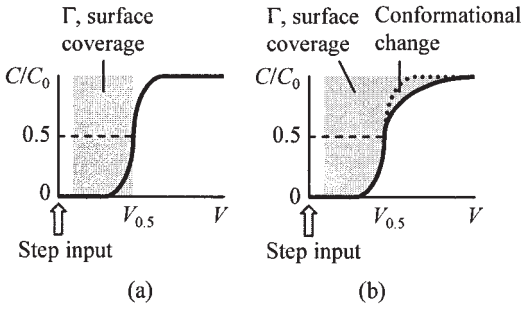

Figure 1. (a) Sigmoidal, and (b) asymmetric breakthrough curves.

Shaded areas correspond to the surface coverage (Γ) determined from frontal analysis or numerical integration.

teins: albumin, hemoglobin, and lysozyme. The adsorption of proteins onto hydrophobic surfaces, such as polystyrene (PS) is commonly modeled as a two-step process: reversible adsorption until the surface is fully covered with protein molecules, followed by irreversible structural changes in the adlayer (Norde and Haynes, 1995). (In reality, the process is almost certainly more complex, with structural changes commencing prior to the surface becoming fully covered with protein molecules.) Within this approximation, the breakthrough curve should have a symmetric sigmoidal shape until the effluent concentration reaches half the input concentration, because this is the point when the surface is fully covered with protein. The surface coverage (adsorbed mass per unit surface area) is calculated by numerical integration (frontal analysis), shown as the shaded area in Figure 1b. We then calculate the area occupied per protein molecule. This result is compared with the molecular dimensions of the protein, considering its association and dissociation characteristics. We are, thus, able to determine the extent of the structural changes that occur during the adsorption process.

Experimental Methods

Materials

Bovine serum albumin (BSA, pI 4.7, molecular dimension $11.6 \times 2.7 \times 2.7$ nm^3, from Sigma Chemical Co., USA, catalog number A-7906), bovine hemoglobin (BHb, pI 7.0, $6.4 \times 5.5 \times 5.0$ nm^3, from Sigma, H-2500), and lysozyme from chicken egg white (LYZ, pI 11.0; $4.5 \times 3.0 \times 3.0$ nm^3, from Sigma, L-6876), were selected as model proteins (Norde and Anusiem, 1992; Yoon et al., 1998b). The partial specific volumes of BSA, BHb, and LYZ at room-temperature are 0.733, 0.739, and 0.721, respectively (Fasman, 1976; Arosio et al., 2002). Weakly carboxylated polystyrene (PS) latex was used as a model particulate substrate. The hydrodynamic particle diameter was 466 nm, as determined by hydrodynamic fractionation. The number density of surface carboxyl groups was 0.530 nm^{-2}, as determined by conductometric titration (the maximum value being 11.0 nm^{-2} for complete surface coverage by carboxyl groups) (Yoon et al., 1996, 1998a). Acetate buffer (pH 4.7) and phosphate buffers (pH 7.0 and 11.0) were used for BSA, BHb, and LYZ, respectively.

Instrumentation

Proteins were dissolved in buffer (final ionic strength of buffer = 0.010), and their concentrations quantified by UV absorbance at 280 nm (Shimadzu UV-160A, Japan). 30.00 mL of PS latex was placed in a stirred cell (Amicon, USA, model 8050). The specific surface area of the particles was 0.190 m^2/mL, and the solid content 1.50% (vol/vol). The particles were trapped within the cell by a 0.3 μm poly(ethersulfone) microfiltration membrane (Omega series, Filtron Technology Co., USA). The particles were equilibrated with buffer (ionic strength = 0.010). Protein solutions were introduced from 1 m above the stirred cell. Eluted fractions were weighed by an electronic balance, and the protein concentrations determined by the absorbance at 280 nm. Protein fouling onto the membrane was evaluated by blank experiments (no polymer particles), in which the concentration was measured before and after elution. The blank experiments showed that 10.0 ± 1.0% of BHb fouled the membranes, while less than 1% of BSA and LYZ fouled them. All experiments were performed at room temperature.

Results and Discussion

Breakthrough curves

Figure 2 shows the breakthrough curves for BSA, BHb, and LYZ onto the PS particles. The pH values were chosen to match the isoelectric point of each protein, to minimize electrostatic interactions between the proteins and the PS latex, and simulta-

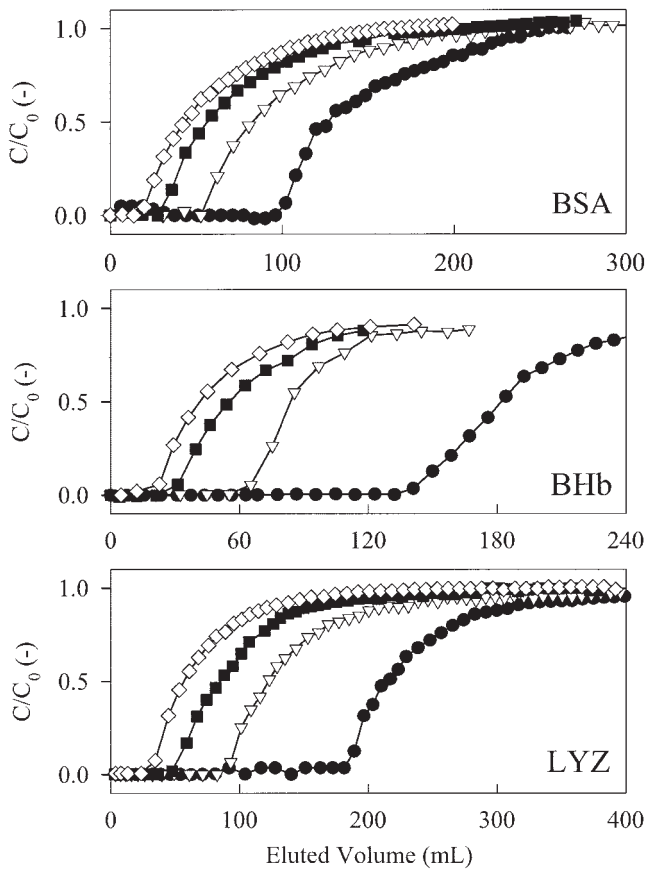

Figure 2. Breakthrough curves for BSA at pH 4.7 (top), BHb at 7.0 (middle), and LYZ at 11.0 (bottom).

Bulk protein concentrations are ● 0.100 mg/mL, σ 0.220 mg/mL, ■ 0.500 mg/mL, ◇ 1.000 mg/mL. Ionic strength = 0.010, specific surface area = 0.190 m^2/mL, total volume = 30.00 mL.

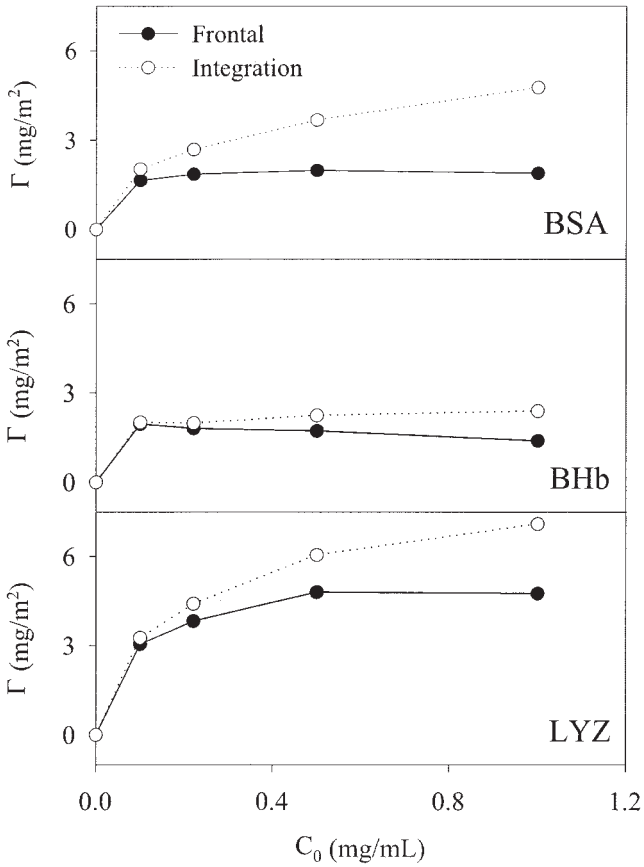

Figure 3. Adsorption isotherms determined from frontal analysis (●) and numerical integration (○).

neously maximize the amount of protein adsorbing through hydrophobic interactions (Yoon et al., 1996). All three sets of breakthrough curves are sigmoidal up to $C/C_0 = 0.5$, but deviations are observed after that, indicating most of the structural changes occur after the surface is fully covered with protein molecules.

Frontal analysis and numerical integration

Frontal analysis was performed as described in Figure 1a. The surface coverage calculated from frontal analysis represents adsorption prior to the surface being completely filled in with protein molecules. Numerical integration was performed until C/C_0 reached 1 for BSA and LYZ, corresponding to complete adsorption of the proteins from solution onto the particle surface. For BHb, control experiments showed that 10% of the injected protein always fouled the membrane surface. Therefore, $V_{0.45}$ was evaluated instead of $V_{0.5}$, and the numerical integration was performed until C/C_0 reached 0.9. As shown in Figure 2, C/C_0 for the breakthrough curves of BHb always saturated to 0.9, regardless of the bulk protein concentration.

Surface coverage calculated from frontal analysis

In Figure 3, the results of frontal analysis are shown as filled symbols, and those of numerical integration as open symbols. The maximum surface coverage (Γ_m) calculated from frontal analysis was 1.99 mg/m^2 for BSA, 1.95 mg/m^2 for BHb, and 4.80 mg/m^2 for LYZ. These values were converted into the adsorbed layer thickness (δ_{exp}) using the following equation (Shirahama and Suzawa, 1985), assuming close-packed monolayer coverage and an ellipsoidal shape for the proteins

$$\delta_{exp} = \frac{3\sqrt{3}}{\pi} \cdot \bar{v}_{\text{protein}} \cdot \Gamma_m$$

Here $3\sqrt{3}/\pi$ is a packing factor for ellipsoids, and $\bar{v}_{\text{protein}}$ is the partial specific volume of the protein. From this equation and partial specific volumes given in the experimental section, we calculated an adsorbed layer thicknesses of 2.41 nm for BSA, 2.39 nm for BHb, and 5.72 nm for LYZ.

These experimental results can be compared with the thickness that would be predicted from the molecular dimensions, assuming a side-on orientation and close-packed monolayer coverage. The predicted thicknesses would be 2.7 nm for BSA, 5.0 nm for BHb, and 3.0 nm for LYZ. The experimental thickness of BSA, 2.41 nm, roughly corresponds to the predicted value, 2.7 nm. The ~10% discrepancy may originate from loose packing of the protein molecules, some flattening of the molecules upon adsorption, and/or experimental error. In contrast, the experimental thickness of BHb, 2.39 nm, is much smaller than the predicted value of 5.0 nm. A possible explanation is that BHb dissociates into its subunits at the interface. The subunits of hemoglobin are, to a large extent, held together by hydrophobic interactions, and are known to dissociate at pH values below 4 and above 10, or at low concentrations. Höök et al. (1998) proposed that interactions between hydrophobic surfaces and protein molecules can lead to dissociation. This implies that BHb can dissociate at neutral pH when it comes in contact with polystyrene, which is hydrophobic. The subunit diameter of 2.5 nm roughly corresponds to the experimental thickness of 2.39 nm, supporting this idea.

The experimental thickness for LYZ, 5.72 nm, is almost twice the smallest molecular dimension (3.0 nm) and significantly larger than the largest dimension (4.5 nm), strongly suggesting that association occurs at the interface. It is well known that LYZ dimerizes at alkaline pH, especially near its isoelectric point, pH 11.0 (Sophianopoulos and Van Holde, 1961; Studebaker et al., 1971). Dimerization would double the postulated thickness to 6.0 nm, slightly thicker than the experimental value of 5.72 nm.

The filled symbols in Figure 3 indicate the surface coverage (Γ) as a function of bulk protein concentration, determined by frontal analysis. For BHb, Γ decreases upon increasing C_0. This indicates the extent of dissociation of BHb is inversely proportional to the bulk concentration, that is, the more protein in the bulk phase, the less dissociation at the interface. For LYZ, Γ increases upon increasing C_0, indicating the association of LYZ is proportional to the bulk concentration, that is, the more protein in the bulk phase, the more association at the interface. Meanwhile, for BSA, Γ is independent of C_0, indicating no significant association or dissociation of BSA at the interface.

Extent of the structural changes

The differences between the values of Γ determined by numerical integration and frontal analysis are plotted against the bulk protein concentration in Figure 4. For all three proteins, the differences depend linearly on their bulk concentrations, and are attributed to structural changes in the adlayer,

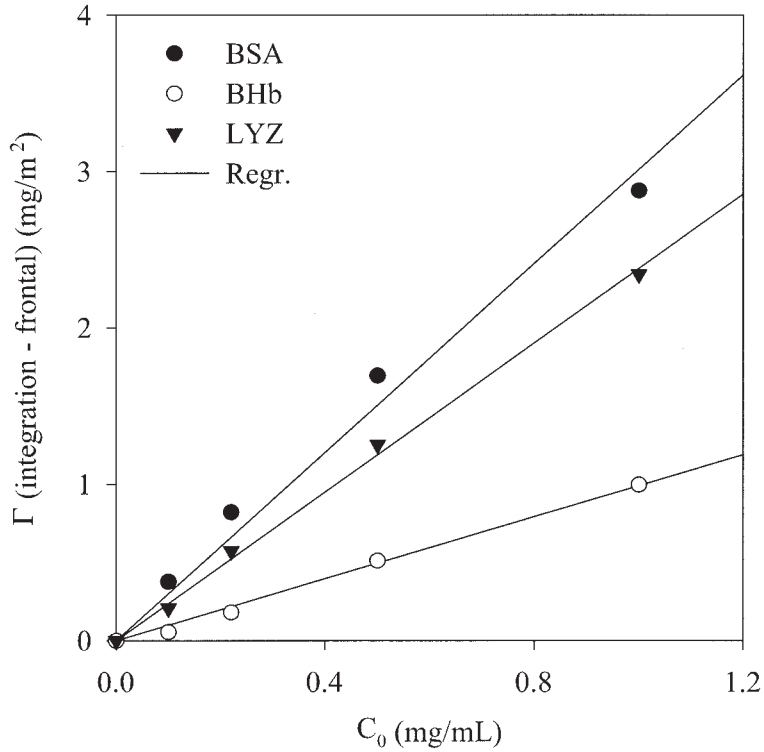

Figure 4. Differences in the surface coverage (Γ) determined by numerical integration and frontal analysis.

including orientation and/or conformational changes, as well as association and dissociation of the adsorbed molecules. The extent of these changes follows the order BSA > LYZ > BHb, and parallels the relative molecular weights of BSA (66,400), dimerized LYZ (14,300 × 2), and dissociated BHb (16,520) (Luo and Andrade, 1998). The observed trend is also consistent with the fact that albumin is more flexible than either dissociated hemoglobin or lysozyme (Peula and de las Nieves, 1993; Kondo et al., 1992; Al-Malah et al., 1995).

Conclusions

We have presented a simple new method for determining the surface coverage and the extent of structural changes in proteins adsorbed on nonporous particulate surfaces. The method utilizes a stirred cell and breakthrough curves analyzed by frontal analysis and numerical integration. The structural changes can be assessed as a function of protein concentration, and may include orientational and/or conformational changes, association and dissociation.

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