Probing Protein Adsorption onto Mercaptoundecanoic Acid Stabilized Gold Nanoparticles and Surfaces by Quartz Crystal Microbalance and ζ-Potential Measurements

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Received December 23, 2006. In Final Form: March 9, 2007

The adsorption characteristics of three proteins [bovine serum albumin (BSA), myoglobin (Mb), and cytochrome c (CytC)] onto self-assembled monolayers of mercaptoundecanoic acid (MUA) on both gold nanoparticles (AuNP) and gold surfaces (Au) are described. The combination of quartz crystal microbalance measurements with dissipation (QCM-D) and pH titrations of the ζ-potential provide information on layer structure, surface coverage, and potential. All three proteins formed adsorption layers consisting of an irreversibly adsorbed fraction and a reversibly adsorbed fraction. BSA showed the highest affinity for the MUA/Au, forming an irreversibly adsorbed rigid monolayer with a side-down orientation and packing close to that expected in the jamming limit. In addition, BSA showed a large change in the adsorbed mass due to reversibly bound protein. The data indicate that the irreversibly adsorbed fraction of CytC is a monolayer structure, whereas the irreversibly adsorbed Mb is present in form of a bilayer. The observation of stable BSA complexes on MUA/AuNPs at the isoelectric point by ζ-potential measurements demonstrates that BSA can sterically stabilize MUA/AuNP. On the other hand, MUA/AuNP coated with either Mb or CytC formed a reversible flocculated state at the isoelectric point. The colloidal stability differences may be correlated with weaker binding in the reversibly bound overlayer in the case of Mb and CytC as compared to BSA.

Introduction

Gold nanoparticles (AuNP), or colloids, stabilized by proteins have wide application in bioassays and cell targeting applications that take advantage of their unique optical, electronic, and chemical properties.1–11 Characterization of the physical processes of protein interactions with Au surfaces and the stability of the resulting particle complexes is of critical importance to these applications.12,13 Previous studies of the binding of bovine serum albumin (BSA) to self-assembled monolayers (SAMs) on Au surfaces reveal nonspecific binding that is sensitive to the outermost chemical groups in the SAM. The order of the adsorption strength was found to be CH3 > R-COO− > R-NH2 > R-OH > ethylene glycol.14–19 Qualitatively, these results indicate that denaturation, which is favored on hydrophobic surfaces, leads to a greater surface interaction than electrostatic binding. Results have also been obtained on bare gold and citrate-coated surfaces based on the combination of quartz crystal microbalance with dissipation (QCM-D) and ζ-potential titrations versus pH.20 These methods have permitted quantification of the number of proteins adsorbed, the binding constant, and the isoelectric point of BSA on gold surfaces and on AuNPs.

The available data14–16,20–22 permit the following mechanistic hypotheses to be put forward. Based on QCM-D data, BSA binds...
to bare Au surfaces by hydrophobic interactions and with about twice the surface coverage as compared to on citrate-coated Au surfaces. This implies that BSA binds by a denaturation process on bare Au surfaces that exposes hydrophobic groups within the protein and also allows multilayer formation. The reduced number of BSA molecules on citrate Au surfaces suggests that citrate is not displaced by BSA adsorption. Binding by an electrostatic mechanism appears to promote formation of monolayers rather than multilayers.

The interpretation of data in the QCM experiments where protein is present in solution (prior to rinsing) requires an understanding of changes in viscosity and density to interpret the adsorbed mass. The experimental effect of solvent and adsorbed macromolecules on the QCM frequency is particularly difficult to distinguish when considering extended layers formed by flexible polymers or polyelectrolytes. Comparative studies of protein adsorption using QCM-D and optical techniques such as ellipsometry, surface plasmon resonance, and optical waveguide light-mode spectroscopy that are not affected by the presence of water within the layer of adsorbed macromolecules have shown that the sensed mass measured by QCM-D is generally higher than the adsorbed mass. For instance, the mass of human immunoglobulin G on hydrophobized gold sensed by QCM is larger by 30% than the adsorbed mass.24,25 For instance, the mass of human immunoglobulin G on hydrophobized gold sensed by QCM is larger by 30–50% than the adsorbed mass detected by the surface acoustic wave technique.26 The contribution of trapped water to the sensed mass is presumably smaller for small compact proteins, and for such systems the complication of dissipative layers is most often ignored.27

Citrato-stabilized AuNPs with adsorbed BSA have high colloidal stability.28 The DLVO theory of colloidal stability defines an electrostatic repulsion and van der Waal’s attraction as two main factors governing stability of the colloidal suspension. In addition to these two balancing potential energy contributions, steric interactions due to the coating play an important role when polymers or proteins are present on the colloidal surface. The solution pH strongly affects the surface charge of colloids with acid/base groups on the surface. Consequently, citrate-stabilized AuNPs were observed to flocculate at around pH 5 in titrations that started at pH 12. The instability with respect to flocculation corresponds to the protonation of the second of three COO⁻ groups in the citrate molecule that has a pKₐ around 4.7. The flocculation of citrate-stabilized AuNPs is expected based on the electrostatic stabilization mechanism. However, BSA/AuNP colloids were stable with respect to flocculation throughout the entire pH range from 12 to 2 and even at pH ~ 4.6.20 Stability at the isoelectric point is indicative of steric stabilization.

In the present study, we have measured the adsorption of the proteins BSA, cytochrome c (CytC), and myoglobin (Mb) on to mercaptoundecanoic acid (MUA) coated Au surfaces and stabilized gold nanoparticles (MUA/Au and MUA/AuNPs), respectively. CytC is the intermediary between complex III and complex IV in the mammalian electron transport chain, and Mb is the chief oxygen storage protein in the cells of mammalian systems. Diffusionless cyclic voltammetry (CV) has been observed for CytC using Au electrodes coated with alkanethiolate monolayers.30–33 Understanding electron transfer at electrode surfaces is of great importance to engineering analytical and biofunctioning materials that use electrical current applied transport involving protein mediation with both in vivo and in vitro applications. Extensive characterization has been performed on CytC/MUA/Au surfaces by various spectroscopic, electrochemical, and scanning probe techniques.31,32,34–36 CytC adsorbs well to long carboxylic-terminated alkane chains (π = 10.435) and loses ideal electrochemical behavior when adsorbed on shorter mercaptoalkanoate chains. On the other hand, diffusionless CVs are not observed for Mb. Moreover, there was no SPR signal when Mb was exposed to various SAMs.19 The difference can be hypothesized to arise from a difference in binding mechanism. CytC has a lysine patch that is important in docking for biological electron transfer. This region of CytC may lead to strong electrostatic interaction with mercaptoalkanoates. Most Mbs lack this electrostatic feature; consequently, they show weaker binding to surfaces. In the following, the surface binding interactions and colloidal stability conferred by the heme proteins, CytC and Mb, is investigated in comparison with BSA using the combination of QCM-D and ζ-potential measurements in order to further elucidate the surface-binding mechanisms for these proteins on MUA/Au surfaces.

Materials and Methods

Ultra fatty acid free BSA (≤0.2 mg/g) was purchased from Roche, Mb and CytC were purchased from Sigma-Aldrich. BSA and Mb were used as received while CytC was purified of dimers, deaminated CytC, and other impurities by the following method at 4 °C: a 0.2 mM CytC solution in potassium phosphate buffer (C = 40 mM; pH = 7.0) was added to a 30 cm carboxymethyl cellulose gel ion-exchange column. Sphining was applied with 70 mM, pH 7 potassium phosphate buffer. Over the course of the next 15–24 h the protein separated into three bands. The first band consisted of deaminated CytC, which appeared as a light red band and was eluted as waste. The following band was CytC monomer, which appeared as a wider dark red band and was collected. Purified CytC is concentrated by Amicon filtration (MWCO = 10 kDa) and stored in glycerol and buffer.

QCM-D measurements were performed with a D300 from Q-Sense, Gothenburg, Sweden, using quartz crystals coated with gold (Q-Sense, Gothenburg Sweden) with a fundamental frequency of 4.95 MHz. All measurements were obtained in a special measuring cell controlled to ±0.02 °C using Peltier elements. The primary data from the measurements is the change in resonant frequency (Δf) and the change in dissipation value (ΔD) where the dissipation is defined as D = E_{diss} / (2πE_{ac}), with E_{diss} being the energy dissipated during one period of oscillation and E_{ac} being the energy stored.

The crystals were placed prior to use in a Harrich plasma cleaner, model PDC-32G, for 15 min on the high setting and placed in 99.5% pure ethanol. From there, the crystals were placed in contact with bichromic acid (BIC) for 3 × 5 min, washed with Millipore water.

References

(resistivity 18.2 MΩ-cm, total organic content <6 ppb), then 99.5% pure ethanol, and dried with N₂ gas. Next, the crystals were placed in 10 mM MUA (Sigma-Aldrich, 95%) dissolved in ethanol for 24 h and then transferred to 99.5% pure ethanol for 24 h prior to use.

Solutions of protein were prepared by addition of the protein to Millipore water, which resulted in a pH of 6.0–6.5, depending on the protein. The solutions were used immediately. After obtaining a stable baseline, adsorption isotherms were obtained by a sequential increase of the protein concentration, using the following concentrations: 100 nM, 250 nM, 500 nM, 750 nM, 1 μM, 25 μM, 50 μM, 75 μM, and 100 μM. The adsorption was followed for 120 min. Next, the crystal was washed with Millipore water and allowed to stand for 15 min, which was sufficient to reach a new stable frequency reading. The same procedure was repeated for all of the protein concentrations. Only the values obtained after rinsing were used for calculations of the sensed mass since after rinsing the bulk density and viscosity remains the same throughout. In another set of experiments, the changes in Δf and ΔD were monitored as a function of time for 100 μM protein solutions. In this case the solutions were allowed to contact the MUA/Au surface for 2 h.

The Sauerbrey equation (37) is commonly used to obtain the sensed mass (Δm) from the change in resonance frequency (Δf). This equation is a good approximation when changes in the dissipation (Δ) are less than 10⁻³ per 5 Hz of Δf. The Johannsmann method (38) is more accurate and informative since it uses QCM data from acoustic impedance and shear modulus of quartz resonators and the adsorbed layer.

ΔDn = √n - 1 ρs Ð 2πΔfn = √n - 1 ρs Ð 2πΔfn  (5)

where ΔD is the harmonic number, Δf is the fundamental frequency in air, and subscripts 0 and n denote pure solvent and solution, respectively. The change in dissipation due to changes in the bulk has been derived by Rodahl and Kasemo (32) and is given by:

Δf = √n - 1 (ρs Ð 2πΔfn)  (4)

where n is the harmonic number, f₀ is the fundamental frequency in air, and subscripts s0 and s denote pure solvent and solution, respectively. The change in dissipation due to changes in the bulk has been derived by Rodahl and Kasemo (32) and is given by:

where \( \Gamma_{\text{max}} \) is the maximum coverage of proteins and \( K_L \) is the binding constant. The Langmuir model assumes equilibrium between solvated and surface-bound proteins, but it has nevertheless been used to analyze similar protein adsorption phenomena. The QCM experiments presented here show that this approximation breaks down. Therefore, the Langmuir model is used only to estimate relative binding interaction and coverage with the caveat that surfaces do not permit equilibrium binding in all cases.

MUA/AuNPs were prepared from AuNPs synthesized by the standard sodium citrate reduction method. Reagents were obtained from Sigma-Aldrich. Particles were characterized by TEM and UV–vis for size, homogeneity, and concentration. The AuNPs were determined to be ~12.8 nm with homogeneity better than commercial particles. MUA/AuNP particles were prepared by adding sufficient excess amounts of MUA to AuNP with 5 μL of 0.1 M NaOH per 1 mL of AuNP. Solutions were allowed to react overnight, and no further purification by centrifugation was found to be necessary.

ζ-potential measurements were performed on a NanoZ SetaProz with a 633 nm He–Ne laser from Malvern Instrument UK, Inc. 44,45 MUA/AuNP samples were prepared with the protein of interest by titrating to pH ≥8.5 using aliquots of 1 M NaOH and a protein to particle ratio of 2000:1. It was noted that if CytC or Mb were added to solutions of MUA/AuNP without increasing the pH the particles tended to flocculate (see Discussion). After several hours mixing time, aliquots of a 1 M NaOH solution were added to achieve pH ~12 at which point the titration was initiated. Aliquots of 1 M HCl were added, and ζ-potential measurements were taken at approximately each unit of pH. Each data point consisted of three samples prepared separately. Each sample was measured three times while each measurement consisted of 30 acquisitions. A 90 s delay between each measurement was used to prevent “charging” of the sample.

\[ \Gamma = \frac{\Gamma_{\text{max}}}{1 + K_L [S]_{\text{free}}} \]  (6)

where the ζ-potential was independent of the frequency in the accessible frequency range. A plot of the equivalent mass against the square of the resonance frequency is independent of the frequency in the accessible frequency range.

\[ \delta f = \frac{1}{\pi \sqrt{\rho_0 \mu_q}} \left( 1 + \frac{J(\delta \tilde{f})}{f_0} \right) \]  (1)

where \( \delta f \) is the shift in the complex frequency, \( \rho_0 \) is the specific density (2.94 kg/m³), \( \mu_q \) is the shear wave velocity (2.95 × 10¹⁰ kg/ms²), and \( J(\delta \tilde{f}) \) is the complex shear compliance. Equation 1 can be rewritten more conveniently by using the equivalent mass \( m^* \) defined by

\[ m^* = -\frac{\sqrt{\rho_0 \mu_q} \delta f}{2\pi f_{\text{ul}} f} \]  (2)

and one thus obtains:

\[ \delta f = \frac{1}{\pi \sqrt{\rho_0 \mu_q}} \left( 1 + \frac{J(\delta \tilde{f})}{f_0} \right) \]  (3)

The sensed mass \( m^* \) was calculated under the assumption that \( J(\delta \tilde{f}) \) is independent of the frequency in the accessible frequency range. A plot of the equivalent mass against the square of the resonance frequency for different overtones gives the sensed mass as the \( \gamma \)-intercept. We note that the sensed mass still contains contribution from both the adsorbing species and solvent oscillating with the crystal. The Johannsmann method is an advantage over the Sauerbrey equation in that it accounts for the viscoelasticity of the adsorbed layer.

At high protein concentrations changes in bulk density (\( \rho \)) and viscosity (\( \eta \)) may induce a change in frequency and dissipation that is not due to the adsorbed layer. The frequency shift can, according to Kanazawa and Gordon, be calculated as

\[ \Delta f = \frac{\sqrt{n^2 f_0^2 \left( \rho_0 \eta_0 \over \sqrt{\rho \eta} \right)^2}}{2 \pi f_0^2} \]  (4)

Charging in this context refers to the observation that without the 90 s delay each subsequent measurement of a sample increased the original \( \zeta \)-potential by about 5–10 mV. All samples were measured at 25 °C with thermal conducting plates and 1 min thermal equilibration. Titrations were carried out from pH 12 to pH 2, unless aggregation occurred, with 1 M HCl. Below pH 2 the proteins, MUA/AuNP, and gold/brass electrodes of the folded capillary cuvettes became unstable or damaged, respectively. The MUA/AuNP and protein/MUA/AuNP samples were investigated at a potential of 150 V optimally set by the computer.

Control experiments consisting of protein only were conducted under the same ionic conditions as the MUA/AuNP/protein mixtures (i.e., with the same concentrations of MUA and NaOH). Controls were needed to determine the setting of the detection threshold on the Zetasizer, thus allowing the removal of the background signal of free protein from samples of protein/MUA/AuNP complexes by setting the threshold sufficiently high. Protein controls were studied at 40 V with 30 s delays between measurements. No charging was noted in successive \( \zeta \)-potential measurements. At higher voltages protein degradation was observed.

The \( \zeta \)-potential was calculated by the Malvern software package using Henry’s equation that relates electrophoretic mobility to \( \zeta \)-potential:

\[
\mu = \frac{2\epsilon_{\infty}e_0}{3\eta}f(\kappa R)
\]

where \( \mu \) is the measured electrophoretic mobility; \( e_0 \) is the dielectric permittivity of vacuum; \( \epsilon \) and \( \eta \) are the dielectric constant and viscosity of the solvent, respectively; and \( \zeta \) is the \( \zeta \)-potential. Henry’s function, \( f(\kappa R) \), is normally approximated by either the Hückel approximation, \( f(\kappa R) = 1.0 \), or the Smoluchowski approximation, \( f(\kappa R) = 1.5 \). Smoluchowski’s expression is valid when \( R/\kappa^{-1} \gg 1 \), \( R \) being the particle radius and \( \kappa^{-1} \) being the Debye length (i.e., for large particles with thin double-layers (high ionic strength)). Hückel’s expression is valid when \( R/\kappa^{-1} \ll 1 \) (i.e., for small particles and extended double layers). Since the protein/MUA/AuNP solutions fit neither of these criteria perfectly, the \( \zeta \)-potentials were calculated with both approximations.

UV-vis measurements were conducted with a HP 8453 UV–vis Chemstation. Titrations were performed just as with the \( \zeta \)-potential measurements but upon aggregation titration with NaOH was used to return the solutions to basic pH. UV–vis spectra were collected initially at pH 12 which served as the control, followed by collection of spectra at acidic pH values where aggregation was noted, and finally after the second addition of NaOH to return to basic pH.

**Results**

The QCM-D results comprise changes in the frequency and dissipation, \( \Delta f \) and \( \Delta D \), obtained from the third overtone. The data shown in Figures 1–3 were obtained for MUA Au-coated quartz crystal resonators with varying concentration of BSA, CytC, and Mb, respectively. The data in Figure 1 show BSA absorption isotherms for both pre-rinse (solid squares) and following a rinsing step (open squares). Protein adsorption occurs already at the lowest BSA concentration (100 nM), but there is a significant shift in frequency at a concentration of 1 \( \mu \)M, which signifies a change in layer structure of adsorbed BSA prior to rinsing. On the other hand, the sensed mass after rinsing increases much more gradually and follows a calculated isotherm (with the caveats provided below). There appears to be little irreversibly bound BSA after rinsing. The large change in sensed mass under the two conditions, pre-rinse and post-rinse, raises the issue of what kind of layer is formed by the loosely bound BSA.

In order to determine if changes in bulk density and viscosity affect the measurements prior to rinsing, the density and viscosity of BSA solutions were measured. It was found that both quantities increased linearly with BSA bulk concentration. The density was found to follow the equation:

\[
\rho_s = \rho_{sl} + 0.01655C
\]

where \( C \) is the concentration in \( \mu \)M and \( \rho_{sl} \) is the bulk viscosity of water at 23 °C, 997.6 kg/m\( \cdot \)s. The corresponding data for the viscosity followed the equation:

\[
\eta_s = \eta_{sl} + 4.558 \times 10^{-7} C
\]

where the bulk viscosity of water at 23 °C is 9.323 \( \times 10^{-4} \) kg/sm. Plots of the data and the corresponding fits are presented in the Supporting Information.

By using these data to evaluate the change in frequency (eq 4) and dissipation (eq 5) due to changes in bulk density and...
viscosity, we conclude that these changes do not affect the QCM response below a BSA concentration of 25 μM. On the other hand, the increase in frequency and dissipation observed before rinsing at [BSA] > 25 μM are largely due to changes in bulk viscosity and density. With this in mind, we conclude that the adsorbed amount of BSA, before rinsing, reaches a plateau value at a BSA concentration of about 25 μM. The sensed mass includes the mass of water associated with the layer. It is generally assumed that the amount of water present in the weakly bound BSA layer is larger than in the irreversibly bound layer. Thus, the fraction of BSA that is removed on rinsing is smaller than indicated by the change in frequency due to rinsing. The correlations obtained using eqs 8 and 9 were used to correct the data for the density and viscosity, respectively, so that the plotted frequency change corresponds to only the adsorbed mass of BSA (triangles in Figure 1B). Figure 1B shows that the corrections for density and viscosity indicate that the measured adsorbed mass is approximately 20% higher than the true adsorbed mass of BSA in the reversibly bound layer formed prior to rinsing.

The change in dissipation for BSA is relatively small (<1.0 × 10⁻⁶) until a concentration of 75 μM has been reached. Thus, at lower concentrations the reversibly bound fraction does not significantly increase the dissipative coupling between the crystal and the bulk solution. At higher concentrations, the dissipation factor before rinsing increases due to changes in bulk viscosity and density, whereas the value obtained after rinsing remains low. We conclude that BSA adsorption results in formation of a rigid layer as well as a loosely bound surface aggregate that can be desorbed by rinsing.

The data obtained for CytC up to a concentration of 1 μM shown in Figure 2A show a relatively small difference in sensed mass before and after rinsing as compared to BSA. Up to a concentration 1 μM the sensed mass approaches a plateau value, which is assumed to indicate the formation of an irreversibly bound layer. However, at concentrations above 1 μM the sensed mass, before rinsing, continues to increase. This additional contribution to the sensed mass is assumed to arise from reversibly adsorbed CytC. The reversibly adsorbed CytC increases with increasing CytC concentration, but it remains significantly smaller than for BSA. As was the case for BSA, a significant part of the increase in dissipation and decrease in frequency, observed before rinsing at concentrations above 25 μM, is attributable to changes in bulk density and viscosity. The change in dissipation value found for CytC is relatively low after rinsing (<1.0 × 10⁻⁶), which leads to the conclusion that the first layer is rigid.

The data obtained for Mb (Figure 3) follow qualitatively the same trends as reported for CytC, but here a plateau value for the adsorbed mass after rinsing is obtained at very low concentrations (<5 μM), and then there is an increase at higher concentration that is consistent with a reversibly bound layer. Again, the large decrease in frequency and increase in dissipation found at high protein concentrations, before rinsing, has a significant contribution from changes in bulk density and viscosity, as discussed for BSA.

Table 1 presents the −Δf and ΔD from Figures 1–3 and the calculated Δm from the Johannsmann model. Γ is calculated by assuming that Δm is equal to the adsorbed mass, which overestimates the amount of protein adsorbed somewhat. Finally, the binding constant Kₙ was estimated using the Langmuir model. The major impetus for the application of the Langmuir model is to obtain a relative estimate of Kₙ. BSA was found to have the highest Kₙ of 1.2 ± 0.12 μM⁻¹ followed by Mb with 0.37 ± 0.07 and then CytC with the lowest value, 0.19 ± 0.05. The values for Γ from Table 1 can be used to obtain an estimate of the layer coverage of a specific protein on the MUA/Au. The size of the proteins, based on X-ray structures, 36–49 are included in Table 2. On the basis of the highest possible Γmonolayer from Table 2, it is logical to assume that BSA is adsorbed by a side-binding mechanism onto MUA/Au surfaces since the measured coverage of 8.52 × 10¹¹ proteins/cm² is less than the known 50 side-binding value of ~2.0 × 10¹². In fact, it has been shown that, for irreversible adsorption without lateral mobility on the surface, the monolayer coverage at the jamming limit is in the range of 0.5–0.55 of full monolayer coverage. 51 Thus, for BSA at the jamming limit one obtains 1.0–1.1 × 10¹¹ proteins/cm², which is close to the measured value. BSA is a flexible protein. However, even with estimates for an elliptically shaped BSA

![Figure 3. QCM-D data from the third overtone obtained by sequential addition of Mb. The solid squares represent the initial measurement when the protein solution was introduced, and the open squares represent the measurement following a wash step. Panel A shows the frequency shift for concentrations in the range from 100 nm to 1 μM. Panel B shows the frequency shift for concentrations in the range from 10 to 100 μM. Panels C and D show the corresponding dissipation shifts (×10⁶).](image)

### Table 1. Experimentally Acquired Data for −Δf max and ΔD max

<table>
<thead>
<tr>
<th>Protein</th>
<th>−Δf max (Hz)</th>
<th>Δm (ng/cm²)</th>
<th>Γ (10⁻¹² cm² × M⁻¹)</th>
<th>Kₙ (μM⁻¹)</th>
<th>ΔD (10⁻⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>13.0</td>
<td>91</td>
<td>0.825</td>
<td>1.2 ± 0.12</td>
<td>0.1</td>
</tr>
<tr>
<td>CytC</td>
<td>42.0</td>
<td>242</td>
<td>11.75</td>
<td>0.19 ± 0.05</td>
<td>0.4</td>
</tr>
<tr>
<td>Mb</td>
<td>63.0</td>
<td>446</td>
<td>15.8</td>
<td>0.37 ± 0.07</td>
<td>0.9</td>
</tr>
</tbody>
</table>

* Δm was calculated by the Johannsmann model and then used to calculate Γ and Kₙ.

| Table 2. Calculated Surface Coverage in Proteins per cm² Based on the Protein Dimensions |
|--------------------------------------------|-----------|-----------------|-------------------|
| molar mass (kDa) | protein dimension (nm) | Γ(proteins/cm² × 10¹²) | end-orientation | side-orientation |
| BSA          | 66         | 5.5 ± 5.5 × 9.0 | 3.3               | 2.0            |
| CytC         | 12.4       | 3.0 × 3.4 × 3.4 | 9.8               | 8.6            |
| Mb           | 17.0       | 3.3 × 3.6 × 6.8 | 8.4               | 4.3            |

with dimensions $2.7 \times 2.7 \times 11.0 \text{ nm}$, the conclusion is still that BSA adsorption is close to the jamming limit. Thus, we conclude that BSA adsorbs to MUA/Au without significant lateral mobility.

CytC and Mb have $\Gamma^*$s of $11.75$ and $15.8 \times 10^{12}$ proteins/cm$^2$, respectively, which are both greater than the maximum coverages of one monolayer, which are $9.8$ and $8.4 \times 10^{12}$ proteins/cm$^2$, respectively (presented in Table 2). For CytC it is plausible that the difference can be accounted for by the fact that water within the adsorbed layer contributes to the sensed mass, which results in an overestimation of the calculated coverage. Since the adsorption clearly exceeds the jamming limit for this protein (i.e., the maximum adsorption achievable if the proteins stick to the surface at the point where they initially attach), we conclude that CytC on MUA/Au display significant lateral mobility that allows a dense layer to form on the surface. For Mb the calculated coverage is larger than a monolayer. According to the adsorbed mass of $446$ ng/cm$^2$ (Table 1) the number of layers of Mb are predicted to be between $1.9$ and $3.7$ for the end-on and side-on binding models, respectively (Table 2). If one were to assume that the adsorbed mass were due to a monolayer of Mb and bound water, the number of water layers would be $\sim 16$. This number arises from the estimate of $4.6 \times 10^{14}$ binding sites/cm$^2$ on Au(111), which corresponds to an adsorbed mass of $\sim 13.8$ ng/monolayer of water/cm$^2$. These considerations suggest that the discrepancy between calculated coverage and monolayer coverage seems to be too large to be explained by water incorporation. The binding isotherm after rinsing has a slight slope at high Mb concentration that is not captured by the fit to the Langmuir model (Figure 3).

In separate experiments solutions of $100 \mu$m protein were allowed to remain in contact with initially bare MUA/Au surfaces for $2$ h. The results from these experiments should be compared with the pre-rinse data seen in Figures 1–3, and we will come back to this point in the Discussion section. The results obtained for the three different proteins are compared in Figure 4, employing $D-f$ plots, and the insert shows $\Delta f$ versus time. $D-f$ plots are useful as they provide information on the energy dissipation per unit mass added to the crystal. These data permit characterization of the evolution of the layer properties during adsorption, and we pay particular attention to the slope of the $D-f$ plots. The slope can be quantified as $R = \partial D/\partial f$, where a small $R$ value is expected to represent a rigid and compact layer.

Table 3 presents the calculated $R$ values. First we note that at the high protein concentration used in these experiments, the major part of the protein layer is formed during the initial injection period, and the time course cannot be observed. Thus, the data in Figure 4 capture the evolution of the protein layer during the later stage of adsorption, with the time-line going from left to right.

Figure 4 shows that the time evolution of the viscoelastic properties of layers formed by the three proteins are very different. The adsorption of BSA is nearly complete after the injection period, and only small changes in frequency and dissipation are observed during the following $2$ h. Thus, the curve for BSA has the smallest slope. It increases gradually, indicating that BSA arriving at the surface at this stage adsorbs by one mechanism and that the proteins attached to the surface last contribute slightly more to the dissipation than the molecules adsorbing just before. On the other hand, the curve obtained for CytC is indicative of the formation of more than a monolayer on the surface. However, since the signal contains a significant contribution from the increase in solvent viscosity, it is not indicative of multilayers but rather a loosely bound overlayer. The data for Mb show a continuous increase in dissipation and frequency with time. The data obtained for Mb are consistent with the formation of more than one layer on the surface.

Figure 5 shows the $\zeta$-potential as a function of pH of 12.8 nm MUA/AuNP. Titrations were stopped below pH 6 because of the onset of particle flocculation.

to lose the steady reading obtained for homogeneous, monodisperse particles. The slight increase in negative charge between pH 12 and pH 8 arises from the formation of salt, which is inherent to the pH titration using HCl as the acid. The distribution of Cl\(^{-}\) formed is in slight excess in the electrical double layer giving rise to a slight increase in the \(\zeta\)-potential of the MUA/AuNPs.

The pH titrations of the \(\zeta\)-potential for BSA, CytC, and Mb with and without MUA/AuNP are displayed in Figures 6–8. The two titrations for BSA and BSA/MUA/AuNP look remarkably similar (Figure 6). Detection thresholds were set so that any signal below a certain intensity compared to the most intense signal would not contribute to the data. AuNP and their complexes scatter several more orders of light than BSA due to their larger size and density. Thus, while BSA, the largest protein studied, did scatter a large amount of light, it was not difficult to remove its background signal in the case of the AuNP suspension. Previous work with citrate AuNP has shown a pI of 4.6 for BSA/citrate AuNP.\(^{20}\) This is comparable to the two pI values at pH 4.8 obtained for BSA alone and BSA adsorbed on MUA/AuNP reported in Figure 6. Since free BSA not associated with the MUA/AuNP surface does not contribute to the signal, this means that BSA imparts its own surface charge characteristics to citrate AuNP and MUA/AuNP when adsorbed onto the surface.

The stability of BSA/MUA/AuNP observed at the isoelectric point is evidence for steric stabilization.\(^{8}\) Thus, BSA adsorption results in a layer with sufficient coverage and thickness to counteract the van der Waals attraction. This is consistent with the large frequency change observed before rinsing by QCM. It also means that bridging interactions that could decrease the free energy when two BSA/MUA/AuNP complexes come into contact are unimportant.

The pH titrations versus \(\zeta\)-potential in Figure 7 show that CytC/MUA/AuNP parallels the behavior of CytC alone. The isoelectric points of CytC/MUA/AuNP and CytC differ by only 0.6 pH units, although the CytC/MUA/AuNP complexes are unstable and tend to flocculate at a pH of 6.3. Moreover, both CytC protein...
and CytC/MUA/AuNPs exhibit a region of relatively small change in ionic potential between pH 6 and pH 9. On the other hand, Figure 8 shows a somewhat different behavior for Mb/MUA/AuNP and Mb alone. The pI for Mb was observed at a pH of 7.8, and as was observed for BSA the change in ionic potential increases steadily with no plateau region. However, no pI was identifiable for Mb/MUA/AuNP particles as flocculation was observed below pH 6.5, but the results demonstrate that the pI for Mb/MUA/AuNP is lower than this value. The flocculation of both CytC/MUA/AuNPs and Mb/MUA/AuNPs was reversible as demonstrated by observation that the addition of 1 M NaOH aliquots resuspended aggregated MUA/AuNP, Mb/MUA/AuNP and CytC/MUA/AuNP samples. Within minutes the samples containing protein had the deep red color typically seen for AuNP. On the other hand, MUA/AuNPs without protein layers were irreversibly coagulated (see Supporting Information). Thus, adsorption of all proteins reduces the attractive interaction between MUA/AuNP, but only BSA is able to infer complete colloidal stability. The measured pls for BSA and Mb are consistent with values obtained elsewhere. However, the pl of 5.7 measured for CytC is not as simple to interpret since the z-potential does not steadily increase with decreasing pH. Instead there is a region of small, but nonzero ionic potential below pH 10 and extending to the zero crossing at pH 5.7. Since the accepted value of the pl for CytC is ~10.4, these results indicate the need to further study the significance of ionic potential data for the determination of the isoelectric point. Most likely this reflects changes in surface conformation of compensating charges. Since CytC is a small protein (see Table 2), the breaking of a single salt bridge on the protein surface can result in significant changes in the surface exposure of titratable amino acid residues such as lysines and aspartates.

**Discussion**

**Adsorption of Globular Proteins at Solid Surfaces.** Adsorption of proteins to solid surfaces is a complex process, which involves structural changes in the protein, dehydration of parts of the protein and the sorbent, and direct interactions (electrostatic, hydrogen bond formation, van der Waals interactions) between the protein and the surface. The review by Haynes and Norde, presented in 1994, is still a highly recommended introduction to the topic. The interpretation of adsorption isotherms is complicated by the fact that protein adsorption is largely irreversible with vastly different adsorbed amounts determined from isotherms obtained by increasing the protein concentration as compared to when isotherms are obtained by sequential rinsing, can be reasonably well-described by the Langmuir model, which is also what was observed for BSA on citrate-coated gold surfaces and on bare Au. The results obtained here can be compared to measurements of BSA on citrate/Au surfaces where $\Gamma = 3.7 \pm 0.2 \times 10^{12}$/cm$^2$ and $\Gamma = 2.8 \pm 0.2 \times 10^{12}$/cm$^2$ was found for BSA before and after rinsing, respectively. Assuming a dimension of 5.5 x 5.5 x 9.0 nm$^3$ for BSA, as given in Table 2, an end-on binding mechanism would accommodate $3.3 \times 10^{12}$ BSA/cm$^2$, whereas a side-binding mechanism would be limited to $2.0 \times 10^{12}$ BSA/cm$^2$. The interpretation in previous work was that BSA binds to citrate-coated surfaces in an end-on orientation. The discrepancy of $0.4 \times 10^{12}$ BSA/cm$^2$ may be due to the increase in viscosity and density as well as water incorporated within the layer, which is not taken into account when interpreting a frequency shift measured in a QCM as being due to solely the adsorbing species. Although BSA and CytC both form monolayers, the adsorption isotherm for Mb cannot be explained without invoking adsorption in more than one layer even after rinsing.

It is interesting that BSA, with a pl of 4.8, has a higher affinity for MUA/AuNPs than the two proteins with significantly higher pl values. However, BSA does have 60 surface lysines that can provide local attractive electrostatic interactions with the surface. BSA also has a relatively low structural stability as compared to CytC and Mb, as evidenced by measurements of the adiabatic compressibility that show the trend BSA $>$ Mb $>$ CytC, which will favor surface denaturation and increase the affinity for the surface. Finally, we note that the coverage of BSA on MUA/Au is lower than on citrate/AuNPs. Our data suggest side-down binding to the extent expected for non-mobile surface layers (the jamming limit) on MUA/Au as opposed to the end-down binding of BSA on citrate/Au. This large difference in layer coverage can only be explained if BSA is mobile on the citrate/Au but not on MUA/Au. The ionic potential of MUA/AuNP and citrate AuNP at pH 12 are $-50$ mV and $-41$ mV, respectively. Thus, the difference in electrostatic attraction is not sufficiently large that it can account for the difference in behavior of BSA on citrate and MUA monolayers. Rather, we propose that it is the mobility of the citrate and MUA stabilizers themselves that account for the difference. The interaction of alkanethiols with gold surfaces is much stronger than the interaction of carboxyl groups leading to slower exchange.

**Changes in Dissipation Values.** The QCM-D allows measurements of both the sensed mass and changes in energy dissipation due to interactions between the oscillating crystal and the environment. The energy dissipation changes can be due to changes at (i) the protein substrate interface, (ii) the protein—
Protein Adsorption on MUA/Au Surfaces

Comparison of Stepwise Adsorption and Adsorption Directly from Solution with High Protein Concentration. Protein adsorption is time dependent. One major reason for this is slow conformational changes that occur after adsorption. Thus, the adsorbed amount of protein is often found to depend on the experimental pathway. It is far from obvious that the same results will be obtained in experiments, such as those shown in Figures 1—3, where the protein concentration is raised stepwise, and those shown in Figure 4, where the surface is exposed directly to a solution with a high protein concentration. To illustrate the possible differences, we compare the results obtained at 100 μM protein concentration before rinsing in Figures 1—3 with the data in Figure 4. For BSA, we find that direct exposure to the solution with high protein concentration (data in Figure 4) results in a more rigidly packed and irreversibly bound layer as compared to the sequential adsorption experiment (Figure 1). This suggests that the BSA layers formed on MUA/Au surfaces by these two processes are very similar. The same conclusion can be drawn for the layers formed by Mb (compare the dark squares in Figure 3 with the Mb data in Figure 4). On the other hand, the layers built from direct adsorption from the 100 μM CytC solution (Figure 4) result in significantly higher sensed mass and dissipation than the corresponding layers formed by sequential adsorption (Figure 2). Thus, in this case adsorption directly from the high protein concentration solution favors formation of a less rigid layer, as evidenced by the higher dissipation value, that presumably contains more water. This process requires considerable time (several hours) as indicated in the inset in Figure 4.

Comparison of Adsorption to Au Surfaces and AuNPs. Au surfaces and AuNPs can be examined by examination of the pre-wash data. In solution the adsorption of proteins to the surfaces and AuNPs can be compared by examination of the inset in Figure 4. As indicated by the linear increase in adsorbed mass (Figures 2B and 3B), respectively. Both this fact and the large dissipation for CytC and Mb provide evidence for layers that have extensive bound water.

Conclusions

QCM-D and ζ-potential titrations versus pH were used to probe adsorption of the proteins BSA, CytC, and Mb on MUA/Au surfaces and MUA/AuNP. All three proteins were adsorbed with irreversible and reversible layers that can be observed by both methods. The irreversibly adsorbed layer is likely to be directly associated with the MUA/Au surface. The reversibly bound layer is likely to arise from protein—protein interactions and may be a second or outer layer. However, the latter point remains to be proven. Measurements of the changes in energy dissipation demonstrate that a rigid layer is formed after rinsing for all three proteins. Analysis of the adsorption isotherms indicates that BSA adsorbs with the highest affinity of the three proteins to MUA/Au surfaces, and with preferential side-down orientation limited by the jamming limit (i.e., BSA is not mobile on the surface). On the other hand, CytC has a significant mobility on the surface, as evidenced from the high adsorption after rinsing that indicates formation of a closely packed monolayer. The surface mobility of CytC results in clear differences between layers formed by sequentially increasing the protein concentration as compared to adsorption directly from a high protein concentration. Mb is the only protein studied that retains a bilayer for all three proteins. Analysis of the adsorption isotherms indicates a high coverage of the MUA/AuNP coated with the studied proteins displays a pH dependence that is similar to that of the proteins themselves. This implies a high coverage of the nanoparticle surface by the protein, a result that is consistent with the QCM data. Adsorption of BSA to form BSA/MUA/AuNPs provides steric stability to the nanoparticles at all pH values measured. The steric stability is likely related to the large reversibly adsorbed fraction of BSA. On the other hand, adsorption of CytC and Mb leads to reversible flocculation of CytC/MUA/AuNPs and Mb/MUA/AuNPs when titrated to their isoelectric points. This is in contrast to the bare MUA/AuNPs, which coagulate reversibly as the pl is approached. The difference in behavior of BSA and the smaller proteins CytC and Mb indicates that the latter proteins have less ability to provide steric stabilization due to their lower conformational flexibility. BSA is a large and flexible protein that has multiple modes of binding and hence provides the best steric stabilization. The

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flexibility also explains why BSA binds tightly via an electrostatic mechanism despite its low isoelectric point. In spite of the overall negative surface charge of BSA at neutral pH, it has 60 surface lysines that provide local regions of positive charge that can bind strongly to both nanoparticle and extended surfaces. The complex nature of interactions of proteins with surfaces and their stabilization of colloidal suspensions is only partially resolved with the methods employed here. There is clearly an important aspect of protein–protein interactions that remains to be more thoroughly explored.

**Acknowledgment.** E.D.K. and S.F. gratefully acknowledge support from NSF Grant CHE-0436467. M.C.J., Z.M.N., J.L.R., and P.K.S. gratefully acknowledge support from NSF Grant REU-0244181. E.D.K. gratefully acknowledges Dr. Wilhelm Glomm of the Norwegian University of Science and Technology, Dr. Scott Brewer of Los Alamos National Labs, Dr. Orlin Velev of North Carolina State University, and Kevin Mattison at Malvern Instruments, USA, for resources, trouble shooting, and advice. We are grateful to Nancy Levinger at Colorado State University for her help in coordinating the international exchange project that took place in this work. P.C., E.B., and T.P. acknowledge financial support from the Swedish Research Council, VR.

**Supporting Information Available:** Absorption spectra showing changes in nanoparticle plasma frequencies that occur upon reversible flocculation. This material is available free of charge via the Internet at http://pubs.acs.org.

LA063725A