Studying Protein and Gold Nanoparticle Interaction Using Organothiols as Molecular Probes

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Supporting Information

ABSTRACT: The protein and gold nanoparticle (AuNP) interfacial interaction has broad implications for biological and biomedical applications of AuNPs. In situ characterization of the morphology and structural evolution of protein on AuNPs is difficult. We have found that the protein coating layer formed by bovine serum albumin (BSA) on AuNP is highly permeable to further organothiol adsorption. Using mercaptobenzimidazole (MBI) as a molecular probe, it is found that BSA interaction with AuNP is an exceedingly lengthy process. Structural modification of BSA coating layer on AuNP continues even after 2 days’ aging of the (AuNP/BSA) mixture. While BSA is in a near full monolayer packing on the AuNPs, it passivates only up to 30% of the AuNP surfaces MBI adsorption. Aging reduces the kinetics of the MBI adsorption. However, even in the most aged BSA-coated AuNP (3 days), 80% of the MBI adsorption occurs within the first 5 min of the MBI addition to the (AuNP/BSA) mixture. The possibility of MBI displacing the adsorbed BSA was excluded with quantitative BSA adsorption studies. Besides MBI, other organothiols including endogenous amino acid thiols (cysteine, homocysteine, and glutathione) were also shown to penetrate through the protein coating layer and be adsorbed onto AuNPs. In addition to providing critical new understanding of the morphology and structural evolution of protein on AuNPs, this work also provides a new venue for preparation of multicomponent composite nanoparticle with applications in drug delivery, cancer imaging and therapy, and material sciences.

INTRODUCTION

Recent experimental evidence shows that when AuNPs are exposed to protein or serum plasma, protein spontaneously accumulates onto the AuNP surface, forming a protein coating layer that is commonly referred to as a protein “corona.”1–3 Multiple works have probed the fundamental mechanism governing the protein interfacial interaction with AuNPs.3–8 However, there are many unsettling questions regarding the protein structure on the AuNP surfaces. For example, to date, binding constants that differ by 4 orders of magnitude (∼10^{-5}−10^{11} M^{-1}) have been reported for BSA binding with AuNPs of 10−40 nm in diameter.4,8−10 Recently, Casals et al. reported that aging enhances the stability of the protein adsorbed on AuNPs, and the protein coating layer evolves from a soft corona to a hard corona. However, the fundamental mechanism of this aging effect and the structural characteristics of the “soft” and “hard” coronas are currently unclear. Important questions such as the possible mobility and the interprotein spacing of the adsorbed protein have not been addressed. Answering these questions is important for our understanding of protein/AuNP interfacial interaction, which will aid future design of protein-functionalized AuNPs for biological and biomedical applications.14

One difficulty in studying the morphology of the protein coating layer on AuNPs is the lack of reliable spectroscopic and imaging techniques for in situ investigation of protein immobilized on AuNPs in solution. While circular dichroism and protein tryptophan fluorescence have been used for understanding the protein/AuNP interactions,2,9,10 background signal from the protein that remained free in solution and the inner filtration effect of the AuNP compromise sensitivity of the techniques and complicate the interpretation of the experimental results. Dynamic light scattering (DLS) techniques measure the change in the hydrodynamic radii of the AuNP induced by protein adsorption. It is unlikely to be sensitive to protein conformational change on the AuNP surfaces. Transmission electron microscopic (TEM), atomic force microscopic (AFM), and optical imaging techniques were applied to study AuNP interfacial interaction with protein; however, the samples have to be dried12 or frozen (cryogenic TEM),13 which likely perturbs the configuration of the protein/AuNP complex.

In this work, we employ mercaptobenzimidazole (MBI), an organothiol, as the molecular probe to investigate the structural characteristics of the protein coating layer on AuNPs. Recent work suggests that organothiols such as glutathione cannot displace BSA adsorbed onto the AuNPs.14 Whether or not an organothiol can be adsorbed onto BSA-covered AuNPs is an open question. Indeed, as it will be shown later, our
quantitative MBI adsorption data revealed that MBI can be readily adsorbed onto the BSA-covered AuNPs without causing BSA displacement. By studying the kinetics and binding capacity of the MBI adsorption onto the AuNP/BSA complex, we are able to obtain critical information regarding the morphology of the protein coating layer and the binding characteristics between BSA and AuNPs. We also investigated the possible binding of the amino acid thiols (AAT) [cysteine (Cys), homocysteine (Hcy), and glutathione (GSH)] onto the BSA-stabilized AuNPs. For simplicity’s sake, we refer to GSH as an AAT even though it is a tripeptide.

**EXPERIMENTAL SECTION**

**Materials and Equipment.** All the chemicals used were purchased from Sigma–Aldrich. BSA with a purity of 97% (lot 064K1251) was used as received. Nanopure water (Thermo Scientific) was used in all our measurements. The SERS spectra were obtained with a LabRam HR confocal Raman microscopy system (Horiba Jobin Yvon, Edison, NJ) using a 632.8 nm HeNe laser for Raman excitation. The RamChip™ slides (Z-S Tech LLC) were used for SERS spectral acquisitions. The UV−vis measurements were measured using an Evolution 300 spectrophotometer (Thermo Scientific, Waltham, MA) or an Olis HP 8452 diode array spectrophotometer (for the time-resolved localized surface plasmon resonance (LSPR) and kinetics of MBI adsorption measurements). DLS measurements were performed on a DynaPro™ NanoStar system (Wyatt Technology, Santa Barbara, CA). Centrifugations were conducted with a Marathon 21000R Fisher Scientific instrument (Pittsburgh, PA).

**Silver and Gold Nanoparticle Synthesis.** Unless specified otherwise, all SERS spectra were acquired using silver nanoparticles (AgNPs) synthesized by the Lee and Meisel method. The gold nanoparticles were synthesized using the citrate reduction method. In brief, 0.0415 g of gold(III) chloride trihydrate was dissolved in 100 mL of distilled water and was heated to reflux with vigorous stirring. Then, 10 mL of 1.14% (w/v) sodium citrate dihydrate aqueous solution was added to the solution right after boiling commenced, and boiling continued for 20 min. The average particle size of the prepared AuNPs was ~15 nm in diameter with peak UV−vis absorption centered at 520 nm (Figure S1). The concentration of AuNPs was calculated as 9.2 nM by assuming that all gold(III) ions are reduced to gold(0), which is also consistent with the concentration of the AuNP estimated using the UV−vis absorbance of the as-synthesized AuNPs.

**DLS Measurements.** DLS measurements were performed with a DynaPro™ NanoStar system equipped with a HeNe laser at 658 nm and an Avalanche photodiode detector. A Wyatt quartz cuvette of path length 1 cm and an active volume of 10 μL was used as a sample holder. After its loading, the sample in the cuvette was left to sit for 5−10 min to allow any turbulence to dissipate before spectral acquisition. The DYNAMICS software package (v.7.1.0) was used to analyze the data. A detection angle of 90° was chosen for all size measurements.

**Ratiometric SERS Quantification of MBI Adsorption on AuNP.** The amount of MBI adsorbed onto the BSA-stabilized AuNP was determined using the ratiometric SERS method we recently developed. After centrifugation removal of the AuNPs together with their surface adsorbates, 100 μL of supernatant was transferred into a vial and then mixed with an equal volume of known concentration of isotope-substituted MBI (MBId4) where the four hydrogen atoms in the benzene ring of normal MBI (MBId0) are substituted with deuterium atoms (Figure S2, Supporting Information). After vortexing for ~1 min, the MBId4 spiked supernatant was transferred to an ultrafiltration tube (MWCO 3500, Millipore (Bedford, MA)) to separate the BSA and AuNP from the isotope-substituted MBI pair. The filtrate was then subjected to SERS spectral acquisition. The removal of BSA is important as it interferes with the SERS acquisition of the MBId4 and MBId0 mixture. The concentration of the free MBI (MBId0) in the ligand binding solution was deduced from the SERS intensity ratio of MBId4 and MBId0 using the ratiometric SERS calibration curve we derived before.

Kinetics of MBI Adsorption onto BSA-Covered AuNPs. MBI adsorption kinetic measurements were carried out with an Olis HP 8452 diode array spectrophotometer. To probe the effect of aging the BSA-covered AuNP on the MBI adsorption, the AuNP was mixed with BSA and the mixture was left to sit at room temperature for a predefined time period. Time-resolved UV−vis spectra were taken immediately after the addition of MBI into the (AuNP/BSA) mixture. The time interval between each consecutive spectral acquisition was 0.5 s. The adsorption kinetics of MBI onto the (AuNP/BSA) complex was determined on quenching of the MBId0−vis absorption upon its adsorption onto AuNP, an effect that will be discussed in the Results and Discussion section.

Quantification of BSA Adsorbed on AuNP. The as-synthesized AuNPs were concentrated using centrifugation precipitation followed by sonication redispersion to a final concentration of 200 nM. The concentrated AuNPs are then mixed with equal volume of 20 μM BSA solution. The amount of BSA adsorbed onto the AuNPs was determined with (AuNP/BSA) mixtures that were aged for 10 min, 12 h, and 48 h, respectively. Three independent samples were prepared for each time point. After incubation, the samples were centrifuged at 9000 g for 90 min (Marathon 21000R, Fisher Scientific), and the amount of BSA that remained free in the supernatant was quantified using the BSA UV−vis absorbance in the 280 nm region.

**RESULTS AND DISCUSSION**

**LSPR Measurement of BSA Adsorption onto AuNPs.** BSA adsorption onto AuNP was monitored using a combination of LSPR, DLS, and quantitative BSA adsorption studies. LSPR is one of the very few techniques appropriate for in situ study of the AuNP interfacial interactions in solution, and it has been used extensively for probing the stability of BSA-covered AuNP. To our knowledge, however, application of LSPR for monitoring the BSA adsorption kinetics has not been reported. Figure 1 shows the time-resolved LSPR spectra of the AuNP/BSA solutions, which demonstrated that immediately following the BSA addition into as-synthesized AuNP, the peak absorbance of the AuNP increased significantly, accompanied by a red shift of the LSPR peak. After the first ~10 min or so of the sample preparation, there is essentially no spectral modification in the LSPR feature, indicating that the BSA adsorption has reached a steady state on the AuNPs. The changes in the AuNP LSPR features may be induced by AuNP aggregation and/or BSA adsorption. Subsequent DLS measurement reveals that the maximum increment of the
AuNPs exposed to BSA is about ~3.9 nm in radius, which is significantly smaller than would be expected from AuNP aggregation. As a result we concluded that the LSPR spectral change is induced by the binding of BSA onto AuNPs.

To investigate the possibility of using LSPR to estimate the binding affinity of BSA onto AuNP, we studied the correlation between the BSA concentration and the AuNP LSPR feature in the AuNP/BSA mixtures (Figure 1B). Compared to the LSPR peak positions, the LSPR peak absorbance correlates much better with the BSA concentration. The LSPR data in Figure 1B showed that the AuNP peak absorbance reached a plateau when the concentration of BSA is equal to or larger than 4 μM, suggesting that BSA reaches saturation adsorption at this concentration.

The kinetic curve and the adsorption isotherm shown in Figure 1 are remarkably similar to the previously reported data obtained with the quartz crystal microbalance (QCM) study of BSA adsorption onto citrate-coated flat gold film. This result suggests that BSA binding onto the citrate-reduced AuNP used in this work is similar to BSA binding onto the citrate-coated gold film. It also indicates that the AuNP LSPR peak absorbance has a strong correlation with the amount of BSA adsorbed, even though the quantitative relationship between these two variables is not currently known. This observation is important as it validates the use of AuNP LSPR absorbance for in situ monitoring of BSA binding onto colloidal AuNP in solution.

Assuming that the BSA adsorption onto AuNP follows a Langmuir isotherm, the results in Figure 1B strongly suggested that the Langmuir binding constant between AuNP and BSA should be between 1 × 10^9 and 5 × 10^7 M⁻¹. It can be shown that if the binding concentration is equal to or smaller than 1 × 10^6 M⁻¹, the BSA packing density on AuNP in the 4 μM BSA sample will be about 20% lower than that in the 50 μM BSA solution. Since a difference would most likely induce a notable difference in the AuNP LSPR feature between these two samples. On the other hand, if the binding constant of BSA is higher than 5 × 10^7 M⁻¹, the packing density of BSA on AuNP should be largely the same between the 1 and 4 μM BSA samples as the amounts of BSA in both solutions are sufficient for a full monolayer BSA packing on the AuNPs, which would make the LSPR feature of these two samples identical.

**Dynamic Light Scattering and Quantitative BSA Adsorption Study.** The time-dependent DLS measurements conducted with equal volume mixtures of 10 μM BSA and as-synthesized AuNP showed that immediately following the sample preparation (the lead time for the DLS measurement is ~5 min for sample handling), the particle size of the AuNP increases from 7.8 ± 0.6 to 11.7 ± 0.5 nm in radius. This 3.9 nm increment in the AuNP radius is consistent with recently reported data. Prolonged incubation (up to 2 days) has no detectable effect on the AuNP size (Supporting Information, Figure S3). This result suggests that BSA reaches a steady-state adsorption onto AuNP within the first 10 min of the preparation of (AuNP/BSA) mixture under our experimental conditions, which is consistent with the time-resolved LSPR result shown Figure 1. However, quantitative BSA adsorption measurements conducted by centrifugation precipitation of AuNP with the adsorbed BSA reveal that aging increases the amount of BSA adsorbed on the AuNP surfaces. The amount of BSA on centrifugation-precipitated AuNP changes from ~21 ± 3 BSA per AuNP in the sample aged for 10 min to ~28 ± 3 BSA per AuNP in the overnight aged (10 h) sample. Further aging of the AuNP/BSA sample has no significant impact on the amount of BSA adsorbed (Figure S4, Supporting Information). The difference between the time courses of BSA adsorption onto AuNP revealed by the LSPR and DLS and quantitative BSA measurements is likely due to the nature of the characterization techniques. While DLS and LSPR are in situ techniques that do not perturb the sample, the quantitative BSA adsorption measurement requires prolonged centrifugation precipitation (90 min) with relatively high centrifugation forces (9000 g) for AuNP removal. It is possible that the binding of some of the freshly adsorbed BSA with AuNPs is not strong enough to sustain the lengthy centrifugation process. As a result they are dissociated from AuNP during the centrifugation. In contrast, the strength of the binding between BSA and the AuNP likely has no detectable effect on the DLS and LSPR measurements. We hypothesize that the higher BSA adsorption onto AuNP in the aged samples is due to the aging-enhanced BSA stability on AuNPs, which is consistent with the recent report that aging enhances the stability of the protein corona on AuNPs formed in serum plasma.

The quantitative ligand adsorption allows us to determine packing density of BSA on AuNPs. The nominal footprint of BSA on AuNPs, calculated using the amount of the BSA adsorbed in the overnight aged (AuNP/BSA) sample, is about 25 nm²/BSA, which is significantly larger than 17.5 nm²/BSA reported previously by De Roe et al. but in agreement with the 27 nm²/BSA determined for BSA adsorbed on citrate-adsorbed gold film. Since native human serum albumin (an analogue of BSA) can be approximated as an equilateral triangular prism with a thickness of 3 nm and side length of 8 nm, our DLS particle size and BSA footprint analysis suggest that BSA most likely lies flat on the AuNPs with a near full monolayer BSA coverage in the overnight aged AuNP/BSA sample.

**Organothiol Adsorption onto BSA-Stabilized AuNP.** Regardless of the age of the AuNP/BSA solutions, the BSA-covered AuNPs exhibit excellent stability in the organothiol and/or the electrolyte containing solutions (Figure 2), while as-synthesized AuNPs aggregate rapidly in the organothiol and/or
Concentration of the AuNP, BSA, and AAT are 2.3 nM, 2.5 μM, respectively (AuNP/BSA) solution was aged for 24 h before the addition of AAT. Pictures were taken after the final component was added for 24 h. (A) MBI, (B) Cys, (C) Hcy, and (D) GSH. For samples (3) and (4), the Na₂HPO₄, 137 mM NaCl, and 2.7 mM KCl.

Photographs of solution of (1) (((AuNP/H₂O)/AAT)/H₂O), (2) (((AuNP/H₂O)/AAT)/PBS), (3) (((AuNP/BSA)/AAT)/PBS), (4) (((AuNP/BSA)/AAT)/H₂O), and (5) (((AuNP/BSA)/AAT)/H₂O)/MBI). The AAT is (A) MBI, (B) Cys, (C) Hcy, and (D) GSH. For samples (3) and (4), the (AuNP/BSA) solution was aged for 24 h before the addition of AAT. Pictures were taken after the final component was added for 24 h. Concentration of the AuNP, BSA, and AAT are 2.3 nM, 2.5 μM, and 8 μM, respectively. The composition of PBS used was 11.9 mM Na₂HPO₄, 137 mM NaCl, and 2.7 mM KCl.

electrolyte containing solutions. Quantitative comparison of the amount of BSA adsorbed onto the overnight aged (AuNP/BSA) mixture with and without subsequent MBI addition (that is subsequently aged for another night) showed that the addition of MBI has no significant effect on the amounts of BSA adsorbed (Figure S5, Supporting Information). This result is consistent with the literature report that GSH is incapable of displacing dye-labeled BSA from AuNPs.

Importantly, the inability of organothiols to displace BSA on AuNPs does not exclude the possibility of organothiol adsorption onto the BSA-covered AuNPs. Such a possibility has not, to our knowledge, been explored before. Our quantitative MBI adsorption experiments, conducted with the ratiometric SERS method we recently reported for MBI quantification, reveal that AuNPs retain ∼80% of their MBI binding capacity after the BSA stabilization (Table 1). This result, combined with our quantitative BSA adsorption onto AuNP with and without subsequent MBI adsorption, indicates that even though BSA maintains a near full packing on the AuNP, it passivates only ∼20% of the AuNP surface, and the rest of the surface is or can be made available for organothiol adsorption. The uptake of AATs by the BSA-stabilized AuNPs was indirectly confirmed from the reduced MBI uptake by AuNPs that were sequentially mixed with BSA and competing AAT (Table 1). The sequential AAT and MBI binding experiments indicate that some of the binding sites that would be available for MBI binding in the BSA-stabilized AuNPs are occupied by the competing AATs, which reduces the MBI binding capacity of the AuNP.

Several lines of evidence support that the MBI adsorbed is directly attached to the AuNP, not on the BSA bonded to the AuNPs. If MBI were bonded through BSA, the amount of MBI adsorbed would be similar to the amount of BSA adsorbed. It is deduced from our quantitative BSA and MBI adsorption results that the amount of MBI adsorbed onto the BSA-covered AuNP is ∼70 times higher than the BSA adsorbed (Table S1, Supporting Information). Such a large difference in the amount of BSA and MBI adsorbed is too high to be attributed solely to the possible MBI/BSA interactions. Further evidence of direct MBI/AuNP interactions is the complete quenching of the MBI UV-vis absorbance upon its adsorption onto the BSA-stabilized AuNP (Figure 3), while the UV–vis spectrum of a BSA and MBI mixture is additive of the BSA and MBI UV–vis spectra. The AuNP quenching of the UV–vis transition of surface adsorbates has been reported before, and it is likely due to the charge transfer between the surface adsorbate and AuNPs.

**Kinetics of the MBI Adsorption onto the BSA-Stabilized AuNPs.** Taking advantage of AuNP adsorption quenching of the UV–vis absorption of MBI and induced spectral changes in the AuNP LSPR features, we studied the kinetics of the MBI adsorption onto the BSA-covered AuNP using a time-resolved UV–vis spectrum obtained with (((AuNP/BSA)/MBI) (Figure 4). Importantly, the time course of the UV–vis change in the MBI adsorption region correlates very well with that of the AuNP LSPR features, indicating that MBI adsorption further modified the AuNP LSPR features.

The possible effects of BSA concentration on the kinetics and amount of MBI adsorption were investigated with (((AuNP/BSA)/MBI) solutions where the concentration of AuNP and MBI were kept constant (3.1 nM and 15 μM, respectively), but the concentration of BSA was varied from 3.3 to 10 to 16.5 μM, respectively (Figure S and Tables 2 and 3). All the kinetic data of MBI adsorption were fitted empirically with two pseudo-first-order reaction functions (eq 1) where k₁ and kₓ refer to the rate constant and the amount of MBI adsorbed, respectively, for the faster, which is also the major process responsible for the MBI adsorption.

\[
M = \Gamma_1 (1 - \exp(-k_1 t)) + \Gamma_x (1 - \exp(-k_x t))
\]  

Aging of the AuNP/BSA complex reduces both the rate and the amount of MBI adsorption (Figure S, Tables 2 and 3). However, the time scale and the magnitude of the aging effect on these two parameters are significantly different. While more than a 10- and a 2-fold reduction in the MBI adsorption time constant of k₁ and kₓ were observed within the first 5 min of sample aging for all the samples, it takes ∼10 h to produce a detectable aging effect on the total amount of MBI adsorbed (∑Γ₁ + ∑Γₓ).

**Table 1. Quantitative MBI Adsorption and DLS Particle Size of AuNP**

<table>
<thead>
<tr>
<th>sample</th>
<th>Γ₅₃₄ (μM)</th>
<th>particle size in radius (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(((AuNP/H₂O)/H₂O)/H₂O)</td>
<td>NA</td>
<td>7.7 ± 0.6</td>
</tr>
<tr>
<td>(((AuNP/BSA)/H₂O)/H₂O)</td>
<td>NA</td>
<td>11.4 ± 0.5</td>
</tr>
<tr>
<td>(((AuNP/H₂O)/H₂O)/MBI)</td>
<td>5.7 ± 0.5</td>
<td>aggregated</td>
</tr>
<tr>
<td>(((AuNP/BSA)/H₂O)/MBI)</td>
<td>4.8 ± 0.5</td>
<td>12.2 ± 0.5</td>
</tr>
<tr>
<td>(((AuNP/BSA)/Cys)/MBI)</td>
<td>1.8 ± 0.4</td>
<td>11.8 ± 0.4</td>
</tr>
<tr>
<td>(((AuNP/BSA)/Hcy)/MBI)</td>
<td>1.2 ± 0.3</td>
<td>11.7 ± 0.5</td>
</tr>
<tr>
<td>(((AuNP/BSA)/GSH)/MBI)</td>
<td>3.5 ± 0.3</td>
<td>11.6 ± 0.4</td>
</tr>
</tbody>
</table>

“*The components in the inner parentheses of (((A/B)/C)/D) were mixed first and aged 2 h before the addition of the third and fourth components. Concentrations of the AuNP, BSA, MBI, and AAT in the solutions are 2.3 nM, 2.5 μM, 8 μM, and 8 μM, respectively. Adsorption of MBI was measured using the ratiometric SERS method after the samples were incubated overnight at room temperature (Supporting Information). Particle size measured by DLS.*
The independence of the rate and amount of MBI adsorption from the BSA concentrations tested in the AuNP/BSA mixtures further supports a relatively high binding constant ($>10^6$ M$^{-1}$) between BSA and AuNP. If the binding constant between BSA and AuNP is smaller than $10^6$ M$^{-1}$, the Langmuir equilibrium packing density of BSA on the AuNP had to be significantly different among the three (AuNP/BSA) samples tested (Tables 2 and 3), which would inevitably induce significant differences in the rates and amounts of MBI adsorption.

The near-perfect fitting of the MBI kinetic data with the two pseudo-first-order reactions indicates that the MBI adsorption can be approximated by two parallel, first-order processes of different rate constants. Conceivably, MBI binding onto the...
BSA-covered AuNP is a two-step process, the diffusion of MBI in bulk aqueous solution (M_b) onto the AuNP surfaces (M_s) and the MBI/AuNP binding (eqs 3–5). While the average rate of the MBI diffusion to the AuNP surfaces is dictated by the morphological features (inter- and intraprotein spacing) of the BSA coating layer on AuNP, the rate of MBI binding onto AuNP is much more complicated. Theoretically, the surface of the BSA-covered AuNP can be divided into three categories (eqs 3–5): (i) void area S_v, i.e., the AuNP surface on which there are no BSA amino acid residues in direct contact, (ii) loosely bonded area S_l, i.e., the area on which BSA amino acid residues are in direct contact, but the binding between these amino acid residues and gold is too weak to prevent MBI displacement, and (iii) tightly bonded area S_L where binding between the BSA amino acid residues with AuNP is strong enough to preclude MBI displacement. Since the binding between MBI and S_L is impossible (k_L = 0), MBI binding onto a BSA-covered surface can be simplified as a combination of two parallel processes, i.e., MBI adsorption onto S_v and S_L.

Step 1:

M_b → M_s

Step 2:

M_s + S_v → M_sS_v

M_s + S_L → M_sS_L

Aging time of (AuNP/BSA) complex before mixing with MBI. Rate constants of MBI adsorption onto (AuNP/BSA) complex extracted by fitting the kinetics data. The concentrations of AuNP and MBI are 3.1 nM and 15 μM, respectively. Measurement results calculated from three independent measurements.

Table 3. Amount of the MBI Adsorption Obtained by Fitting the Kinetic Data of MBI Adsorption onto (AuNP/BSA)

<table>
<thead>
<tr>
<th>t^a (min)</th>
<th>[BSA] = 3.3 μM</th>
<th>[BSA] = 10 μM</th>
<th>[BSA] = 16.5 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>k_{b1}^s (μM)</td>
<td>k_{b2}^s (μM)</td>
<td>k_{b1}^L (μM)</td>
</tr>
<tr>
<td>0.08</td>
<td>4.9 ± 0.4</td>
<td>1.2 ± 0.2</td>
<td>3.5 ± 0.2</td>
</tr>
<tr>
<td>0.25</td>
<td>4.7 ± 0.3</td>
<td>1.7 ± 0.5</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>1.00</td>
<td>4.5 ± 0.3</td>
<td>1.6 ± 0.2</td>
<td>4.1 ± 0.4</td>
</tr>
<tr>
<td>5.00</td>
<td>4.6 ± 0.3</td>
<td>1.2 ± 0.2</td>
<td>4.2 ± 0.5</td>
</tr>
<tr>
<td>10.00</td>
<td>4.5 ± 0.4</td>
<td>1.2 ± 0.1</td>
<td>4.9 ± 0.1</td>
</tr>
<tr>
<td>30.00</td>
<td>4.5 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>60.00</td>
<td>4.2 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>120.00</td>
<td>3.4 ± 0.2</td>
<td>1.7 ± 0.2</td>
<td>3.7 ± 0.1</td>
</tr>
<tr>
<td>2400.00</td>
<td>3.2 ± 0.2</td>
<td>1.7 ± 0.1</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td>4300.00</td>
<td>3.4 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>3.2 ± 0.1</td>
</tr>
</tbody>
</table>

Aging time of (AuNP/BSA) complex before mixing with MBI. Rate constants of MBI adsorption onto (AuNP/BSA) complex extracted by fitting the kinetics data. The concentrations of AuNP and MBI are 3.1 nM and 15 μM, respectively. Measurement results calculated from three independent measurements.
Although BSA has a nominal footprint of 25 nm²/protein on MBI adsorption onto the BSA-stabilized AuNPs (Table 3). Between BSA and AuNP is garnered on the basis of the amount capacity in 3-day-aged AuNP. Evidenced by the reduced MBI adsorption rate constants and enough to undergo additional structural modification as stability after 1 day of the (AuNP/BSA) aging, it remains fresh prepared sample to void or loosely bonded to BSA amino acid residues that allows there is still a large fraction of the AuNP surface that remains void or loosely bonded to BSA amino acid residues that allows subsequent, high-capacity MBI adsorption.

The fact that there is still a notable aging effect on MBI adsorption in the 2-day-aged AuNP/BSA mixtures indicates that BSA deformation on the AuNP is a lengthy process. Otherwise, the MBI binding kinetics and capacity would be the same for the AuNP/BSA samples that are aged 2 days or more. The ability for BSA to change structure on the AuNP has a significant implication for the BSA structure on the AuNP surface. Although BSA on AuNP is “hardened” in terms of its stability after 1 day of the (AuNP/BSA) aging, it remains “soft” enough to undergo additional structural modification as evidenced by the reduced MBI adsorption rate constants and capacity in 3-day-aged AuNP.

Additional important insight into the binding characteristics between BSA and AuNP is garnered on the basis of the amount of MBI adsorption onto the BSA-stabilized AuNPs (Table 3). Although BSA has a nominal footprint of 25 nm²/protein on the AuNPs in the fully aged (AuNP/BSA) mixtures (aged 1 day or more), BSA passivates only ~9 nm² of the AuNP surface against MBI adsorption in the 72-h-aged sample. The rest of the surface is either void (~10.6 nm²) or linked to loosely bonded BSA amino acids that can be replaced by MBI (~5.3 nm²), as estimated from the amount of MBI adsorbed in Table 3. Taking into consideration that the average van der Waals radius for the amino acids is 3.3 Å, and the necessary spacing between different protein anchoring points, this 9 nm² passivated surface indicates that for each adsorbed BSA, the number of amino acid residues that can be in direct contact with the AuNP should be smaller than 26. This conclusion is consistent with the recent NMR study of ubiquitin bonded onto AuNPs which shows there are only five amino acids residues involved in the AuNP/protein interaction.29

Taking all the experimental results into consideration, we propose the following mechanism explaining structural evolution of BSA on AuNPs and its impact on subsequent organothiol adsorption (Figure 6). When BSA is initially adsorbed onto the AuNPs, the interprotein spacing between the surface-adsorbed BSAs is likely large. As a result MBI penetrates rapidly through the BSA coating layer and is adsorbed onto the AuNPs. With aging, however, the adsorbed BSA spreads on AuNP, which on one hand increases the BSA stability on the AuNP while on the other hand reduces the speed and quantity of the subsequent organothiol adsorption onto the BSA-covered AuNPs. It is important to note, however, even for the most aged (3 days) AuNP/BSA sample, there is still a large amount of void space between the AuNP and BSA coating layer, and the protein coating layer remains exceedingly permeable. Indeed, the data in Figure 5 showed that for all the samples tested, over 80% of MBI adsorption occurs within the first 5 min of MBI mixing with (AuNP/BSA) complex (Table 3).

### CONCLUSION

Organothiol is a powerful molecular probe for studying the structural evolution, morphology, and binding stability of protein on AuNPs. With this technique, we found that BSA interaction with AuNP is an exceedingly lengthy process. While BSA adsorption per se is rapid and is largely completed within 10 min of the sample preparation, the conformational modification continues for at least 2 days. Even though BSA forms a nearly full monolayer packing on AuNP, it passivates only a small fraction of the AuNP surfaces against further organothiol adsorption. In addition to shedding critical new insights on the morphological feature and structural evolution of protein on AuNPs, the results from our organothiol adsorption study also have significant implications for AuNP applications. For example, the ability of the protein-covered AuNP to uptake organothiols with high binding capacity opens a convenient avenue for fabrication of multicomponent (nano, bio, and organo) composite materials that can be exploited in AuNP applications including nanoparticle drug delivery, nano/biomaterial fabrication, and biosensor development. This finding also raises new nanotoxicity concerns of AuNPs. Given the biological significance of the amino acid thiols to human health30,51 and their relative high abundance in biofluids including serum plasma, the possibility for inclusion of organothiol into protein-covered AuNPs and its potential impact on the toxicity and functionality of the protein-covered AuNP should not be overlooked in biological/biomedical applications of AuNPs.

### ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures which include AgNP and AuNP synthesis, ratiometric SERS quantification of MBI, LSPR, and DLS measurements, and quenching of MBI
absorbance on AuNP. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
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