Video Article

Luminophore Formation in Various Conformations of Bovine Serum Albumin by Binding of Gold(III)

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Abstract

The purpose of the presented protocols is to study the process of Au(III) binding to BSA, yielding conformation change-induced red fluorescence (λ_{em} = 640 nm) of BSA-Au(III) complexes. The method adjusts the pH to show that the emergence of the red fluorescence is correlated with the pH-induced equilibrium transitions of the BSA conformations. Red fluorescent BSA-Au(III) complexes can only be formed with an adjustment of pH at or above 9.7, which corresponds to the "A-form" conformation of BSA. The protocol to adjust the BSA to Au molar ratio and to monitor the time-course of the process of Au(III) binding is described. The minimum number of Au(III) per BSA, to produce the red fluorescence, is less than seven. We describe the protocol in steps to illustrate the presence of multiple Au(III) binding sites in BSA. First, by adding copper (Cu(II)) or nickel (Ni(II)) cations followed by Au(III), this method reveals a binding site for Au(III) that is not the red fluorophore. Second, by modifying BSA by thiol capping agents, another nonfluorophore-forming Au(III) binding site is revealed. Third, changing the BSA conformation by cleaving and capping of the disulfide bonds, the possible Au(III) binding site(s) are illustrated. The protocol described, to control the BSA conformations and Au(III) binding, can be generally applied to study the interactions of other proteins and metal cations.

Video Link

The video component of this article can be found at https://www.jove.com/video/58141/

Introduction

A BSA-Au compound exhibiting an ultraviolet (UV)-excitable red fluorescence, with remarkable stokes shift, has been originally synthesized by Xie *et al.*[1]. The unique and stable red fluorescence can find various applications in fields such as sensing[2,3,4], imaging[5,6,7], or nanomedicine[8,9,10,11,12,13]. This compound has been studied extensively by many researchers in the field of nano-science in recent years[14,15,16]. The BSA-Au compound has been interpreted as Au_{25} nanoclusters. The goal of the presented method is to examine this compound in detail and to understand the origin of the red fluorescence. By following the presented approach, the presence of multiple Au binding sites, and the origin of fluorescence, alternative to the single-site nucleation of Au_{25} nanoclusters, can be illustrated. The same approach can be employed to study how other proteins[17,18,19] complexed with Au(III) can change their intrinsic fluorescent properties.

The synthesis of the red-fluorescent BSA-Au compound requires a narrow control of the molar ratios of BSA to Au (BSA:Au) to maximize the intensity of the fluorescence and the location of the peaks in the excitation-emission map (EEM)[20]. It can be shown that multiple binding sites exist for Au(III) to bind, including the Asparagine fragment (or Asp fragment, the first four amino acid residues at the N-terminus of BSA)[21,22]. The 34th amino acid of BSA (Cys-34) is also shown to coordinate Au(III) and to be involved in the mechanism of the red fluorescence([Cys34-capped-BSA]-Au(III))[20]. Upon cleaving all Cys-Cys disulfide bonds and capping all thiols, red fluorescence is not produced ([all-thiol-capped-BSA]-Au(III)). This indicates the necessity of Cys-Cys disulfide bonds as the Au(III) binding site to produce the red fluorescence.

Protein chemistry techniques have not been widely used to study the BSA-Au(III) complexes in the nano-science community. However, it would be valuable to employ these techniques to understand certain aspects of these complexes, as well as to gain detailed understanding of the Au(III) binding sites in BSA. This article is intended to show some of these techniques.

Protocol

1. Synthesis of BSA-Au(III) Complex

1. Dissolve 25 mg of BSA in 1 mL of high-performance liquid chromatography (HPLC) grade water in a 5 mL reaction vial.
   NOTE: The solution should appear clear.
2. Dissolve gold (III) chloride trihydrate (chloroauric acid) to a concentration of 5 mM in HPLC grade water.
   NOTE: The solution should appear yellow. Chloroauric acid solution prepared at this concentration will result in a BSA to Au ratio of 1:13.

1. Alternatively, prepare a solution of chloroauric acid with a concentration of anywhere between 0.38 mM (BSA:Au = 1:1) to 20 mM (BSA:Au = 1 : 50) in HPLC grade water.
   NOTE: Different ratios of BSA to gold will result in drastically different red fluorescence patterns of the excitation-emission map.

3. Place the reaction vial of BSA in a 37 °C water bath and vigorously stir at 750 rpm using a magnetic stirrer.
4. Immediately after the stirring begins, add 1 mL of chloroauric acid to the solution. The color of the solution should transform from clear to yellow.
5. Stir the mixture for 2 min at 37 °C and at 750 rpm using a magnetic stirrer.
6. Into the reaction vial, add 100 µL of 1 M NaOH to the solution to bring the pH to 12.
   NOTE: Immediately after NaOH is added, the solution should darken slightly to a yellow-brown and then turn back to yellow.
7. Continue to stir at 750 rpm for 2 h and at 37 °C. The solution should slowly change from yellow to a dark yellow/brown color. This color change indicates the formation of the red fluorescing BSA-Au(III) complex.
8. Allow the sample to sit at room temperature for 2 days, and the solution will continue to darken to an amber brown and the fluorescence intensity will increase.
   1. Alternatively, let the sample continue to stir at 37 °C for 12 more h as the color of the solution evolves to an amber brown.

2. Synthesis of BSA-Cu(II)-Au(III)

1. Dissolve 25 mg of BSA in 1 mL of HPLC grade water. The solution should appear clear.
2. Dissolve copper (II) chloride dihydrate in HPLC grade water to a concentration of 5 mM. The solution should appear light blue.
3. Add 1 mL of the aqueous BSA solution to a 5 mL reaction vial and place in a water bath at 37 °C. Stir the mixture at 750 rpm.
4. Immediately add 0.5 mL of the copper (II) chloride dihydrate solution to the reaction vial and mix for 2 min. The solution will remain light blue.
5. Add 75 µL of 1 M NaOH to bring the pH to 12 and allow to mix for 2 h. The solution will become purple.
6. Dissolve chloroauric acid in HPLC grade water to a concentration of 5 mM.
7. Add 0.5 mL of aqueous chloroauric acid to the reaction vial and adjust the pH back to 12 using 1 M NaOH.
8. Stir the reaction mixture for 2 h.
   NOTE: The solution should evolve to a brown color.

3. Synthesis of BSA-Ni(II)-Au(III)

1. Dissolve 25 mg of BSA in 1 mL of HPLC grade water. The solution should appear clear.
2. Dissolve nickel (II) chloride hexahydrate in HPLC grade water to a concentration of 5 mM. The solution should appear light green.
3. Add 1 mL of the aqueous BSA solution to a 5 mL reaction vial and place in a water bath at 37 ^{o}C. Stir the mixture at 750 rpm.
4. Immediately add 0.5 mL of the nickel (II) chloride hexahydrate solution to the reaction vial and mix for 2 min.
   NOTE: The solution will remain light green.
5. Add 75 µL of 1 M NaOH to bring the pH to 12 and allow to mix for 2 h.
   NOTE: The solution will become dark yellow.
6. Dissolve chloroauric acid in HPLC grade water to a concentration of 5 mM.
7. Add 0.5 mL of the aqueous chloroauric acid to the reaction vial and adjust the pH back to 12.
8. Stir the reaction mixture for 2 h.
   NOTE: The solution should evolve to a brown color.

4. Synthesis of [Cys34-capped-BSA]-Au(III)

1. Dissolve 2 mg of N-ethylmaleimide (NEM) in 1 mL of phosphate buffered saline (PBS, pH 7.4).
2. Dissolve 2 mg of BSA in 1 mL of PBS-NEM solution.
3. Transfer the solution to a 5 mL reaction vial and stir at 20 °C at 500 rpm for 1 h.
4. Dialyze the solution using 12 kDa dialysis tubing in 500 mL of PBS, stirring at 50 rpm with a magnetic stirrer overnight to remove unreacted NEM.
5. Dissolve chloroauric acid in PBS to a concentration of 0.4 mM.
   NOTE: The solution will be a faint yellow.
6. Transfer the reaction vial to a water bath at 37 °C. Stir at 750 rpm.
7. Immediately add 1 mL of chloroauric acid solution to the reaction vial and allow to mix for 2 min.
8. Add 75 µL of 1 M NaOH to the reaction vial to bring the pH to 12 and allow to mix for 2 h.

5. Synthesis of [all-thiol-capped-BSA]-Au(III)

1. Prepare a solution of 2 M urea and 50 mM ammonium bicarbonate (NH_4HCO_3, pH 8.0) in HPLC grade water.
2. Dissolve 3.3 mg of BSA in 1 mL of the above solution and transfer to a 5 mL reaction vial.
3. Make a stock solution of 0.25 M tris(2-carboxyethyl)phosphine (TCEP) by dissolving 62.5 mg of TCEP in 1 mL of HPLC water.
4. Add the stock solution of TCEP to the reaction vial until the final concentration of TCEP is 8 mM.
5. Incubate the solution in a water bath for 1 h at 50 °C. Stir at 500 rpm using a magnetic stirrer.
6. Allow the solution to completely cool to room temperature.
7. Prepare a stock solution of 100 mM NEM by dissolving 12.5 mg of NEM in 1 mL of HPLC grade water.
8. Add the stock solution of NEM to the reaction vial until the final concentration of NEM is 16 mM.
9. Allow the solution to combine for 2 h at 20 °C. Stir at 500 rpm.

10. Dialyze the solution using a 12 kDa dialysis tubing, stirring the solution at 50 rpm with a magnetic stirrer overnight in 500 mL of 50 mM NH_4HCO_3 to remove excess TCEP, NEM, and urea.
11. Move the reaction vial to a water bath at 37 °C and stir at 750 rpm.
12. Dissolve chloroauric acid in HPLC grade water to a concentration of 0.66 mM.
13. Immediately add 1 mL of chloroauric acid solution to the reaction vial and allow to mix for 2 min.
14. Add 1 M NaOH until the pH of the solution is 12 and allow the solution to continue to mix for 2 h.

Representative Results

From the fluorescence of the BSA-Au(III) complex, it has been observed that the conversion of the intrinsic blue fluorescence of BSA (λ_{em} = 400 nm) to red fluorescence (λ_{em} = 640 nm) occurs at about pH 9.7 through an equilibrium transition (**Figure 1**). EEM of BSA-Au(III) at different BSA to Au molar ratios is shown in **Figure 2**, and this data shows how altering the molar ratios yields the same emission wavelength at different excitation wavelengths. Cu(II), Ni(II), and Au(III) competitively bind to a known site (Asp fragment) in BSA (**Figure 3**). The Cys34-capped BSA shows a change in EEM peak patterns upon Au(III) binding, and these results show how altering of specific binding sites alters fluorescence patterns. The all-thiol-capped BSA shows no red fluorescence and reveals Cys-Cys disulfide bonds as possible binding sites to produce the red fluorophore (**Figure 4**).

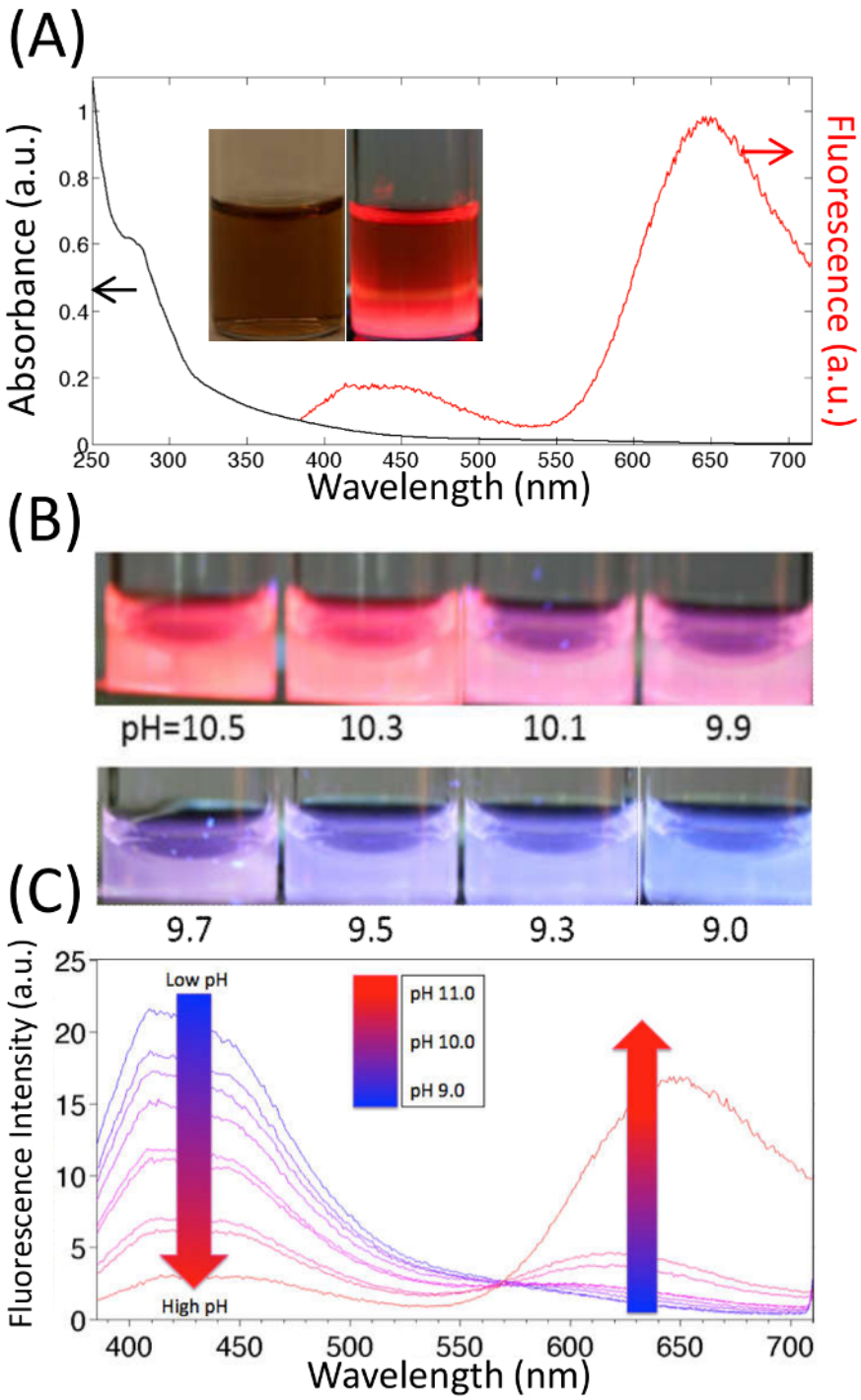

Figure 1. Fluorescence of BSA-Au(III) and the conformational induced change from blue to red. (**A**) The absorbance and fluorescence (λ_{ex} = 365 nm) of BSA-Au(III). (**B**) Red fluorescence beings to emerge at around pH 9.7, at which the conformation of BSA changes. (**C**) Blue fluorescence decays as red fluorescence emerges. Please click here to view a larger version of this figure.

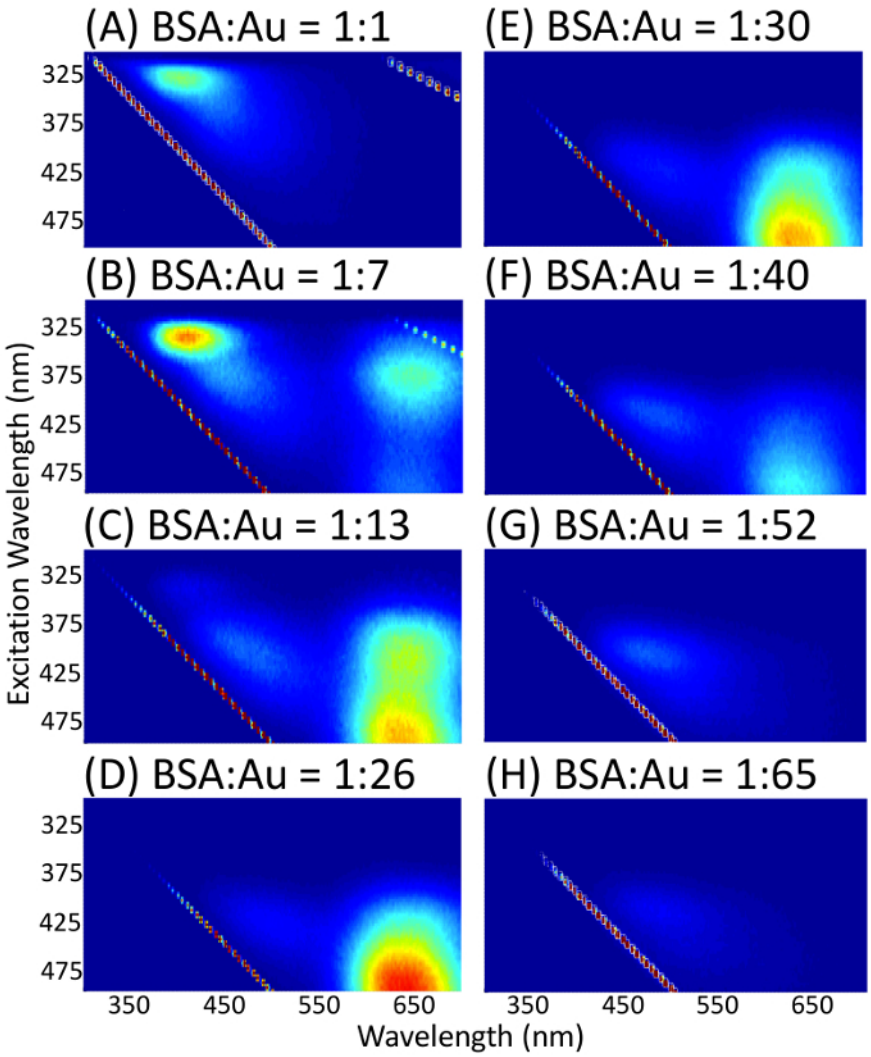

Figure 2. Ratio-metric excitation-emission map (EEM) measurements of BSA-Au(III). EEM of BSA-Au(III) complex synthesized using the standard protocol while adjusting the ratio of BSA to gold. (**A**) BSA at pH 12, (**B**) BSA:Au = 1:1, (**C**) BSA:Au = 1:7, (**D**) BSA:Au = 1:13, (**E**) BSA:Au = 1:26, (**F**) BSA:Au = 1:30, (**G**) BSA:Au = 1:40, (**H**) BSA:Au = 1:52. Please click here to view a larger version of this figure.

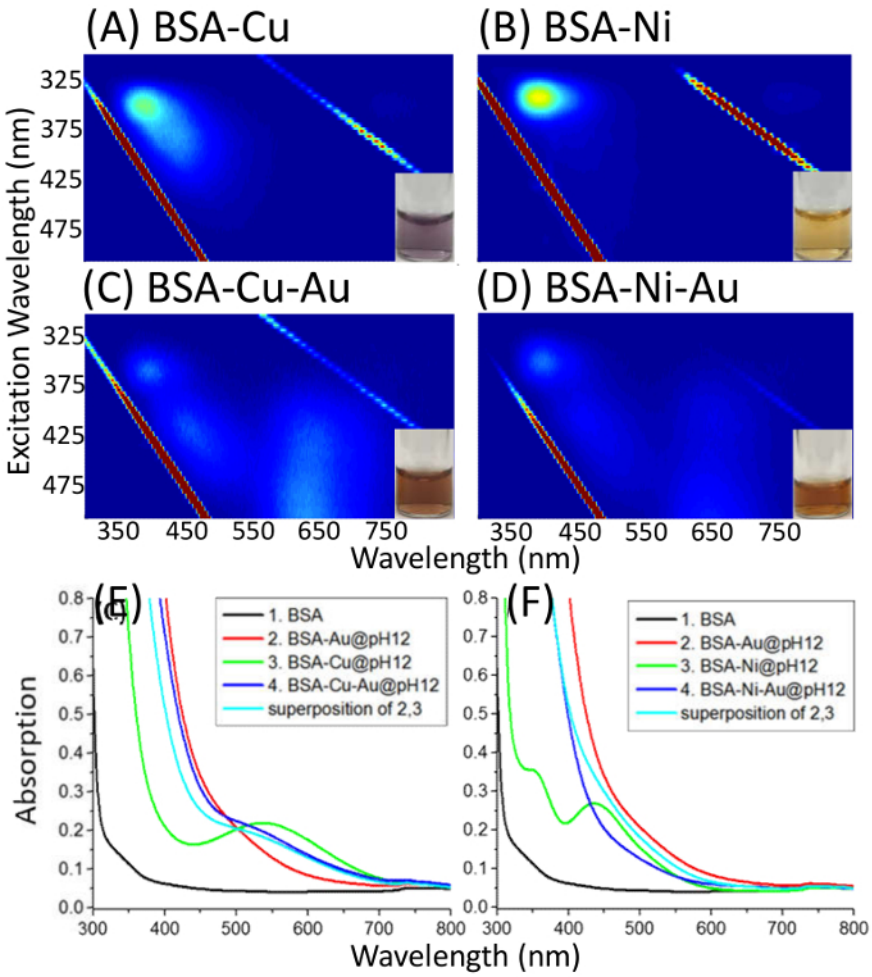

Figure 3. EEM of BSA-Cu(II)/Ni(II)-Au(III) complexes. Excitation-emission maps (excitation: 290 - 500 nm; emission: 300 - 850 nm) of BSA complexed with Cu(II)/Ni(II) at pH 12 (**A** and **B**), BSA complexed with Cu(II)/Ni(II) and then with Au(III) at pH 12 (**C** and **D**), and absorption spectra comparing BSA, BSA-Au, BSA-Cu(II)/Ni(II) and BSA-Cu(II)/Ni(II)-Au(III) (**E** and **F**). Curve 4 is compared with the superposition of Curves 2 and 3. This figure has been modified from Dixon, J. M. & Egusa, S. *J. Am. Chem. Soc.* **140** 2265-2271, (2018). Please click here to view a larger version of this figure.

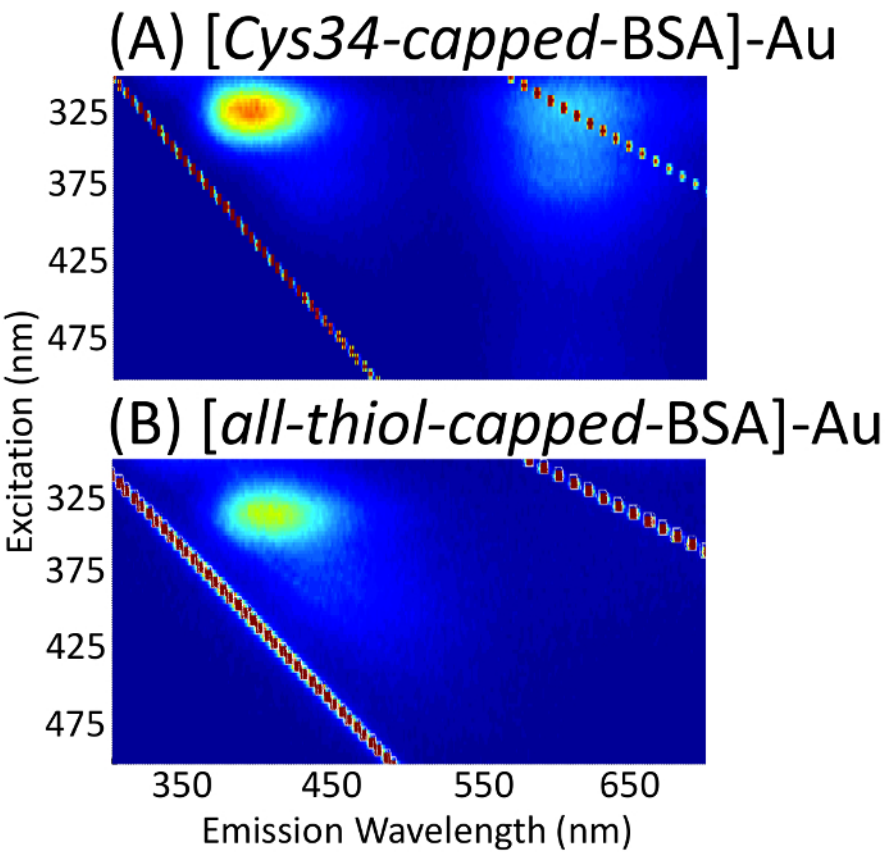

Figure 4. EEM of Cys34 capped and all thiols capped. EEM (excitation: 300 - 500 nm; emission: 300 - 700 nm) of (**A**) Cys34-capped BSA reacted with Au at pH 12. In (**B**), all Cys-Cys disulfide bonds in BSA were cleaved and then the all-thiol-capped-BSA was reacted with Au at pH 12. This figure has been modified from Dixon, J. M. & Egusa, S. *J. Am. Chem. Soc.* **140** 2265-2271, (2018). Please click here to view a larger version of this figure.

Discussion

The BSA-Au(III) compounds prepared at pH 12 exhibit red fluorescence at an emission wavelength of λ_{em}= 640 nm when excited with ultraviolet (UV) light λ_{ex}= 365 nm (**Figure 1A**). The emergence of red fluorescence is a slow process and will take a few days at room temperature to increase to a maximum intensity. Running the reaction at 37 °C will yield the optimum results, though higher temperature can be used to produce the red fluorescence faster. Irreversible degradation of the protein can occur at temperatures above 45 °C[23]. The adjustment of pH so that BSA transforms into its aged (pH > 10) conformation[21] ("A-form") is critical for red fluorescence; pH is finely adjusted from neutral to basic to determine the threshold of the occurrence of the red fluorescence (**Figure 1B, C**). For maximum red fluorescent intensity, the pH should be adjusted above 11. For red fluorescence, the pH can be adjusted beyond 11, although extremely basic (pH > 13) conditions can denature BSA and cause red fluorescence to disappear.

Varying the stoichiometric ratio of BSA and Au can illustrate the binding of Au to BSA. The fluorescence spectra of the BSA-Au(III) compounds vary depending on the stoichiometric ratios of BSA to Au (**Figure 2**). As the BSA to gold ratio is adjusted to 1:26, a maximum in red fluorescence intensity is observed at λ_{ex}= 500 nm. On the other hand, as the BSA:gold ratio is adjusted to 1:7, red fluorescence is observed primarily at λ_{ex}= 365 nm. No red fluorescence can be detected at a ratio of BSA to Au less than 1:7 or above 1:52. The minimum number of gold cations required to produce the red fluorescence is less than 7 and more than 1, and the maximum number for the loss of the red fluorescence is greater than 52 (**Figure 2B, C**). Additionally, the reduction of all aforementioned samples will occur under excess sodium borohydride, elucidating that all samples still contain cationic Au(III). Furthermore, the addition of excess amounts of gold beyond 20 mM can cause the solution to become too acidic and denature the protein. If protein denaturation occurs due to high acidity, reduce the concentration of BSA and Au relatively to mediate this issue.

Competitive binding of Au and other metal cations to BSA can illustrate the binding sites in BSA. It is known that Cu(II) and Ni(II) both bind to the Asp fragment at the N-terminus of BSA[24,25,26,27]. Through the addition of Cu(II), a strong binder to the Asp-fragment, followed by the addition of Au(III), the absorbance spectra of BSA-Cu(II)-Au(III) and the absorbance spectra of BSA-Au(III) and BSA-Cu(II) are the same - indicating that gold and copper do not compete for the same binding site at the Asp-fragment (**Figure 3C**). Ni(II) binds weakly to the Asp-fragment and therefore Au(III) competes with Ni(II) as gold is added; it has been observed that the absorbance spectra of BSA-Ni(II) and BSA-Au(III) does not correlate with that of BSA-Ni(II)-Au(III) (**Figure 3F**). Through the above protocol, one can show how Au(III) binds to the known binding site of BSA. This technique also requires the adjustment of pH above 11 and the technique is modified by adding twice the concentration to BSA but at half volume, thus this should be performed at low BSA to Au ratios to maintain the protein conformation.

Modifying Cysteine residues in BSA can further elucidate the Au binding sites. Gold is known to have a high affinity for thiol[28] and BSA possesses a surface accessible thiol on (Cys34)[21]. Through the blocking of this thiol, secondary binding sites can be elucidated. The blocking of this cysteine is carried out prior to the addition of Au(III) to the sample and shows an altered fluorescence pattern of BSA-Au(III), indicating a possible transfer pathway involved in the mechanism of the red fluorescence (**Figure 4A**). It is imperative to add the thiol blocking agent, in this case NEM, at a neutral pH. Cleaving all disulfide bonds and the subsequent capping of their free thiol groups reveals no red fluorescence (**Figure 4B**). These results indicate that a disulfide bond is required to produce a red fluorescent complex.

We have demonstrated in various protocols, through the use of spectroscopic and protein chemistry techniques, a method to analyze the BSA-Au(III) complexes. Protein chemistry techniques presented herein have not been widely used in the protein-based nano-materials research[14]. These techniques can be generally applicable and be valuable to understand the metal binding process and the possible binding sites in other, if not all, proteins such as trypsin, pepsin, lysozyme, and transferrin[17,29]. Proteins are dynamic, yet very precise "nano-materials". Detailed understanding of the metal binding site could pave the way towards new protein-based materials with controlled optical properties with myriad of potential applications.

Disclosures

The authors have nothing to disclose.

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