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Gold:Protein Stoichiometric Analysis of Albumin-Stabilized Gold Nanoclusters

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Michel

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14. ABSTRACT <p>The field of bionanomaterials presents an opportunity to leverage the unique functionality inherent within the individual bio and nanocomponents to enable novel hybridized functionalities. Protein-stabilized nanoclusters are a primary example of such a bionano hybrid system, where enhanced coupled functionality has been demonstrated for sensor applications. Currently, however, the factors that control whether a protein component retains its activity are not well understood, but are believed to primarily relate to the location of the cluster stabilization within the host protein. In this work, foundational efforts toward identifying the stabilizing ligands coupled to the nanocluster are performed. Specifically, the gold:protein stoichiometry of a bovine serum albumin-nanocluster hybrid is characterized and purification strategies are optimized to allow for subsequent ligand identification routes.</p>					
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1. Introduction

Fluorescent gold-nanoclusters (AuNCs) have the potential to be used as novel imaging agents¹ and sensors^{2,3} due to their distinct highest occupied molecular orbital–lowest unoccupied molecular orbital transition than better-studied gold nanoparticles.⁴ Among the AuNCs, protein-stabilized nanoclusters (PNCs) are subnanometer-sized particles that are formed via a simple one-pot reaction process that is initiated with a protein of interest.⁵ A number of applications for PNCs have been suggested including as fluorescence sensors of in situ cellular processes because of their lack of toxicity in biological settings.⁶ Currently, however, the molecular properties of PNCs are not well understood, and there is a limited understanding of their local structure. The field of PNCs is fairly small, with only handful of published examples with varying degrees of characterization.⁷ Within these examples, there is variability with respect to protein activity. In some cases, the protein retained native structure and activity when converted to the PNCs, and in others, the activity was diminished.⁸ The factors that control PNC activity are not well understood, including the gold:protein stoichiometry. Here, we report the synthesis and characterization of a bovine serum albumin (BSA)-AuNC using of a combination of liquid chromatographic and mass spectrometric application to determine gold:BSA stoichiometry.

2. Experimental Procedure

2.1 Materials

Milli-Q water was used for all solutions. All chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri) and used without further purification except unspecified one. Primary chemicals are hydrochloric acid, chloroauric acid (HAuCl₄), ascorbic acid, and sodium hydroxide (NaOH).

2.2 Synthesis of BSA-AuNCs

As shown in Fig. 1, the synthesis of BSA-AuNCs is a straightforward procedure involving the direct mixing of protein and metal precursors at select ratios and mixing at an elevated pH.

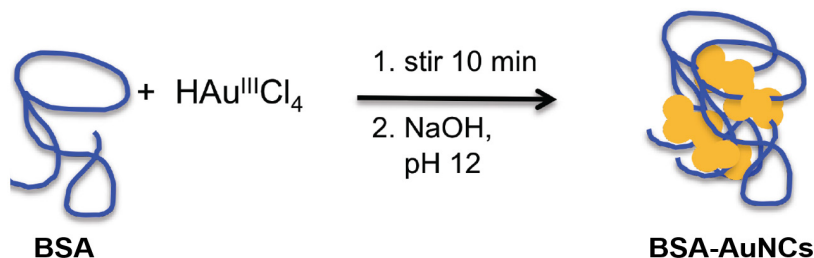


Fig. 1 Synthesis scheme of BSA-AuNCs

In brief, a mixture solution of 2 mL of 50-mg/mL BSA with 2 mL of 10-mM HAuCl_4 was stirred vigorously at 37 °C for 10 min and the measured solution pH was 3. The pH of the mixture was adjusted to 12 by adding 10 μL of 6-M NaOH and stirred vigorously for 2 h at 37 °C. After 2 h, to exclude excess gold salts, the reaction mixture was spin-filtered by 4-mL Amicon ultra centrifugal filter (30 kDa MW cutoff) by tabletop centrifuge with 5,000 g's for 30 min. A filtered solution of BSA-AuNCs was diluted up to 1 mL by water and stored at 4 °C.

2.3 UV-Visible (UV-Vis) Spectroscopy and Circular Dichroism

Full scanned absorption spectra were obtained via a Perkin-Elmer Lambda 25 UV-Vis spectrophotometer with screw-capped quartz cuvette (Starna Cells). The far-UV circular dichroism (CD) analyses were performed via a JASCO-810 spectropolarimeter using a 1-mm path length quartz rectangular cuvette (Starna Cells). CD data were collected over the wavelength range of 180–280 nm, with a scan rate of 100 nm/min at 25 °C. Five scans were obtained for each point, and the average was determined and smoothed.

2.4 Emission Fluorescence Measurement

To measure emission fluorescence of synthesized BSA-AuNCs, an ISS K2 spectrofluorometer, configured in the L format, was utilized. Emission scans were performed at a range of 560–700 nm with the excitation wavelength/bandpass at 365 nm/1 nm in water solution at room temperature. The cuvettes used were Spectrosil far-UV quartz window fluorescence cuvettes (Starna Cells).

2.5 Protein Quantitation of BSA-AuNCs

Coomassie Plus Reagent (Pierce) for Bradford protein assay was performed by following the product manual. A series of diluted BSA samples was used to generate standard protein curve in range of 0.125–1.50 mg/mL.

2.6 Direct Measurement of [^{197}Au] via Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

ICP-MS was used to measure [^{197}Au] of BSA-AuNCs. The concentration of gold ion in isolated protein samples was determined by injecting samples into an Agilent 7700X ICP-MS instrument (Agilent Technologies, Santa Clara, California), [^{197}Au] ions were determined using an RF power of 1550 W, helium gas flow of 4.3 mL/min, an argon gas flow of 0.99 L/min, octopole RF of 190V, and an OctP bias of -18V . The AuNC sample was directly infused using the 7700X peristaltic pump with a speed of 0.1 rps and a micromist nebulizer. The [^{197}Au] concentration was derived from a calibration generated by a series of dilution of atomic absorption standard (Fluka Analytical) prepared in the same matrix. Data analysis was performed via Agilent's MassHunter software.

2.7 Liquid Chromatography Coupled ICP-MS

To separate the gold-containing BSA-AuNCs, high performance liquid chromatography (HPLC) equipped with size exclusion column (SEC) is directly coupled with an ICP-MS. The stock BSA-AuNC sample was diluted by 10-mM ammonium acetate at pH 7.4 and injected into an Agilent 1260 HPLC equipped with a BIO SEC-3 Guard column (3 μm , 300 \AA , $4.6 \times 50\text{ mm}$) and an AdvancedBio SEC column (2.7 μm , 300 \AA , $4.6 \times 50\text{ mm}$) in series. Eluent from HPLC was directly connected to the 7700X ICP-MS peristaltic pump with a micromist nebulizer. The [^{197}Au] concentration was then measured and data analysis conducted by MassHunter software (Agilent).

3. Results

3.1 UV-Vis and Circular Dichroism

Synthesized BSA-AuNCs resulting from the reported synthetic procedure were scanned by a Perkin Elmer UV-Vis photospectrometer and a JASCO far-UV circular dichroism. As shown in Fig. 2, UV-Vis absorbance of synthesized BSA-AuNC showed a different absorption pattern at the 280-nm region with its reference protein due to an AuNCs formation. The CD experiment result demonstrated that the BSA-AuNC secondary structure is conserved after forming AuNCs with BSA. Although other protein-stabilized AuNCs results support protein secondary structure loss upon formation of AuNCs, this result, unlike smaller molecular weight proteins such as trypsin (23 kDa) and insulin (5.8 kDa), suggests that the BSA-AuNCs secondary structure can be similar to its reference BSA (66 kDa). The conserved secondary structure of BSA-AuNCs implicates that the

protein used for stabilizing AuNCs might have their physiological functions after formation of AuNCs.

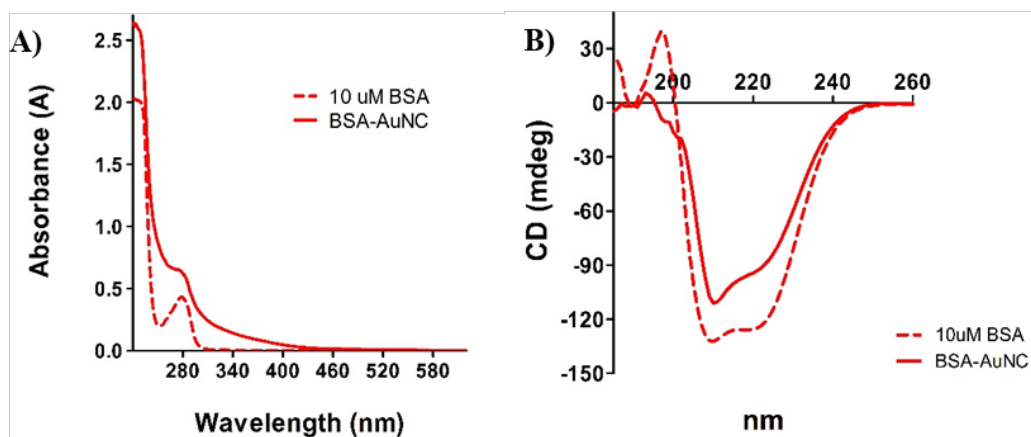


Fig. 2 A) UV-Vis absorption pattern comparison between reference BSA and BSA-AuNCs. Formation of BSA-AuNCs causes different UV-absorption pattern in 280-nm region. B) The far-UV CD scans were performed to examine the secondary structural change by the formation of AuNCs.

3.2 Emission Spectrum of BSA-AuNCs

To confirm that the synthesized BSA-AuNC was fluorescently active (Fig. 3b), emission scanning was performed using an ISS K2 spectrofluorophotometer. As previously published, the synthesized BSA-AuNC exhibited an emission wavelength peak at 630 nm (excitation at 365 nm). This emission wavelength is dependent on pH. It is observed at high pH (11–12) and lost as the pH is lowered (Fig. 3a). BSA is known to maintain its structure between pH 7 and 7.5 and lose structure at pH 12. How the structure is affected in the context of the nanocluster requires further study.

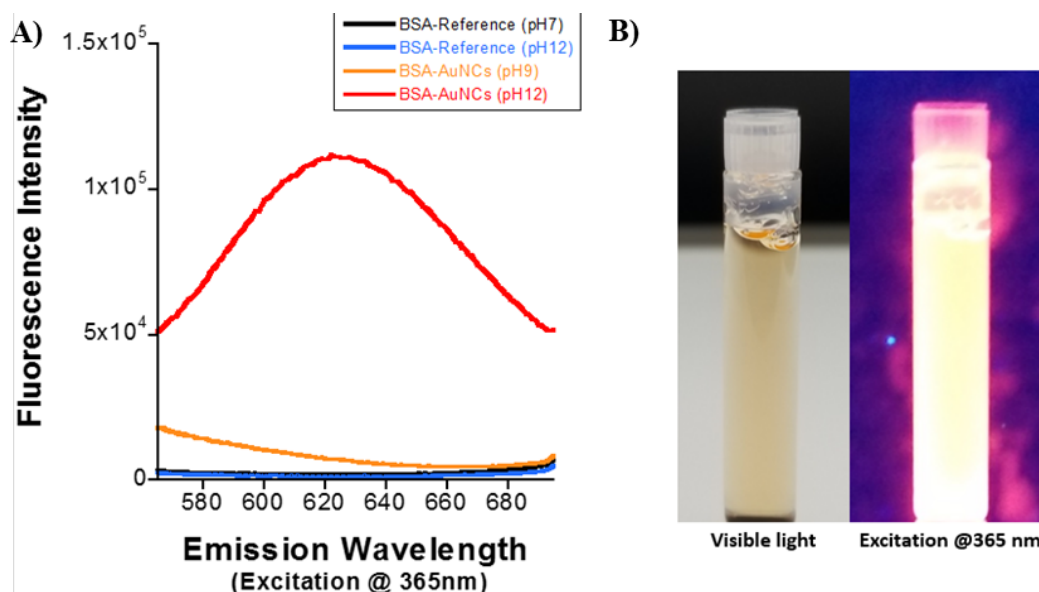


Fig. 3 A) Emission wavelength peak at 630 nm is a typical feature of the BSA-AuNCs formed. Emission of BSA-AuNCs is dependent on solution pH. B) Visual change in BSA-AuNCs under visible light (left), and its emission fluorescence excited at 365 nm (right).

3.3 Direct Measurement of [¹⁹⁷Au]

To quantify the gold content of the PNC, ICP-MS was performed using diluted BSA-AuNC samples. The dilution factor was 41,250 due to the detection upper limit of [¹⁹⁷Au] by 50–100 ppb. The Bradford method was used to quantify the protein. By comparing the gold and protein concentrations, the stoichiometry was then determined. The results shown in Table 1 are similar to those that have been reported using electrospray ionization mass spectrometry (ESI-MS).

Table 1 [Au]:[BSA] equivalents of BSA-AuNC estimation based on [¹⁹⁷Au] and BSA concentration measured by direct ICP-MS and Bradford protein assay

ICP-MS [¹⁹⁷ Au] (ppb)	ICP-MS [¹⁹⁷ Au] (μM)	Bradford assay [BSA] (μM)	[Au] vs. [BSA] (equivalents)	Reported BSA-AuNC equivalents
22.01 ^a	4069.3	556.7	7.31	9

^a Dilution: 1/41,250

3.4 Liquid Chromatography Coupled ICP-MS

To determine the purity of the gold-containing BSA-AuNCs, an Agilent 1260 HPLC with SEC, directly coupled with ICP-MS, was used. Multiple protein-containing species (based upon a 280-nm protein absorbance) were observed. These data show that additional purification may be necessary to purify PNCs.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to confirm this separation for the fractions (Fig. 4).

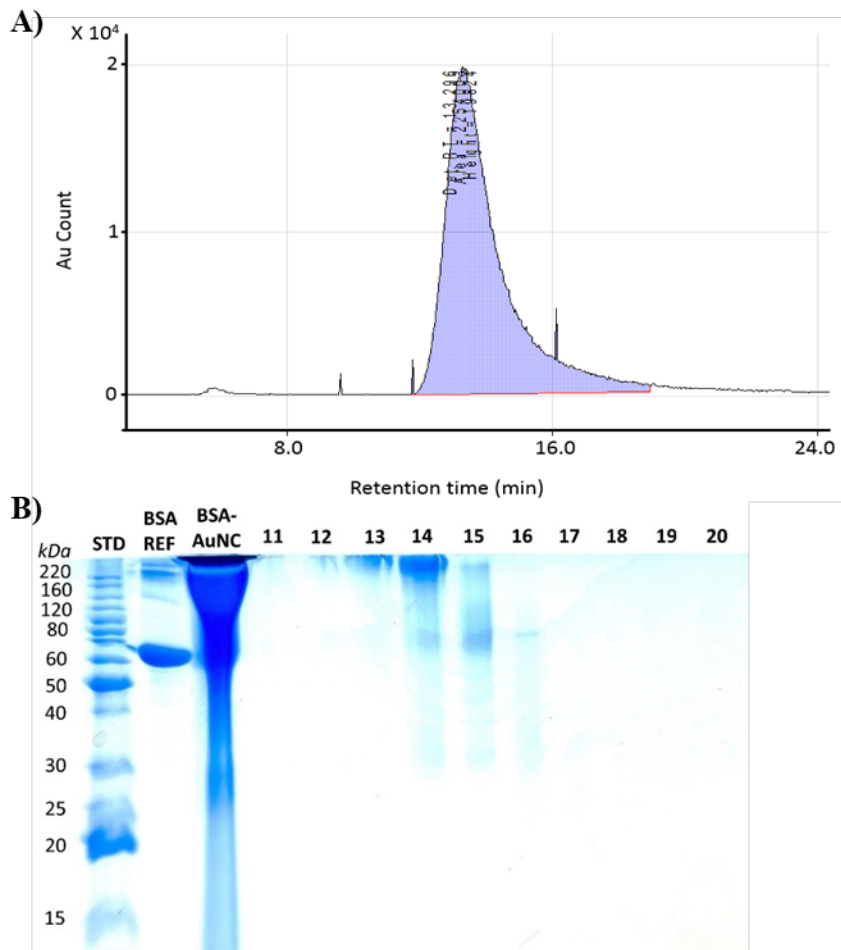


Fig. 4 A) Liquid chromatograph ICP-MS analysis of diluted BSA-AuNC sample. The majority of [^{197}Au] were detected at 12–15 min of retention time. B) Different molecular weight distributions among the reference BSA, BSA-AuNC, and size exclusion chromatography fractions of BSA-AuNCs by SDS-PAGE.

4. Conclusions

In this report, we synthesized BSA-AuNC and analyzed the gold distribution using ICP-MS coupled with size exclusion chromatography. The AuNCs formed with BSA retained the secondary structure confirmed by CD. It may be dependent on the size of the host protein (BSA: 66 kDa), suggesting that PNC's functional structure is able to be maintained. In addition, BSA-AuNCs showed a pH dependent emission fluorescence. This result implicates that lowering pH can disrupt AuNCs formation, isolating the pH setting as an important factor for future application of PNCs. A direct measurement of [^{197}Au] in BSA-AuNCs estimated the stoichiometry between gold and BSA is around 7.3, which is in comparable

agreement to the resultant ratio of 9 using ESI-MS analysis. The liquid chromatography ICP-MS and SDS-PAGE results supported the evidence of BSA-AuNCs have multiple-sized protein formed with AuNCs and presents the purification pathway required to allow for subsequent gold-bound ligand identification.

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List of Symbols, Acronyms, and Abbreviations

Au	gold
AuNC	gold nanocluster
BSA	bovine serum albumin
CD	circular dichroism
ESI-MS	electrospray ionization mass spectrometry
HAuCl ₄	chloroauric acid
HPLC	high performance liquid chromatography
ICP-MS	inductively coupled plasma mass spectrometry
NaOH	sodium hydroxide
PNC	protein nanocluster
RF	radio frequency
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	size exclusion column
UV	ultraviolet
Vis	visible

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