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Single bovine serum albumin molecule can hold plural blue-emissive gold nanoclusters: A quantitative study with two-photon excitation

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ABSTRACT

Two-photon luminescence properties of gold nanoclusters (Au_8NCs) encapsulated in bovine serum albumin (BSA) were investigated. A new synthesis protocol is reported to control the number of Au_8 clusters in one BSA. Both one- and two-photon excitation of BSA with multiple Au_8NCs gave an identical blue luminescence. The two-photon absorption (TPA) cross-section value for BSA with one Au_8NCs was obtained to be 190 GM (Goeppert-Mayer) and 540 GM for BSA with three Au_8NCs, at 740 nm excitation wavelength. This study shows that BSA with multiple Au_8NCs may be used as a biocompatible luminescence probe for two-photon *in-vivo* imaging.

1. Introduction

Synthesis of small noble metal nanoclusters (NMNCs) with controllable number of metal atoms has been an active area of research due to potential applications of such NCs in bioimaging, sensing, photonics and catalysis [1,2]. Atomically precise small NMNCs are traditionally prepared using thiols, dendrimers or specific oligonucleotide sequences as a capping agent [3–7]. Such ligand-stabilized NCs have size-tunable optical properties and show nonlinear optical (NLO) phenomena [6,8]. Two-photon absorption and emission properties of NMNCs have been studied in detail in connection with two-photon excitation imaging and fundamental investigations of NLO effects observed in NMNCs [8,9]. High two-photon absorption (TPA) cross-section values were reported and theoretically predicted for Au and AgNCs [10–12]. Goodson's group reported TPA cross-section value of 2 700 GM Goeppert-Mayer, 1GM = (10^{-50} cm^4 s $photon^{-1}$) for thiol-stabilized Au_{25}NCs, at 1290 nm excitation wavelength measured in hexane. In the near-infrared region (800 nm) TPA cross-section for the same cluster reached 427 000 GM. Antoine et al. reported TPA cross-section values for small glutathione-protected Au_{15} and Ag_{15}NCs [9,11]. The TPA cross-section for $Au_{15}SG_{13}$ was reported to be 65 700 GM and 64 GM for $Ag_{15}SG_{11}$ at 780 800 nm excitation wavelength. Such high TPA cross-section values, especially for AuNCs, make them promising candidates as fluorescent probes in two-photon excitation microscopy (TPEM) [13]. TPEM is especially attractive for *in-vivo* imaging as it offers a higher penetration depth, and reduced cellular autofluorescence and photodamage [14,15]. As a consequence, development and characterization of small, biologically friendly two-photon fluorescent probes with high stability and brightness is highly desired.

Biologically compatible, water soluble NMNCs have been prepared using capping with specific oligonucleotide sequences. Complicated and lengthy procedures to prepare such NCs prompted researchers to utilize proteins as a host matrix for synthesis of small metal NCs. Among various proteins, bovine serum albumin (BSA) or human serum albumin (HSA) has often been used as a host matrix [16–20]. Xie et al. reported synthesis of fluorescent BSA by mixing BSA with a gold precursor [17]. Blue-

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emitting Au_8 nanoclusters were obtained at acidic pH, and red-emitting Au_{25}NCs at highly alkaline pH. Recently, Chuang et al. reported microwave-assisted formation of blue- and red-emitting gold NCs in BSA [21].

Tunable emission properties of BSA-encapsulated AuNCs (AuNCs@BSA) make them attractive for use as fluorescent labels in *in-vivo* imaging, particularly for studying cellular transport of lipids or therapeutic drugs [22]. Despite many reports on AuNCs@BSA, little is known about their two-photon absorption/emission properties. Raut et al. reported a quadratic relation between excitation power and emission intensity for the red-emitting, plasmonic Au_{25}NC but no TPA cross-section has been determined, due to the difficulty of calculating the concentration of NCs in solution [23].

Here, we overcame this difficulty by careful control of $HAuCl_4$ concentration and of pH during synthesis. The concentration of BSA was kept constant while the concentration of $HAuCl_4$ was varied. This synthesis protocol allowed us to control the number of blue-emitting Au_8NCs encapsulated in a single BSA molecule and to calculate their TPA cross-section. The number of Au atoms present in one BSA was derived with the help of matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS). TPA cross-section values were thus evaluated for BSA with one, two and three Au_8NCs using a two-photon-induced emission measurement technique.

2. Experimental

2.1. Chemicals

Gold (III) chloride hydrate ($HAuCl_4 \times 3H_2O$, 99.99%), quinine sulfate (98%) and H_2SO_4 (98%) were purchased from Sigma-Aldrich. Bovine serum albumin, fatty acid free (98%), ascorbic acid (98.6%), sodium hydroxide (NaOH) and 2-(4-hydroxyphenylazo) benzoic acid (HABA) were obtained from Wako Chemical Co. All chemicals were used without further purification. Ultrapure water with a specific resistance of 18.2 MΩ was used throughout the experiments.

2.2. Synthesis of AuNCs@BSA

In the synthesis 5 ml of an aqueous $HAuCl_4$ solution was dropped slowly into an aqueous BSA solution (5 ml, 20 mg/ml) while stirring at 200 rpm. The synthesis details are shown in Scheme 1. The synthesis protocol is similar to that used by Guevel et al. but $HAuCl_4$ concentration is different (Guevel's, 10 mM) [16]. NCs were prepared with three different $HAuCl_4$ concentrations; 1.45, 2.89 and 5.86 mM. The initial pH of BSA solution before adding $HAuCl_4$ was around 7. Subsequent addition of $HAuCl_4$ lowered the pH to 4.4 for the 1.45 mM solution, 3.7 (for 2.89 mM); and 3.0 (5.86 mM). Afterwards, 50 μl of ascorbic acid (0.35 mg/ml) were added. In the next step, pH was raised back to 7 by slowly dropping NaOH (1 mM in water). During pH adjustment the mixture turned "milky" around the isoelectronic point of BSA ($pI = 4$) and gradually became clear upon returning to pH = 7. The solution was further stirred for 5 h at 37 °C, under a dark cover. After the reaction was finished, samples were dialyzed against water using a dialysis membrane with a molecular weight cut-off (MWCO = 2000 Da) for 12 h to remove free Au nanoclusters.

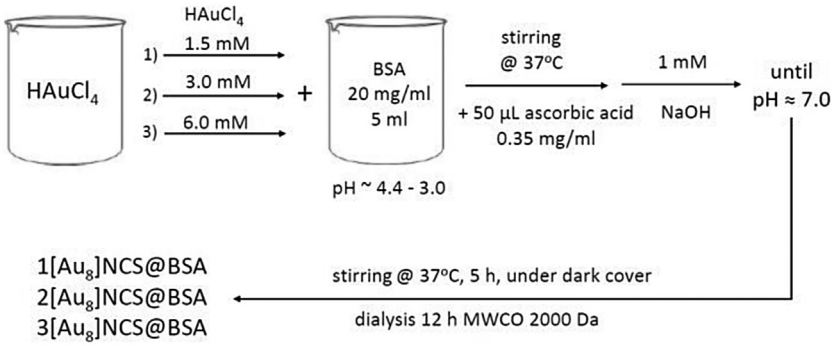
When preparing small AuNCs@BSA it is important to standardize the molarity of the $HAuCl_4$ used in the synthesis and to maintain pH so it never exceeds 7. In this work, concentration of $HAuCl_4$ was standardized by measuring the absorbance value of the solutions and calculating unknown concentration using the Beer-Lambert's law and reported extinction coefficient of $AuCl_4^-$ at 226 nm as 25600 M^{-1} cm^{-1} in acidic solutions [24].

2.3. Measurements

UV–vis absorption spectra were measured using a UV-3100 PC, Shimadzu spectrophotometer; and one-photon emission spectra using a Hitachi F-4500 fluorescence spectrophotometer. A solution of quinine sulfate in 0.1 N H_2SO_4 was used as a reference for quantum yield calculation.

For the measurement of two-photon emission spectra, samples were excited with a Ti sapphire laser Mai-Tai, Spectra-Physics (tunable 690–1040 nm, 155 fs, 80 MHz). The laser beam was focused on a solution contained in a quartz cuvette using lens with $f = 70$ mm. Two-photon emission spectra and decay curves were measured with a picosecond streak camera (Hamamatsu C10627, Hamamatsu Photonics Inc.). The fluorescence was filtered by the 2 cm path length of a 1 M $CuSO_4$ solution to completely remove residual excitation illumination.

Mass spectra of the synthesized AuNCs@BSA were measured by MALDI-TOF mass spectrometer, Shimadzu Axima CFR plus instrument, operated in a linear configuration mode. A pulsed N_2 laser of 337 nm was used to initiate ionization. The laser intensity was adjusted to the level at which no lower mass fragments were observed in the spectrum. A solution of 2-(4-hydroxyphenylazo) benzoic acid (HABA) (10 mg/ml in 50% ethanol) was used as a matrix. All mass spectra were recorded in the positive-ion mode, and averaged over 100 shots. In each experiment, a single drop (0.2 μl) of sample solution was dropped on the target plate well, followed by a drop (0.8 μl) of the matrix solution. The solution was mixed and dried before inserting a target plate into mass spectrometer unit. Mass calibration was done before series of experiments using BSA solution. Each mass spectrum was smoothed using five-point adjacent averaging in Origin software. The subsequent spectra were fitted using Gaussian fit. The mass resolution for m/z 66456 (BSA) was 800 (FWHM).



Scheme 1. Schematic representation of the formation of BSA with controlled number of Au_8NCs.