

Microwave-assisted synthesis and mechanistic study of multicolor emissive Au nanoclusters using thiol-containing biomolecules

Yong Yu¹, Preston Yi Jie Ching^{1,2}, Yen Nee Tan^{1,3*}

¹*Institute of Materials Research and Engineering, A*STAR (Agency for Science, Technology and Research),*

²*Fusion polis Way, 138634 Singapore.*

²*National Junior College, 37 Hillcrest Road, 288913 Singapore.*

³*Department of Chemistry, Faculty of Science, National University of Singapore, 3 Science Drive 3, 117543 Singapore.*

*Corresponding author

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Abstract

Developing effective synthesis method for few-atom gold nanoclusters (AuNCs) with tunable emissions is vitally important to gain better understanding of their formation mechanism and provide more design flexibilities to suite for various applications such as multiplex cellular imaging and/or light-emitting diodes. This paper reports a fast method (<30 minutes) of preparing multicolour (red, orange, near infrared) emissive AuNCs via a microwave-assisted biotemplating synthesis approach. A series of analytical tools such as UV-vis and photoluminescence spectroscopies, transmission electron microscopy (TEM) and polyacrylamide gel electrophoresis (PAGE) have been utilized to characterize the resultant AuNCs and unravel the formation mechanism that lead to their multicolour emissive properties. It was found that the surface charge of the thiol-containing peptide reagent and the peptide-to-Au ratio are crucial factors to control the size and emission colour of the resultant AuNCs. By bringing the solution pH to near the isoelectric point of peptide ligand (~3), the red ($\lambda_{em} = 725$ nm) emissive AuNC which is initially stable at alkaline conditions (pH12) tends to aggregate due to deficiency of surface charge, thus forming a larger and orange ($\lambda_{em} = 640$ nm) emissive AuNC. By further applying an even lower peptide-to-Au ratio to fine-tune the protection power of thiolate ligands, a near-infrared ($\lambda_{em} = 846$ nm) emissive AuNC has been synthesized. Compared to other microwave-assisted synthesis of AuNCs, current study is featured by its simplicity, rapidity, and versatility to tune the emission wavelength of resultant AuNCs in a much broader range (up to 206 nm). Copyright © 2018 VBRI Press.

Keywords: Gold nanoclusters, tunable emission, microwave-assisted synthesis, biotemplating.

Introduction

Gold nanoclusters (AuNCs) with a few to a hundred Au atoms have shown great promise in biological sensing [1-4] and imaging [5-9] due to their ultra-small size (<2 nm), bright photoluminescence, good photo stability, and low toxicity. These important applications further stimulated the interest of developing effective synthesis methods for fluorescent AuNCs [10-13]. For instance, a biotemplating synthesis approach which utilizes biomolecules such as peptides and proteins to direct the synthesis of fluorescent AuNCs is of particular interest thanks to its inherent biocompatibility and easy functionalization for biological applications [14-15]. In a typical biotemplating method, Au ions are first sequestered by the functional groups present in the protein or peptide, followed by reduction enabled by the biomolecule itself without the use of an exogenous reducing agent. However, such synthesis process usually takes a relatively long reaction time (hours to days), which restricts the fast acquiring and

implementation of fluorescent AuNCs for the intended applications.

More recently, microwave irradiation has been introduced in the biotemplating method for more effective synthesis of fluorescent AuNCs. In a microwave-assisted synthesis, microwave energy is directly coupled with the polar molecules (e.g., water and reagents present in the reaction mixture) to produce efficient internal heating. As such, ultrahigh temperature can be achieved at ambient conditions in a few seconds which greatly accelerates the reaction kinetics to complete the synthesis within a short period of time (from minutes to an hour). Although there are a few studies of combining microwave-assisted method with biomolecules as templates for the synthesis of AuNCs, most of them produce only the AuNCs with single emission color which limit the understanding of their formation mechanism and design flexibilities for specific applications. For example, red emissive AuNCs were synthesized by using lipoic acid [16] or BSA/HSA [17-18] as template. Yellow emissive NCs were

reported by alloying Ag in BSA-templated AuNCs [19]. Recently, Zhang et al monitored the emission wavelength of a series of AuNCs collected during the course of a microwave-assisted synthesis and observed that the emission of AuNCs could be varied from red (~670 nm) to orange (~605 nm) as the irradiation time increased from 10 minutes to 3 hours [20]. However, the quantum yield of those intermediate AuNCs was much lower (e.g., QY 0.42% for AuNC collected at 10 minutes) than that of the final product (i.e., AuNC collected at 3 h; QY 2.4%). In addition, the tunable range in emission wavelength was only 65 nm.

Herein, we report a rapid microwave-assisted synthesis of multicolor emissive AuNCs (~30 min) with a broad range of tunable emission wavelength (up to 206 nm) using simple thiol-containing biomolecules, e.g., tripeptide glutathione (GSH) as the template. The control of emission color is achieved by varying the pH of reaction solution to regulate the overall surface charge of the peptide reagent and the peptide-to-metal ratio. By lowering the solution pH to near the isoelectric point of the GSH peptide, the red emissive AuNC ($\lambda_{em} = 725$ nm) initially stable at a higher solution pH is transformed to orange emissive one ($\lambda_{em} = 640$ nm). By decreasing the peptide-to-metal ratio further, the emission color of AuNC can be tuned to the near infrared (NIR) region ($\lambda_{em} = 846$ nm), a biological window where adverse effect of tissue auto fluorescence can be greatly minimized for bio imaging application. The size and optical properties of AuNCs corresponding to different reaction conditions are examined closely to reveal mechanistic insights for the change of emission color. The effect of microwave cleavage of peptide ligand was also studied by replacing GSH with its thiol containing subunits. Present below are the details of this study.

Experimental

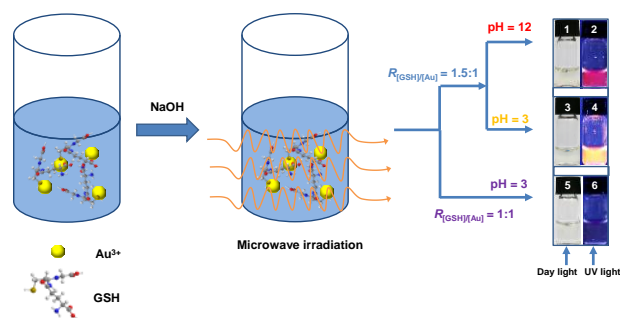
Materials

Gold (III) chloride solution (HAuCl₄, 30% in dilute HCl), glutathione reduced (GSH, 97%), and L-cysteine (Cys, 97%) were purchased from Sigma-Aldrich. Customized peptides Cys-Gly and γ -Glu-Cys were supplied by Sigma-Aldrich. All reagents were used as received and without further purification. All glassware was washed with Aqua Regia (HCl: HNO₃ volume ratio = 3:1) and rinsed with ethanol and ultrapure water. (Caution: Aqua Regia is a very corrosive oxidizing agent, which should be handled with great care.) Ultrapure water with a specific resistance of 18.2 M Ω was used throughout the experiment.

Material synthesis

Tripeptide γ -Glu-Cys-Gly or GSH was selected as a model thiol-containing biomolecule for the synthesis of multicolor emissive AuNCs. Aqueous solutions of HAuCl₄ (25 μ L, 20 mM) and GSH (25/37.5/50 μ L, 20 mM) were mixed with a GSH-to-Au ratio ($R_{[GSH]/[Au]}$) of

1:1, 1.5:1 or 2:1 in a 1.5 mL glass vial under vigorous, constant stirring. The reaction mixture was topped up to 1 mL with ultrapure water. After that, the solution pH was adjusted with NaOH (1 M) to 3, 9 and 12 prior to microwave irradiation. The synthesis was carried out in a domestic Cornell DMO-4099 Microwave Oven under intermittent irradiation for 4 \times 1 minute at 385 W. The synthesis conditions and appearance of resulting AuNCs are summarized in **Scheme 1**. Specifically, the red emissive AuNC (r-AuNC) was formed with $R_{[GSH]/[Au]} = 1.5:1$ at pH12 and was collected right after microwave heating. The orange emissive AuNC (o-AuNC) was formed with a same $R_{[GSH]/[Au]}$ as the red emissive one but at a lowered solution pH (i.e., 3), and was collected 30 minutes after irradiation. The near-infrared emissive AuNC (NIR-AuNC) was synthesized with a lower $R_{[GSH]/[Au]}$ (1:1) at pH3 and was also collected immediately after microwave irradiation. All samples are centrifuged at 8,000 rpm for 10 minutes to remove any large particles.



Scheme 1. Schematic diagram of the microwave-assisted synthesis of (1, 2) red, (3, 4) orange, and (5, 6) near-infrared emissive AuNCs.

Characterizations

Unless otherwise specified, UV-vis absorption and photoemission spectra of gold nanoclusters were obtained on a Tecan microplate reader. The photoemission spectrum of near infrared emitting AuNC was measured on a Horiba Jobin Yvon Fluorolog-3 spectrofluorometric. The quantum yield (QY) of AuNCs was measured using a 370 nm Xenon laser as light source and 4-(Dicyanomethylene)-2-methyl-6-(4-dimethylaminostyryl)-4H-pyran (DCM) as reference. The QY of AuNCs was calculated based on a comparative method and using Equation (1) below.

$$Q_S = Q_R \times \frac{k_S}{k_R} \times \frac{n_S^2}{n_R^2} \quad (1)$$

where Q_R is the known quantum yield of the reference compound, k is slope of the lines obtained from the plot of the integrated fluorescence intensity ($\lambda_{ex} = 370$ nm) vs. absorbance (at 370 nm), n is the refractive index of solvent, and the subscripts S and R refer to sample and reference, respectively. Transmission electron microscopy (TEM) images were taken on a JEOL JEM-2010 microscope operating at 200 kV. Native

polyacrylamide gel electrophoresis (PAGE) was carried out on a Bio-Rad Mini-PROTEAN Tetra Cell system. Stacking and resolving gels were prepared from 4 and 30 wt % acrylamide monomers, respectively. The electrophoresis was allowed to run for ~ 2.5 h at a constant voltage of 150 V.

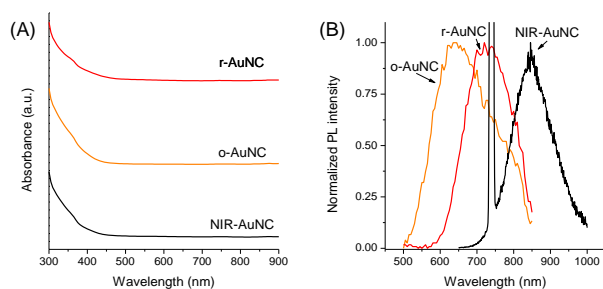


Fig. 1. (A) UV-vis and (B) photoemission spectra of red, orange, and near infrared emissive Au NCs synthesized by a microwave-assisted method using GSH tripeptide as template.

Results and discussion

Fig. 1A shows the UV-vis absorption spectra of the red, orange, and near-infrared emissive AuNCs synthesized by the microwave-assisted method using GSH as the model bimolecular template in this study. It is well known that large Au nanoparticles (>2 nm) show a distinct peak at ~ 520 nm on the photo absorption spectra due to the surface plasma resonance (SPR) of their conduction electrons [21]. However, no obvious peaks were observed on the UV-vis absorption spectra of any AuNCs in this study, which suggest the AuNCs are really small in size such that the Au atoms in these AuNCs are so few to support SPR. The molecular-like emissions of these AuNCs were further characterized by the photoluminescence measurement. The emission peaks were observed at an emission maximum (λ_{em}) of 725 nm, 640 nm, and 846 nm for the red, orange, and near-infrared emissive AuNCs, respectively (**Fig.1B**). These emission peaks were not observed in the control experiments with only GSH, or gold chloride included in the reaction solution, which confirmed that the emissions were exclusively from the as-formed AuNCs in the respective reaction mixture. The quantum yield of the red, orange, and near-infrared AuNCs was calculated to be 2.56%, 3.12%, and 2.69%, respectively.

Fig. 2 show the TEM images and size distribution histograms of the as-synthesized red, orange, and near-infrared emissive AuNCs. As expected, all the three types of AuNCs are very small in size and most particles are within the sub-2 nm size range. Further analysis of the size distribution gave an average size of 0.53 ± 0.04 nm, 0.84 ± 0.06 nm, and 1.03 ± 0.03 nm for the red, orange, and near-infrared emissive AuNCs, respectively. Note that high resolution TEM images are not shown because the AuNCs tended to fuse into larger particles when they were shone with an electron beam with higher energies. Surprisingly, the sizes of the red, orange, and near-infrared AuNCs does not follow

the trend suggested by the previously reported Jellium model [22], in which the emission energy (E) of AuNCs is proposed to be inversely proportional to their sizes (N) via the relation $E = E_F / N^{1/3}$, where E_F is the Fermi energy of the bulk metal (i.e., Au).

The unique size-emission correlation of these multicolor emissive AuNCs has motivated us to further examine the synthesis conditions and their formation mechanism. GSH is a naturally occurring tripeptide (γ -Glu-Cys-Gly) having two $-\text{COOH}$, one $-\text{NH}_2$, and one $-\text{SH}$ group. The thiol group is well known for its reducing capability to reduce Au(III) to Au(I) [23]. As a result, stable Au(I)-SG complexes are formed at ambient conditions (SG denotes the deprotonated thiolated anion of GSH). As microwave irradiation is extremely efficient, super heating can be built up in as short as seconds, these Au(I)-SG complexes could absorb the energy from the microwave irradiation, leading to the activation of the functional groups on the thiolates to donate electrons to Au(I) in forming Au (0). Thus, ultra-small AuNCs could be formed in minutes by the aggregation of these in-situ formed Au atoms.

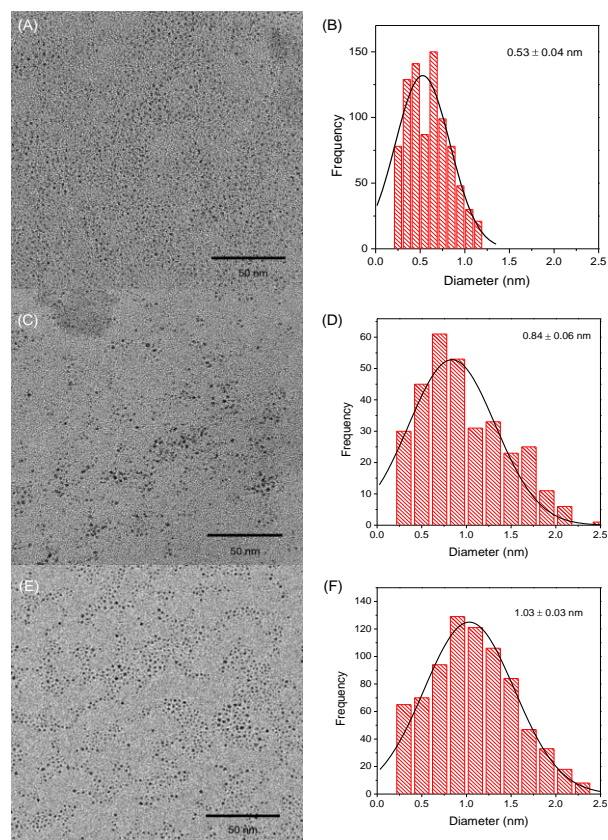


Fig. 2. (A, C, E) TEM images and (B, D, F) size histograms of (A, B) red, (C, D) orange, and (E, F) near infrared emissive Au NCs.

As GSH has three types of functional groups with different pK_a values (**Fig. 3A**), the impact of the solution pH to the formation of different emissive AuNCs was further examined. Based on the molecular structure, GSH has an isoelectric point of 2.85. When the solution pH was 3, only the $-\text{COOH}$ group in Glu was deprotonated and the net charge of GSH was 0. As

a result, the GSH-protected AuNCs tend to aggregate into larger ones due to their poor dispersity arising from their charge neutral surface.

Interestingly, a red \rightarrow orange emission change (725 nm \rightarrow 640 nm) of the AuNC prepared at $R_{[\text{GSH}]/[\text{Au}]} = 1.5:1$ and pH3 was observed in about 30 minutes (**Fig. 3B**). Note that blue-shift in emission is typical for AuNCs with aggregation induced emission (AIE) behaviors. Clustering of the primary AuNCs was also observed in the TEM image of the orange emissive AuNC (**Fig. 2B**). Polyacrylamide gel electrophoresis (PAGE) analysis further confirmed the AIE properties of the orange emissive AuNC. A series of AuNCs with different sizes (i.e., $\text{Au}_{15}(\text{SG})_{13}$, $\text{Au}_{18}(\text{SG})_{14}$, $\text{Au}_{22}(\text{SG})_{16-17}$, $\text{Au}_{25}(\text{SG})_{18}$, $\text{Au}_{29}(\text{SG})_{20}$, $\text{Au}_{33}(\text{SG})_{22}$ and $\text{Au}_{39}(\text{SG})_{24}$) by using a previously reported protocol [24] were used as a reference to estimate the size of the orange emissive AuNCs. As shown in **Fig. 3C**, the orange emissive AuNC (lane II and IV) contains two prominent bands with a migration distance smaller than $\text{Au}_{29}(\text{SG})_{20}$ but larger than $\text{Au}_{39}(\text{SG})_{24}$, suggesting that these two species contain more than 29 but less than 39 Au atoms.

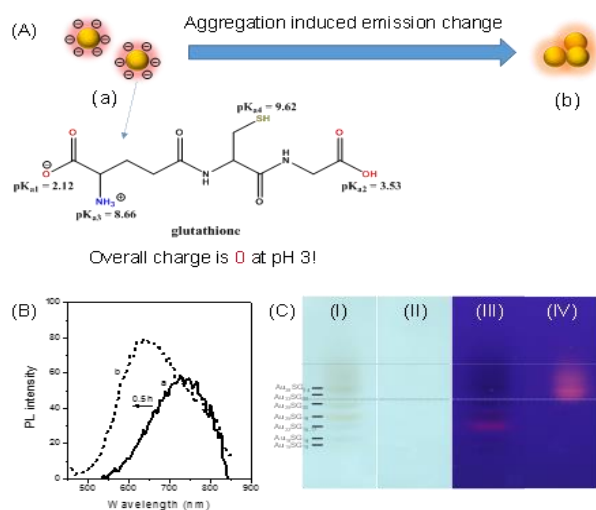


Fig. 3. (A) Scheme showing the emission change of AuNCs effected by charge induced aggregation (Reaction conditions: $R_{[\text{GSH}]/[\text{Au}]} = 1.5:1$, pH 3; a-immediately synthesized, b- after aging for 30 minutes). (B) Corresponding UV-vis spectra of the AuNCs in (A). (C) PAGE gels of (lane I, III) mix-sized $\text{Au}_n(\text{SG})_m$ prepared according to a published protocol, and (lane II and IV) the orange emissive AuNCs under (I, II) visible and (III, IV) UV light.

A smeared band with an even greater migration distance than $\text{Au}_{39}(\text{SG})_{24}$ was also observed, which suggested that some larger species were also presented in the orange emissive AuNC, but they couldn't be separated by PAGE at the investigated conditions. Nonetheless, all these species showed similar orange emissions regardless of their size difference, which is another characteristic of AIE-type AuNCs [25]. Therefore, it is concluded that the red emissive AuNCs were less stable at the isoelectric point of GSH, which gradually aggregate and reorganize into orange emissive AuNCs under the acidic conditions (pH 3).

When the solution pH was 12, all of the functional groups of GSH were deprotonated with a net charge of -2 . In this case, the negatively charged red emissive AuNCs is found to be more stable at high pH conditions (pH 12) with no color change observed during the course of reaction. It is therefore speculated that the surface charge of the AuNCs is critical to determine the emission color of the final product.

When $R_{[\text{GSH}]/[\text{Au}]}$ was further decreased to 1:1 while pH was kept as 3, NIR emissive AuNCs with the largest atomic size were observed. As the amount of GSH is relevant to the polymerization degree of the Au(I)-thiolate complexes, a lower $R_{[\text{GSH}]/[\text{Au}]}$ ratio will render the Au(I)-thiolate complexes to get reduced easier in forming more Au(0). On the other hand, a lower $R_{[\text{GSH}]/[\text{Au}]}$ ratio also gives less thiolate motifs available to cap on the in-situ formed Au(0), and thus a lesser protection to prevent the growth of in-situ formed AuNCs into larger particles. As a result, a higher chance of these Au(0) to collide with each other in the presence of limited protecting motifs lead to the formation of larger AuNCs that give the NIR emissions.

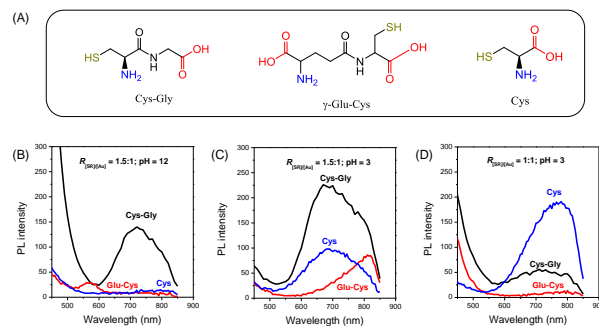


Fig. 4. (A) Molecular structures of Cys-Gly, γ -Glu-Cys, and Cys. (B-D) PL spectra of AuNCs synthesized with corresponding peptides/amino acids at various different reaction conditions (B: $R_{[\text{SR}]/[\text{Au}]} = 1.5:1$, pH12; C: $R_{[\text{SR}]/[\text{Au}]} = 1.5:1$, pH3; D: $R_{[\text{SR}]/[\text{Au}]} = 1:1$, pH3).

Other than the surface charge and relative strengths of the particle growth and passivation, the possible decomposition of GSH under microwave irradiation may also be accounted for the formation of multicolor emissive AuNCs. Failure *et al.* discovered that reduced GSH could be decomposed at sterilization temperatures to release amino acids other than forming oxidized form of GSH, i.e., GS-SG [26]. The super high temperature created by microwave irradiation in this work could also lead to the decomposition of GSH via cleavage at different amide bond sites to produce dipeptides or amino acids that might participate in the formation of AuNCs. To investigate the possible impact of these GSH derived peptides or amino acids on the formation of AuNCs, we replaced GSH with three possible thiol-containing compounds, i.e., Cys-Gly, Glu-Cys and Cys (only thiol-containing compounds were selected due to the essential role of strong Au-S interactions in the formation of AuNCs, see their molecular structures in **Fig. 4A**) in the microwave-assisted synthesis. At the same synthesis conditions for r-AuNC with GSH (i.e.,

$R_{[SR]/[Au]} = 1.5:1$, pH12; here H-SR denotes the respective thiol-containing compound), only Cys-Gly led to the formation of a red emissive AuNC with an emission maxima at ~ 720 nm while the other two did not produce any intensely fluorescent species (**Fig. 4B**). While at the same conditions for o-AuNC with GSH (i.e., $R_{[SR]/[Au]} = 1.5:1$, pH3), both Cys-Gly and Cys produced red emissive AuNCs with an emission maxima at ~ 685 nm, while a NIR emissive AuNC with an emission maxima at ~ 815 nm was formed for Glu-Cys under the same reaction conditions (**Fig. 4C**). Note that a blue-shift in emission maxima was observed for Cys-Gly when reaction pH was changed from 12 to 3, which is similar to the phenomenon observed for GSH. As Cys-Gly possesses no net charge at pH3 either, it might also arise from the surface charge mediated aggregation. At the same synthesis conditions for NIR-AuNC with GSH (i.e., $R_{[SR]/[Au]} = 1:1$, pH3), a NIR emissive AuNC with an emission maximum at 780 nm was also observed for Cys, the wavelength of which is 95 nm longer than that produced with Cys with a higher peptide-to-metal ratio at the same solution pH (i.e., $R_{[SR]/[Au]} = 1.5:1$ and pH3, **Fig. 4D**). This phenomenon is also corroborated with that observed in the case of GSH, which is due to weaker protection of less thiolate ligands. Only a weakly red emissive AuNC was formed for Cys-Gly ($\lambda_{em} = \sim 720$ nm) while no fluorescent species was formed for Glu-Cys. These results suggest the overall surface charge of peptide reagent and the peptide-to-metal ratio still dominate the emission color of final products even cleavage of GSH could occur under microwave irradiation.

Conclusion

In summary, we presented here the one-pot synthesis of multicolor (red, orange, and NIR) emissive AuNCs with a simple bio-occurring peptide in a microwave-assisted method. It was found that the overall charge of peptide played an important role in controlling the emission color of resultant AuNCs. When the solution pH was regulated near the isoelectric point and thus there was no net charge for the peptide ligand, the red emissive AuNC which was stable at a higher solution pH (12) aggregated into larger and orange emissive AuNC. Transmission electron microscopy (TEM), photoluminescence (PL) and polyacrylamide gel electrophoresis (PAGE) studies revealed that the blue-shift in emission color was due to the mechanism of aggregation induced emission (AIE). The emission color of AuNCs was further tuned to the near infrared (NIR) region by varying the peptide-to-metal ratio to control the relative strength of protecting thiolate ligands. Investigations of replacing GSH with its thiol-containing subunits suggest that the overall surface charge of peptide and peptide-to-metal ratio other than the sequence are more critical factors to control the emission color of resultant AuNCs. This study is of interest not only because it can supply AuNCs with tunable emissions in a simple and rapid way, but also

because it exemplifies the important role of overall surface charge of peptide reagent in determining the emission color of AuNCs, which can be simply controlled by varying the solution pH.

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Author's contributions

Conceived the plan: YY, YNT; Performed the experiments: YY, YJC; Data analysis: YY, YNT; Wrote the paper: YY, YNT. Authors have no competing financial interests.

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