

# Novel trimethyl lock based enzyme switch for the self-assembly and disassembly of gold nanoparticles†

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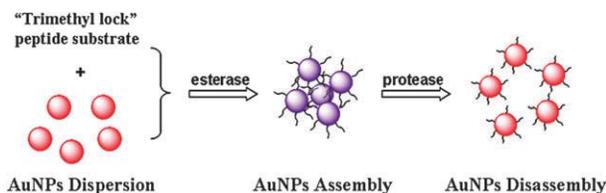
**A novel trimethyl lock based dual enzyme-responsive gold nanoparticles (AuNPs) conjugate was developed to control the self-assembly and disassembly of AuNPs in one population of nanoparticles, which can be easily monitored by naked eye, simple spectrophotometer and surface enhanced Raman scattering (SERS) measurements.**

The programmed self-assembly of gold nanoparticles (AuNPs) with controlled surface chemistry has attracted considerable attention for their potential applications in drug delivery and medical diagnosis.<sup>1</sup> A number of specific biomolecular recognition motifs such as complementary oligonucleotide hybridization, biotin-avidin binding and enzymatic catalytic reactions have been extensively exploited for the control of AuNPs assembly, which provide a simple and specific sensing platform for the systematic identification of a variety of molecular analytes including DNAs,<sup>2</sup> bacterial toxins,<sup>3</sup> proteins and enzymes<sup>4</sup> by performing colorimetric or surface enhanced Raman scattering (SERS) measurements. However, most of the molecular recognitions were mainly based on one-step AuNPs aggregation or dispersion and these assembly or disassembly processes were usually achieved in a separate population of gold nanoparticles. A few recognition processes with various degrees of continuous two-step self-assembly of AuNPs have been reported, in which the triggered changes in their assembled states were driven by a physical or chemical perturbation such as pH,<sup>5a-c</sup> temperature,<sup>5d</sup> light,<sup>5e</sup> concentration of inorganic/organic molecules,<sup>5f-h</sup> or fuelling oligonucleotides.<sup>5i-l</sup> However, the development of AuNPs network where the self-assembly and disassembly are associated with multiple enzyme stimuli within one population of nanoparticles has not been well-exploited yet.

In this study, we present a novel “trimethyl lock” based dual enzyme-responsive AuNPs conjugate which can be utilized to control the self-assembly and disassembly of AuNPs in the same population of nanoparticles (Scheme 1). The enzyme-responsive AuNPs conjugate in our design consists of two sections: (1). A unique “trimethyl lock” lactonization section

to release the peptide linker upon the esterase treatment, leading to the self-assembly of AuNPs; (2). A protease active section to cleave the peptide and induce further AuNPs disassembly (Scheme 2). Both esterase and protease are known to be abundant in nature and are essential for many important biological processes,<sup>6</sup> and they are also involved in various disease states such as HIV, cancer, Alzheimer’s and heart diseases.<sup>7</sup> Based on the specific enzymatic hydrolysis, both the self-assembly and disassembly of AuNPs can be achieved in the same nanoparticles system and this sequential two-step process will be easily monitored by naked eye, simple spectrophotometer and surface enhanced Raman scattering (SERS) measurements.

To demonstrate the proof of concept, we designed the self-assembled functionalized AuNPs network based on dual enzyme reactions. As shown in Scheme 2, a flexible 4-mercapto-phenylacetic acid modified thermolysin cleavable peptide (SH-Ph-CH<sub>2</sub>-Gly-Gly-Gly↓Phe-Gly-Gly-Lys(NH<sub>2</sub>)-CO-NH<sub>2</sub>) was connected to the carboxyl on the acetylated *o*-hydroxyphenolpropionic acids derivatives (See the ESI†), which are well known as “trimethyl lock” commonly used in “prodrug” development and fluorescent imaging,<sup>8</sup> to afford the thiol ester and amide bond at each end of the peptide sequence. The advantage of introducing “trimethyl lock” in this investigation is to achieve the effective esterase hydrolysis for the further release of the peptide linker based on lactonization reaction, and also to significantly minimize the self-aggregation of AuNPs caused by enzyme substrate itself as reported in previous study.<sup>4d</sup> Typically, the “trimethyl lock” peptide substrate (8.5 μM) was firstly incubated with esterase (1.25 μg mL<sup>-1</sup>) in monodispersed bis (*p*-sulfonatophenyl) phenylphosphane stabilized AuNPs suspension (pH 7.4) for 2 h. The enzyme hydrolysis catalyzed by esterase resulted in the cleavage of the thiolester bond and removal of acetyl group from the “trimethyl lock” peptide substrate. Unmasking of phenolic oxygen would facilitate the lactone formation

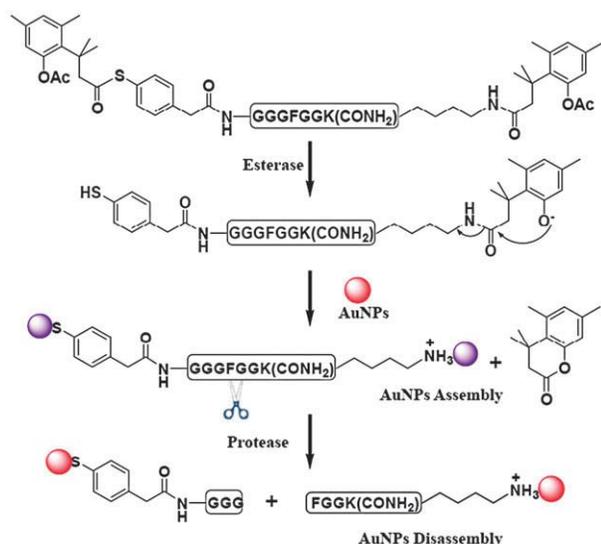


**Scheme 1** Schematic illustration for the self-assembly and disassembly of based on the dual enzyme reactions.

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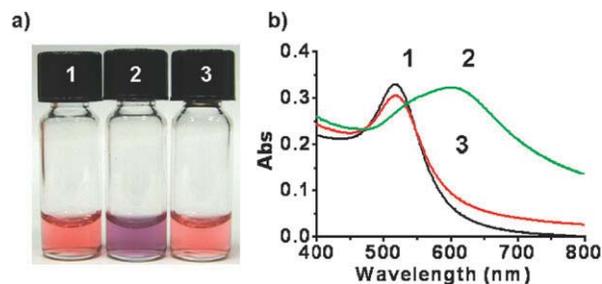
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**Scheme 2** Mechanism illustrating the self-assembly and disassembly of AuNPs by “trimethyl lock” peptide substrate, catalyzed by esterase and protease enzymes.

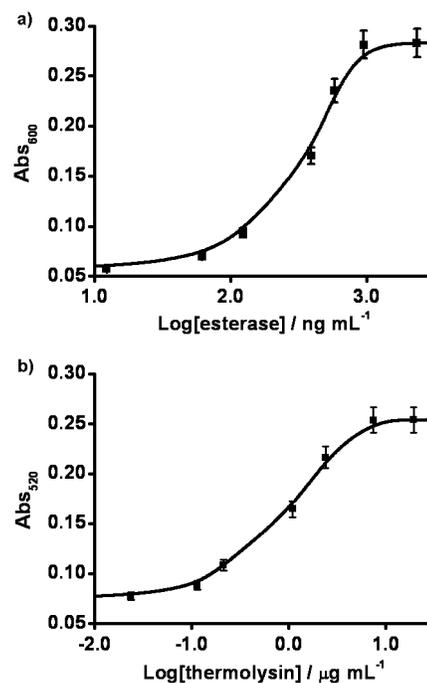
with concomitant release of peptide moiety previously attached to the carboxyl groups. The free thiol and positively charged amino group at each end of released peptide initiated the formation of aggregated AuNPs clusters through Au–S bond and electrostatic interactions with the functional groups on the surface of bis (*p*-sulfonatophenyl) phenylphosphane stabilized AuNPs,<sup>9</sup> thus resulting in the significant color change from red to blue. Meanwhile, both an increased absorption band at 600 nm and a decreased absorption at 520 nm were observed in the UV-Vis spectrum (Fig. 1, state 2). As a control, the incubation of AuNPs with only intact “trimethyl lock” peptide substrate could not induce any further color change and spectral shifts (Fig. 1, state 1), indicating the substrate was stable and the self-assembly of AuNPs was mainly from the esterase reaction. The state of AuNPs was observed to be highly aggregated with higher concentration of esterase and the absorbance reached a plateau when the enzyme concentration was higher than  $1 \mu\text{g mL}^{-1}$  (Fig. 2(a)). The minimum concentration of esterase that could induce the AuNPs self-assembly was found to be  $18.5 \text{ ng mL}^{-1}$ . In order to examine whether the second enzyme could disassemble the aggregated AuNPs, the aggregated AuNPs were further treated with protease thermolysin ( $60 \mu\text{g mL}^{-1}$ ) at  $37^\circ\text{C}$  for 4 h. The amide bond between Gly and Phe in the peptide sequence which was connecting to the AuNPs would be selectively recognized and cleaved. The AuNPs aggregation was interrupted and the disassembly of AuNPs resulted in the color change from blue to red and the maximum plasmon resonance peak shifted back from 600 nm to 520 nm (Fig. 1, state 3). The results demonstrated that addition of thermolysin could induce the redispersion of aggregated AuNPs in the solution. This disassembled process could be easily achieved with the thermolysin enzyme concentration as low as  $34.1 \text{ ng mL}^{-1}$ . The hydrolysis of “trimethyl lock” peptide substrate catalyzed by esterase or thermolysin was monitored by RP-HPLC (see the ESI†). All the results confirmed the active site of



**Fig. 1** Color image (a) and UV/Vis spectrum (b) for the self-assembly and disassembly of AuNPs induced by the enzyme reactions. State 1: AuNPs with “trimethyl lock” peptide substrate ( $8.5 \mu\text{M}$ ) only; State 2: AuNPs with esterase ( $1.25 \mu\text{g mL}^{-1}$ ) treated substrate ( $8.5 \mu\text{M}$ ); State 3: Aggregated AuNPs in state 2 treated with thermolysin ( $60 \mu\text{g mL}^{-1}$ ).

peptide substrate towards enzyme cleavage and the continuous AuNPs color change in one population of nanoparticles was from the specific enzyme recognitions. This process can be influenced by the concentration of trimethyl lock peptide substrate (see the ESI†).

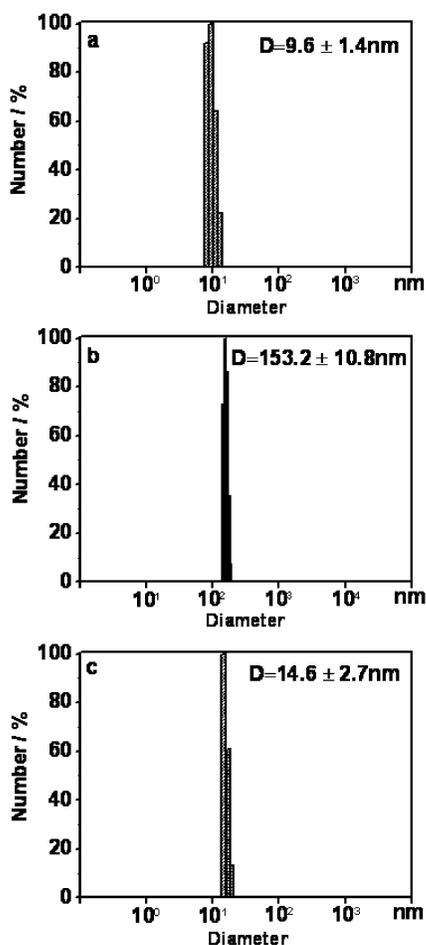
Further evidence of the AuNPs self-assembly and disassembly based on the dual enzyme reactions was obtained from dynamic light scattering (DLS). As shown in Fig. 3, the “trimethyl lock” peptide substrate itself was unable to induce the assembly of the AuNPs and the hydrodynamic size of the monodispersed AuNPs was determined to be 9.6 nm (Fig. 3(a)). Upon the esterase treatment, the specific lactonization of the “trimethyl lock” triggered the release of thiol and amino groups at each end of the peptide, which initiated the distinctive self-assembly of AuNPs. The nanoparticles



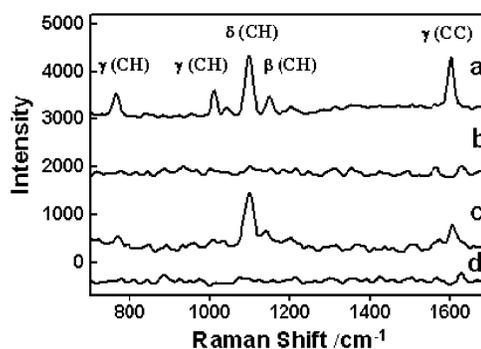
**Fig. 2** (a) Esterase concentration versus absorbance at 600 nm of AuNPs aggregation after addition of esterase treated “trimethyl lock” conjugate. (b) Thermolysin concentration versus absorbance at 520 nm of AuNPs redispersion upon hydrolysis of peptide linker.

aggregated into larger clusters with the hydrodynamic diameter increasing to 153.2 nm (Fig. 3(b)). Following the addition of thermolysin into the assembled AuNPs for 4 h, DLS showed a population of well-dispersed AuNPs with the average size reducing to 14.6 nm, indicating the disassembly of AuNPs based on the protease cleavage. Compared to the monodispersed AuNPs, the slight size increasing in the disassembled particles revealed the presence of cleaved peptide fragment on the surface of redispersed AuNPs (Fig. 3(c)). Transmission electron microscope (TEM) measurements were also performed to determine the different self-assembly and disassembly states of AuNPs (See the ESI†). The data revealed the dual enzymatic reactions were crucial for the continuous assembly and disassembly of AuNPs, which is consistent with the results in DLS and previous measurements.

It has been well known that resonant surface plasmon excitation of the free electrons in metal nanostructures can enhance localized electromagnetic fields around the surface. This enhancement effect becomes particularly strong in the interstitial spaces of aggregated nanoparticles, which is very suitable as platform for surface enhanced Raman scattering (SERS) studies.<sup>2b,c</sup> In order to monitor the self-assembly and



**Fig. 3** The hydrodynamic size and size distribution of AuNPs. (a), AuNPs with “trimethyl lock” peptide conjugate (8.5  $\mu\text{M}$ ); (b), AuNPs with esterase (1.25  $\mu\text{g mL}^{-1}$ ) treated “trimethyl lock” peptide conjugate (8.5  $\mu\text{M}$ ); (c), Aggregated AuNPs with thermolysin treatment (60  $\mu\text{g mL}^{-1}$ ).



**Fig. 4** Raman spectra of (a)  $10^{-1}$  M dipotassium bis (*p*-sulfonatophenyl) phenylphosphine dihydrate solution; (b) AuNPs stabilized with dipotassium bis (*p*-sulfonatophenyl) phenylphosphine; (c) Stabilized AuNPs with esterase treated substrate and (d) Aggregated AuNPs treated with thermolysin, Laser wavelength: 633 nm.

disassembly processes of AuNPs induced by the multiple enzyme interactions, the SERS enhancement measurements were performed directly by using stabilizer, dipotassium bis (*p*-sulfonatophenyl) phenylphosphine dihydrate as molecular reporter. As depicted in Fig. 4(b), the stabilized AuNPs without esterase treatment did not have plasmonic coupling and SERS signals from these dispersed particles were very weak. However, intense signals were detected from AuNPs aggregation upon the addition of “trimethyl lock” peptide conjugate treated with esterase (Fig. 4(c)). The observed SERS signals included the  $\nu$  (CH) at  $765\text{ cm}^{-1}$ ,  $\delta$  (CH) at  $1098\text{ cm}^{-1}$ , and  $\nu$  (CC) at  $1603\text{ cm}^{-1}$  which were in accordance with characteristic bands of stabilizer in aqueous solution (Fig. 4(a)).<sup>10</sup> After further treatment with thermolysin, the peptide sequence in the aggregate was cleaved and the aggregated AuNPs went back to the dispersed state and no SERS signals could be observed as shown in Fig. 4(d).

In conclusion, we have demonstrated a unique design for the control of self-assembly and disassembly of AuNPs on the basis of dual enzyme reactions in one population of nanoparticles. This process is illustrated by a series of enzymatic reactions in which randomly dispersed AuNPs are first converted into an aggregated structure, and subsequently to a redispersed state upon different enzyme treatments. Based on the consecutive color change from red to blue and finally to red again, visualization of the self-assembly and disassembly of AuNPs in the same nanoparticles system can be observed by the naked eye, simple spectrophotometer and surface enhanced Raman scattering (SERS) measurements. With more sophisticated design of the substrate or peptide sequence, this strategy could also be readily extended to other enzymatic systems. This sequential two-step assembly and disassembly process initiated by specific enzyme reactions can help us to understand the mechanisms of biomolecular recognitions. It may also have the potential to serve as a valuable platform for multiple enzyme detection and drug screening in biological studies or clinical settings.

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## Experimental

### Synthesis of “trimethyl lock” peptide substrate

To a stirred suspension of acetylated “trimethyl lock” compound (15.6 mg, 0.06 mmol) in 200  $\mu\text{L}$  anhydrous dichloromethane, thionyl chloride (43  $\mu\text{L}$ , 0.59 mmol) was added dropwise at 0  $^{\circ}\text{C}$  (ice bath). After addition, the reaction mixture was allowed to warm to room temperature and stirred for 18 h. The solvent was evaporated in vacuum to give the oil product without purification. Then, the purified thermolysin cleavable peptide (SH-Ph-CH<sub>2</sub>-Gly-Gly-Gly-Phe-Gly-Gly-Lys(NH<sub>2</sub>)-CO-NH<sub>2</sub>) (8.6 mg, 0.012 mmol) and triethylamine (13  $\mu\text{L}$ , 0.093 mmol) in 150  $\mu\text{L}$  anhydrous DMF were added into the oil product under nitrogen atmosphere and the reaction mixture was stirred for 12 h. After removing DMF, the crude product was purified by reversed-phase semi-preparative HPLC with 20%–80% water–acetonitrile gradient system containing 0.1% TFA to give the final product 7.4 mg (yield 53.1%). ESI-MS Found  $[\text{M} + \text{H}^+]$ : 1220.63,  $[\text{M} + \text{Na}^+]$ : 1242.85; calculated 1219.6.

### Colorimetric assay for enzyme hydrolysis of “trimethyl lock” substrate

To the solution of 0.4 mL of monodispersed dipotassium bis (*p*-sulfonatophenyl) phenylphosphane stabilized AuNPs (10 nm) (See the ESI<sup>†</sup>),<sup>9</sup> the peptide substrate and esterase in 0.05 mL of PBS buffer (pH 7.4) were added to afford their final concentrations of 8.5  $\mu\text{M}$  and 1.25  $\mu\text{g mL}^{-1}$ , respectively. The mixture was incubated at 37  $^{\circ}\text{C}$  for enzyme hydrolysis 2 h. Gold nanoparticles were induced to highly aggregated clusters which displayed purple-blue color and surface plasmon resonance shifts from 520 nm to 600 nm. Then, the aggregated gold nanoparticles were further incubated with thermolysin (60  $\mu\text{g mL}^{-1}$ ) for 4 h, the aggregated gold clusters were driven disassembly, which displayed red color and surface plasmon resonance shifts back from 600 nm to 520 nm again. This self-assembly and disassembly process were monitored by naked eyes and UV-Vis spectrophotometer.

### Dynamic light scattering (DLS) measurements for AuNPs

Dynamic light scattering (DLS) measurements were performed using a 90 Plus particle size analyzer (Brookhaven Instruments Corporation). The DLS instrument was operated at 25  $^{\circ}\text{C}$ , 90 degree detector angle with an incident laser wavelength of 660 nm. Size and size distribution of gold nanoparticles were determined in solution. All the samples were measured for 3 min, and the reported values are the average of five repeated consecutive measurements.

### Surface enhanced Raman Scattering (SERS) measurements

Dipotassium bis (*p*-sulfonatophenyl) phenylphosphane stabilized gold nanoparticles solution dropped on glass slides (approximately 10  $\mu\text{L}$ ) was used for SERS measurement. The spectra were excited using 3.5 mW of power at He-Ne 633 nm laser. The laser beam was then focused onto the sample *via* a dichroic mirror and through an Olympus

0.90 NA microscope objective. Raman signals were collected and focused into a 400  $\mu\text{m}$  optical fibre (Ocean Optics, Inc.) which delivered the signals to a single-stage monochromator (DoongWo, Inc.). Spectrum acquisition was started and an integration time is 40 s for all SERS measurements.

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