Specific detection of cysteine and homocysteine: recognizing one-methylene difference using fluorosurfactant-capped gold nanoparticles†

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Aggregation of fluorosurfactant-capped gold nanoparticles could be induced selectively by cysteine and homocysteine and, when solution ionic strength was low, the kinetics of homocysteine-induced aggregation of large size nanoparticles (~40 nm) was much faster than that induced by cysteine, leading to specific detection of homocysteine in the presence of excess cysteine.

Cysteine (Cys) and homocysteine (Hcy) are naturally occurring thiol-containing amino acids, and they are structurally similar (only one methylene difference) and metabolically linked. In biological fluids, the total Cys (tCys) and total Hcy (tHcy) refer to the sum of protein-bound, free-oxidized and free-reduced species of Cys and Hcy, respectively. Abnormal levels of these aminothiols in human plasma and urine are associated with a number of clinical situations.[1,2] Currently available methods for their determination are based on chromatography or immunoassay.[3–5] The development of simpler assay methods is attractive. Some recent efforts on selective derivatization of Cys and Hcy for their photometric and fluorescent detection have been reported.[4,6]

Gold nanoparticles (GNPs) have emerged as important colorimetric materials for their strongly distance-dependent optical properties. The analyte-induced aggregation of GNPs shifts the surface plasmon resonance absorption peak towards longer wavelength, based on which methods for the detection of DNA,[7] proteins,[8] and metal ions[9] have been proposed. The interactions of amino acids with GNPs have also been investigated.[10] Gold nanorods stabilized by cationic surfactant ligands[11] and Nile Red-adsorbed GNPs[12] were used for the detection of small biothiols. Although these methods could probably sense biothiols selectively in an amino acid pool, it is still difficult to detect a specific species.

Recently, we synthesized GNPs capped with fluorosurfactant ligands (*i.e.* Zonyl® FSN), and the colloid solution was used as a postcolumn reagent for HPLC assay of Hcy.[13] The FSN-capped GNPs exhibit high stability in aqueous solutions over a wide pH range, even in the presence of high salt (up to 1 M), and the responses of the colloid towards Cys and Hcy are highly selective in biological matrices. In the present study, it was found that the aggregation kinetics of FSN-capped GNPs could be altered significantly by tuning particle size and solution ionic strength, which made it possible to recognize one-methylene difference between Cys and Hcy. Therefore, the specific determination of both species in biological fluids could be achieved without the aid of separation techniques, even though the concentration of tCys is usually ~10–20 times higher than that of tHcy in these samples.

12 nm and 40 nm FSN-capped GNPs were used here. Color change of the colloid solutions occurred upon the addition of Cys or Hcy. New absorption peaks at ~650 nm and ~790 nm appeared for 12 nm and 40 nm GNPs, respectively, indicating the formation of GNP aggregates. Under the same experimental condition, the colloids did not respond to other standard amino acids and biomolecules such as glutathione, cysteinylglycine and glucose. The highly specific response of the GNPs towards Cys and Hcys could be attributable to the unique features of the fluorosurfactant ligands (other types of surfactants, such as Triton and Tween, have also been used to stabilize GNPs; however, these ligands could not provide high response specificity).[13]

Solution ionic strength and the GNP size had significant effects on Cys- and Hcy-induced aggregation. Generally, the increase of salt concentration resulted in a more rapid solution color change (Fig. 1), which could be attributable to the shielding of the interparticle electrostatic repulsion by higher ionic strength. For the 12 nm GNPs, the kinetics of colorimetric evolution induced by Cys and Hcy was almost identical, especially under high-salt conditions. For the 40 nm GNPs, however, Cys-induced color change was much slower in low-salt solutions. For example, the 40 nm GNP colloid containing 2 mM phosphate (pH ~ 6) did not respond to 50 μM Cys for at least 24 h, while the addition of 5 μM Hcy led to the color change from deep-red to blue within 20 min. The kinetic difference was reduced as the solution ionic strength increased.

Several pathways of amino acid-induced aggregation of GNPs have been proposed on the basis of cross-linking mechanism, where the bound amino acid molecules act as cross-linking agents to establish connection between GNPs *via* either hydrogen

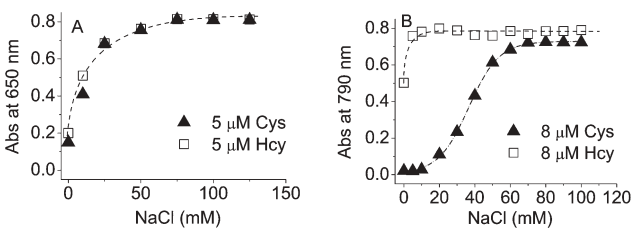

Fig. 1 Salt effect on Cys- or Hcy-induced absorbance changes of 12 nm (A) and 40 nm (B) FSN-capped GNPs. The solutions contained 5 mM phosphate. The values were acquired at 300 s and 1200 s after the addition of the analytes for 12 nm and 40 nm GNPs, respectively.

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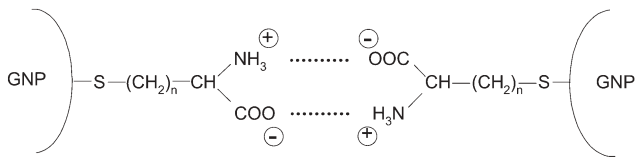
bonding or electrostatic interaction.[10a,d,11] The GNP-bound aminothiols existed as the zwitterionic forms under our experimental conditions (pH ~ 6.0), and the GNP aggregation could result from a two-point electrostatic interaction between the amino acids on a pair of particles (Scheme 1). It is interesting to note that, in low salt solutions, one methylene difference between Cys and Hcy led to significant variation in aggregation kinetics of the 40 nm GNPs. Compared with Cys-Cys linkage, the connection afforded by Hcy would provide a two-methylene longer interparticle distance, which facilitated the aggregation by alleviating more effectively the electrostatic repulsion as well as FSN steric hindrance.

When solution ionic strength was high, the electrostatic linkages afforded by bound Hcy or Cys species would be suppressed, leading to a weaker cross-linking effect. The fact that much more rapid aggregation occurred and the kinetic difference of the aggregation induced by Cys and Hcy was not obvious, suggests a non-cross-linking mechanism. We believe that the displacement of some of FSN ligands by aminothiol molecules made the colloidal solution unstable, and the aggregation could be driven by the London–van der Waals attraction force under high-salt conditions.

Since both Cys and Hcy could induce rapid aggregation of the 12 nm GNPs under high-salt condition, their determination may be achieved by measuring the absorbance change of the colloidal solution. In our experiments, little kinetic difference was observed for the aggregation induced by Cys and Hcy of the same concentration, which enabled the colorimetric determination of the total concentration of Cys and Hcy.

At low ionic strength, the 40 nm GNPs exhibited colorimetric response towards Hcy only; therefore, highly specific detection of Hcy could be expected. However, it has been found that Cys significantly interfered with the sensing of Hcy. As shown in Fig. 2A, the absorbance change induced by Hcy slowed down remarkably in the presence of Cys. This should be attributable to the competitive binding of Cys and Hcy on the GNPs. Since the Cys-attached GNPs were relatively stable in low-salt solutions, the aggregation was greatly retarded.

To solve this problem, the solution ionic strength was increased to make both the Cys- and Hcy-attached GNPs less stable. As shown in Fig. 1B, when 5 mM NaCl was added to the 40 nm GNP solution, the aggregation induced by Hcy proceeded faster while that induced by Cys was still not obvious. Fig. 3B shows that, under this condition, the response towards 1 μM Hcy could be clearly detected in the presence of an excess of Cys. The displacement of FSN ligands by Cys molecules made the GNPs much less stable in the solution with a mild level of salt (but did



Scheme 1 Possible cross-linking of Hcy or Cys-bound GNPs under low-salt conditions (the adsorbed FSN ligands are not shown).

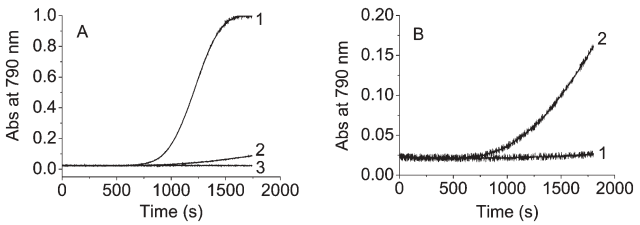


Fig. 2 Time courses of extinction of 40 nm FSN-capped GNPs under various conditions. (A) Upon the addition of 8 μM Hcy and x μM Cys; x = 0 (1), x = 8 (2), x = 16 (3); the solutions contained 5 mM phosphate. (B) Upon the addition of 10 μM Cys (1), or 10 μM Cys and 1.0 μM Hcy (2); the solutions contained 5 mM phosphate and 5 mM NaCl.

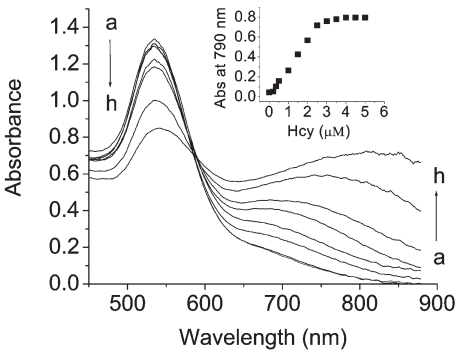


Fig. 3 UV-vis spectra of 40 nm FSN-capped GNPs upon the addition of the mixtures of Cys and Hcy (acquired at 1200 s). Cys : Hcy (μM): a. 5 : 0; b. 4.8 : 0.2; c. 4.65 : 0.35; d. 4.5 : 0.5; e. 4 : 1; f. 3.5 : 1.5; g. 3 : 2; h. 2.5 : 2.5. The solutions contained 10 mM phosphate and 20 mM NaCl. Inset, the absorbance at 790 nm as a function of Hcy concentration.

not aggregate), and the response sensitivity of Hcy was significantly enhanced. Therefore, by tuning the solution ionic strength, highly sensitive and selective response of the 40 nm GNPs towards Hcy could be achieved. Fig. 3 shows the spectral changes of the 40 nm GNPs in responding to a mixture of Hcy and Cys with a total concentration of 5 μM and different Cys : Hcy ratios. The inset shows the absorbance at 790 nm as a function of Hcy concentration.

Overall, by using the 12 nm GNPs, the total concentration of Hcy and Cys could be measured; while the amount of Hcy in the presence of an excess of Cys could be determined by measuring the aggregation kinetics of the 40 nm GNPs. These measurements will lead to the specific detection of both Hcy and Cys.

To demonstrate the validity of the proposed method, the levels of Cys and Hcy in a human urine sample were measured. Calibration curves were obtained by using spiked urine samples. In order to make the calibration more accurate, the small amounts of free-reduced Cys and Hcy (~1–5% of tCys and tHcy) in the sample have been eliminated by oxidation before the standard spiking. The calibration curve for the total concentration of Cys and Hcy was obtained by using the 12 nm GNPs, as shown in Fig. 4. A linear increase of the absorbance at 650 nm was observed in the concentration range of 1.0 to 4.5 μM. The lower limit of detection was ~0.8 μM with a signal-to-noise ratio of 3. After reducing the sample with tris(2-carboxy-ethyl)phosphine, the total concentration of Cys and Hcy was determined (Table 1). Since the dynamic range was narrow, it was important to ensure the

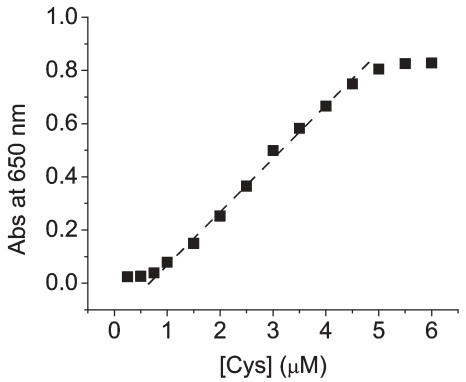


Fig. 4 Absorbance (at 650 nm) of 12 nm FSN-capped GNPs versus Cys concentration in the diluted urine samples (dilution factor, ~78). The solutions contained 5 mM phosphate and 100 mM NaCl.

Table 1 Assay results of a human urine sample

X	Sample[a]/μM	Added/μM	Measured[a]/μM	Recovery (%)
tCys	183.5 ± 1.2	50	51.3 ± 1.2	102.6
		100	98.7 ± 1.0	98.7
tHcy	27.8 ± 0.5	10.0	9.87 ± 0.23	98.7
		20.0	19.82 ± 0.17	99.1

[a] Mean ± standard deviation of three measurements.

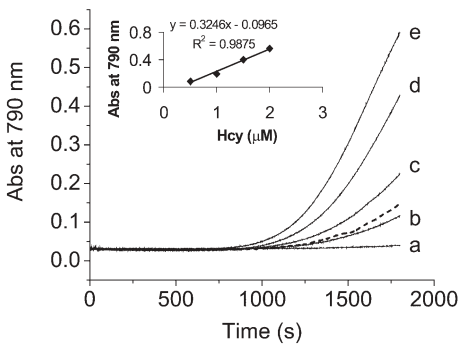


Fig. 5 Time courses of extinction (at 790 nm) of 40 nm FSN-capped GNPs upon the addition of the calibration samples containing different ratios of Cys and Hcy (totally ~5 μM). Cys : Hcy: a. 5 : 0; b. 4.5 : 0.5; c. 4 : 1; d. 3.5 : 1.5; e. 3 : 2. The dashed line was obtained with the dilute urine sample after reduction. The solutions contained 10 mM phosphate. Inset, the absorbance (at 1800 s) as a function of Hcy concentration.

analytical signal fall within the linear range by diluting the samples appropriately.

Fig. 5 shows the time courses of the absorbance changes of 40 nm FSN-capped GNPs in responding to the calibration samples with different Cys : Hcy ratios (total concentration, 5 μM). The limit of detection for Hcy was ~0.2 μM, corresponding to a Cys : Hcy ration of ~24. After the urine sample was reduced and injected to the colloidal solution (diluted to make the total concentration of Cys and Hcy 5 μM), the time course was measured and the concentration of tHcy was determined (Fig. 5 and Table 1).

In conclusion, the highly specific interactions of the FSN-capped GNPs with Cys and Hcy and their significant size- and ionic strength-dependent aggregation behavior led to the selective detection of these aminothiols. The results obtained in the analysis of human urine samples indicate that the method is promising for the detection of Cys and Hcy in biological fluids.

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