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Selective determination of homocysteine levels in human plasma using a silver nanoparticle-based colorimetric assay

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ABSTRACT

The first use of silver nanoparticles (AgNPs) for the rapid, simple, and selective determination of homocysteine (Hcy) levels in human plasma was studied. Hcy and five other amino acids, including cysteine (Cys), could be distinguished by their different aggregation kinetics, which caused a change in the visible color and a shift in the UV–vis absorption spectra. The difference in the cross-linking (aggregation) rate between Hcy and Cys was used as the basis for developing a selective probe for Hcy and allowed the detection of Hcy in the linear range of 2–12 μM (R^2 = 0.9936). The limits of detection and quantification were found to be 0.5 μM and 1.7 μM, respectively. To investigate its selectivity and potential applicability, this AgNP-based method was successfully applied for the determination of Hcy levels in actual biological (human plasma) samples, where the determined levels of Hcy were within the error range of the measured level using the traditional chemiluminescence microparticle immunoassay (CMIA). Thus, the use of AgNPs is a feasible and potentially reliable method for the determination of Hcy levels in biological samples.

1. Introduction

Homocysteine (Hcy), a sulfur-containing amino acid, is an intermediate formed during the conversion of methionine into cysteine (Cys). Hcy exists in various forms in plasma, including disulfide, mixed disulfides and protein-bound homocystine, as well as the free (unattached) reduced form of Hcy. Hence, the measurement of total Hcy levels is typically completed after the chemical reduction of the disulfides. The normal concentration of Hcy in plasma ranges from 5 to 15 μM, with levels of Hcy above 15 μM defined as hyperhomocysteinemia. Hyperhomocysteinemia is categorized into three groups: moderate (15–30 μM), intermediate (30–100 μM) and severe (over 100 μM) [1]. High plasma levels of Hcy are associated with several diseases such as cardiovascular disease [2], Alzheimer's disease [3], neural tube defects [4], and osteoporosis [5].

Hcy and Cys both contain a free thiol and are structurally similar [6], and thus analytical methods for the determination of Hcy have been extensively investigated after prior separation techniques to remove the Cys, for instance, gas chromatography with mass spectrometry (GC–MS) [7], high performance liquid chromatography (HPLC) [8,9] or capillary electrophoresis (CE) [10,11] separation methods then coupled with electrochemical [12], UV–vis [13] and fluorescent [14] detection. However, these methods are rather expensive, complicated and time-consuming [15]. Recently, colorimetric assays based on nanoparticle (NP) assembly have received considerable attention for the detection of Hcy and Cys [16–20] because of their ease of operation, detection using optical methods, suitability for diagnosis, simplicity, and high sensitivity [21,22]. Gold nanoparticles (AuNPs) have been intensively developed as probes or sensors for the determination of aminothiols [16,23] based on the fact that AuNPs aggregate upon the addition of aminothiols. The interparticle forces between AuNPs and aminothiols include electrostatic interactions [24], hydrogen bonding [25–28], zwitterionic forces [19,24] and van der Waals forces [25]. The aminothiol-induced aggregation of AuNPs leads to a decrease in the plasmon resonance absorption peak and the formation of a red-shifted band [29]. The colorimetric changes of AuNPs in the presence of aminothiols such as Hcy [15,30,31] and

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Cys [32,33], have been reported. The structurally similar Cys and Hcy molecules, which only differ by one methylene group, can be selectively detected using modified AuNPs based on the differences in their aggregation kinetics [16]. Recently, silver nanoparticles (AgNPs) have been used as an alternative source for colorimetric sensors. The cost of the methods using AgNPs is lower when compared with AuNPs. Moreover, AgNPs have gained in popularity owing to their chemical and physical properties [34,35]. The advantage of AgNPs is that the molar extinction coefficient of AgNPs is approximately 100-fold greater than that for AuNPs, which leads to improved visibility based on the difference in optical brightness and increased sensitivity when using absorption spectroscopy [36]. Furthermore, AgNPs illustrate a narrower plasmon resonance band in the visible range than AuNPs do, at around 400 nm [37]. Hence, a colorimetric method based on AgNPs is a potential alternative approach for the determination of Hcy levels, but this approach has not yet been evaluated.

In this paper, we report the first use of AgNPs for the rapid, simple, and selective determination of Hcy based upon aggregation kinetics. Under optimum conditions, the red-shifted band resulting from Hcy-induced aggregation of AgNPs was observed at 525 nm, whereas Cys gave no significant wavelength shift. Furthermore, the current AgNP-based method was successfully applied for the actual determination of Hcy levels in biological human plasma samples, where the experimentally determined levels were within the error range of the levels measured for the same samples using the traditional clinical diagnostic chemiluminescence microparticle immunoassay (CMIA).

2. Experimental

2.1. Chemicals

Hcy, glutathione (GSH), and tris(2-carboxyethyl)phosphine (TCEP) were purchased from Sigma–Aldrich (Steinheim, Germany). Cys, methionine (Met), histidine (His), and alanine (Ala) were obtained from KASEI (TCI, Tokyo, Japan). Trichloroacetic acid (TCA), sodium chloride, and disodium hydrogen phosphate were purchased from Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid (EDTA) and potassium chloride were obtained from Fluka (Buchs, Switzerland) and Univar (New South Wales, Australia), respectively. Potassium dihydrogen phosphate was acquired from BDH laboratory supplies (Poole, England). AgNPs, prepared following the reported procedure [38], were obtained from the Sensor Research Unit at the Department of Chemistry, Faculty of Science, Chulalongkorn University. All reagents were of analytical grade, and water purification was conducted using a Millipore Milli-Q purification system throughout this experiment.

2.2. Apparatus

AgNPs were suspended in water at 10–40 ppm as indicated, and the UV–vis spectra were recorded in a quartz cuvette using a UV–vis spectrophotometer (UV-vis 2401 PC, Shimadzu). Transmission electron microscopy (TEM) was performed with a JEM-2100 (JEOL, Japan) microscope operating at 200 kV. A tabletop centrifuge (4000 Kubota) was used for sample preparation.

2.3. Detection of Hcy levels

Aqueous solutions of each amino acid were freshly prepared before use. A stock solution of Hcy (10 mM) was prepared in 1 M phosphate buffer saline (PBS, pH 7.4), and the working solutions of Hcy were prepared by serial dilution of the stock solution with PBS. One thousand microliters of each working solution was added to 1000 μL of a suspension of AgNPs within the range of 10–40 ppm, from which the optimal concentration was selected (20 ppm; see Section 3) and was used thereafter. The UV–vis absorption spectrum of each AgNP suspension was recorded as detailed above at the indicated time (range 0–60 min) after mixing at the room temperature (25 °C). Consequently, the calibration standard solutions (0–12 μM) were equilibrated for the optimum incubation time (1 min; see Section 3). To investigate the selectivity of AgNPs for Hcy, five different amino acids (Cys, GSH, Met, Ala and His) were assayed as individual solutions. The difference in the kinetics between the Hcy/AgNPs and Cys/AgNPs interactions was observed by plotting the absorbance at 525 nm *vs.* time.

2.4. Characterization of Hcy-induced aggregation of AgNPs

AgNPs were synthesized following the reported procedure using the chemical reduction process [38]. An aqueous solution of sodium borohydride reducing agent with methyl cellulose solution as a solvent was prepared. Subsequently, an aqueous solution of silver nitrate with methyl cellulose, which was used as a stabilizer, was gradually added to the sodium borohydride solution under a vigorous mixing. The dark cloud solution visually and dramatically changed to the bright yellow solution. The solution was finally turned to the dark brown when all reactants were completely added. The different sizes of AgNPs were also synthesized using the chemical reduction process but the different mole ratio between silver nitrate and sodium borohydride was carried out. AgNP preparations with different mean sizes were obtained from the Sensor Research Unit at the Department of Chemistry, Faculty of Science, Chulalongkorn University. The actual shapes, particle size distributions, and absorbance spectra of the AgNPs with nominal mean diameters of 10, 30, 35, 45 and 50 nm were studied using TEM (shape and size) and UV–vis spectroscopy (absorbance), respectively. For the TEM characterization of AgNP aggregation, AgNPs and Hcy were mixed at 20 ppm and 10 μM final concentrations, respectively, and after 1 min, a drop of the colloidal suspension was placed on a carbon-coated copper grid, dried at room temperature and operated as described in Section 2.2.

2.5. Sample preparation

Fresh human blood samples (2.0 mL) to which EDTA had been added were obtained from volunteers of the local hospital and centrifuged at 4000 rpm for 10 min. The supernatant, which contained proteins and amino acids amongst other components, was used as the source of the plasma. For the reduction of disulfides, TCEP (100 g/L) was added to the plasma samples, which were then incubated for 30 min at room temperature. The interference of proteins and other substances in the plasma was removed by adding 900 μL TCA (100% (w/v)) containing 1 mM EDTA [39]. The sample was mixed immediately and centrifuged at 4000 rpm for 10 min. The supernatant, which contained Hcy and other components including other amino acids, was used for further analysis, and the unknown amount of Hcy was estimated. The CMIA was performed as a validation method.

3. Results and discussion

3.1. Aggregation of AgNPs induced by Hcy

The most attractive colorimetric sensors are based on the aggregation of metal NPs, typically AuNPs, due to the high molar absorptivity of the color changes resulting from aggregation, these molar absorptivities are several orders of magnitude greater than those of traditional organic chromophores. Metal NPs are also favored because of their simplicity and biocompatibility. AuNPs have been applied for the determination of the level of aminothiol

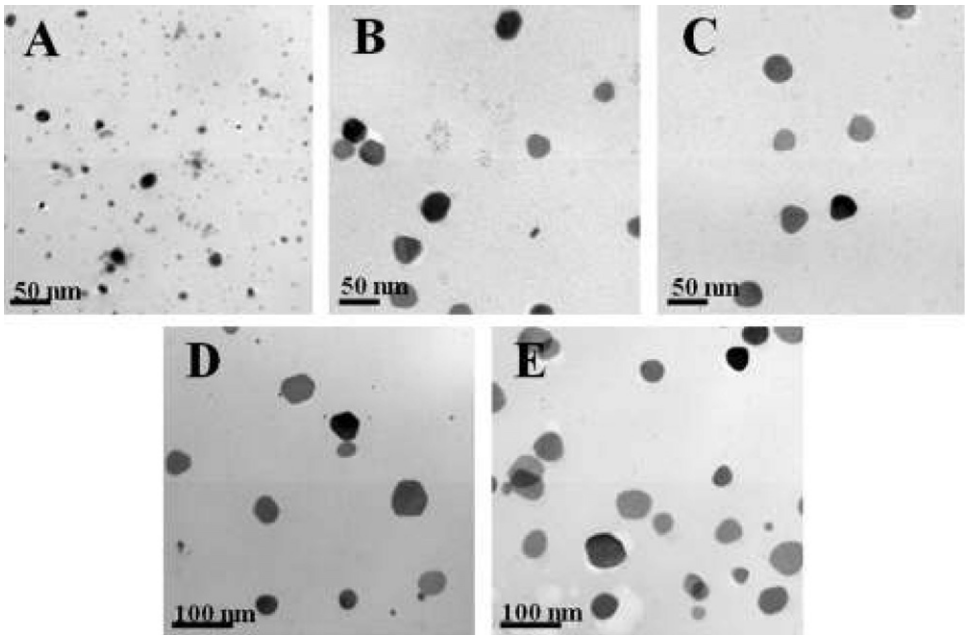

Fig. 1. TEM images of AgNPs with nominal average diameters of (A) 10 nm, (B) 30 nm, (C) 35 nm, (D) 45 nm and (E) 50 nm.

compounds because of their strong affinity for amino and sulfur compounds.

Unfortunately, AuNPs require surface modifications to achieve selective cross-linking (and thus aggregation) between the sulfur-containing amino group (aminothiols) and the amino group. AgNPs are, therefore, an alternative source of NPs for colorimetric sensors because they do not require such surface premodification. In spite of this advantage, the practical application of AgNP aggregation for Hcy and Cys colorimetric detection remains almost unexplored. Only one published paper has reported the selective determination of Cys using triangular AgNP aggregation without surface modification. Therefore, we first studied the effect of the size of the AgNPs on aggregation and the absorbance wavelength shift in the presence of Hcy using AgNPs with nominal average diameters of 10, 30, 35, 45 and 50 nm. TEM evaluation of each AgNP preparation confirmed the different diameters of the AgNPs (Fig. 1).

The UV–vis spectra of the AgNPs varied with the AgNP size, with absorbance wavelength peaks at 400, 500, 520, 550 and 600 nm for 10, 30, 35, 45 and 50 nm average diameter AgNPs, respectively (Fig. 2), these peaks are ascribed to the surface plasmon absorption of AgNPs. These wavelength changes were also visually detectable as color changes of the AgNP suspensions from yellow (10 nm average diameter AgNPs) through orange and purple to light blue (50 nm average diameter AgNPs), as shown in Fig. 2.

Although a new absorption peak appeared at a longer wavelength (~525 nm), and a visible color change from yellow to red occurred with the 10 nm average diameter AgNPs after the addition of Hcy or Cys, no significant difference was noted with the larger-sized (30–50 nm average diameter) AgNPs. This change in color and shift of the wavelength suggests that the average particle size of the NPs increased, the aggregate size increased or there was shorter inter-particle spacing [40]. However, the absorption peaks of Hcy and Cys at the new peak of 525 nm were essentially indistinguishable and thus not useful for the diagnostic detection of Hcy in plasma samples. Therefore, 10 nm average diameter AgNPs were selected as the optimal size and were used in further experiments to optimize the resolution of the system.

To confirm the mechanism of the interaction between Hcy and 10 nm AgNPs, the AgNPs were examined by TEM. Representative micrographs of 10 nm AgNPs in the presence of Hcy are shown in Fig. 3 and clearly show the aggregation of AgNPs in the presence of Hcy. The aggregation and change in the size and shape of AuNPs induced by Cys has been reported previously [41,42].

3.2. Selectivity of AgNPs for Hcy

The ability to determine Hcy levels in human plasma samples is of interest because the level of Hcy in biological samples is associated with many diseases. The selectivity of AgNP-based colorimetric sensors is directly affected by the assay conditions, such as the concentration of the AgNPs and the reaction time. Because Cys, which is found in plasma samples, has the structure most similar to that of Hcy, the assay conditions were first optimized in terms of the AgNPs concentration and the reaction time with respect to the ability to distinguish between Hcy and Cys. The aggregation of different concentrations of AgNPs with 10 μM Hcy and Cys was investigated using absorption spectra in Fig. 4. The difference between the absorption bands at 525 nm for Hcy and Cys was insignificant at the AgNP concentration of 10 ppm (Fig. 4A). Although the absorbance at 525 nm of Hcy at 30 and 40 ppm AgNPs were higher than those of Cys, their peaks were ambiguous (Fig. 4C and D). The absorbance at 525 nm of 20 ppm AgNPs in the presence of Hcy was remarkably higher than that for Cys, as shown in Fig. 4B. Thus, 20 ppm AgNPs was selected as the optimum concentration for the selective detection of Hcy.

The incubation time between AgNPs and either Hcy or Cys also lead to a different response in terms of the change in the optical spectra. The kinetic distinction between Hcy and Cys, which is the difference in the speed of the spectral evolution between these two amino acids, was observed by plotting the absorbance at 525 nm *vs.* time (Fig. 4). The Hcy-induced aggregation of AgNPs was found to be much faster than that induced by Cys. The aggregation may involve cross-linking, where Hcy or Cys act as cross-linking agents to link AgNPs together through hydrogen bonding, electrostatic interactions [16,23,40], van der Waals forces [43], or a mixture of any of these forces. Regardless of the interaction mechanism, the results are in accord with those reported in the literature, which show that the cross-linking rate of AuNPs with Hcy is much faster than that with Cys [16,43]. These results clearly show that the difference in the AgNP cross-linking rates for Hcy and Cys can be employed

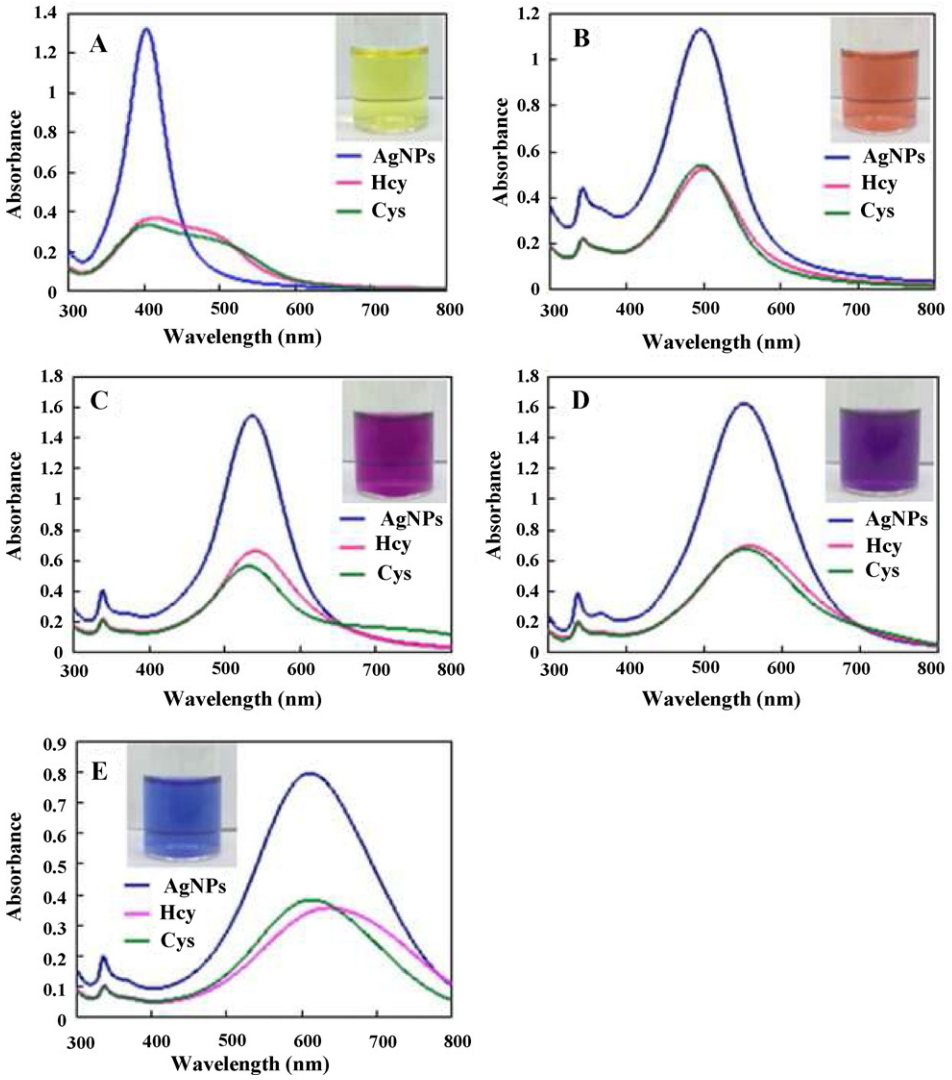

Fig. 2. UV–vis spectra of AgNPs with nominal average diameters of (A) 10 nm, (B) 30 nm, (C) 35 nm, (D) 45 nm and (E) 50 nm, and the same sized AgNPs after incubation with 10 μM of either Hcy or Cys for 1 min. The inserts display the visible color of each AgNP suspension.

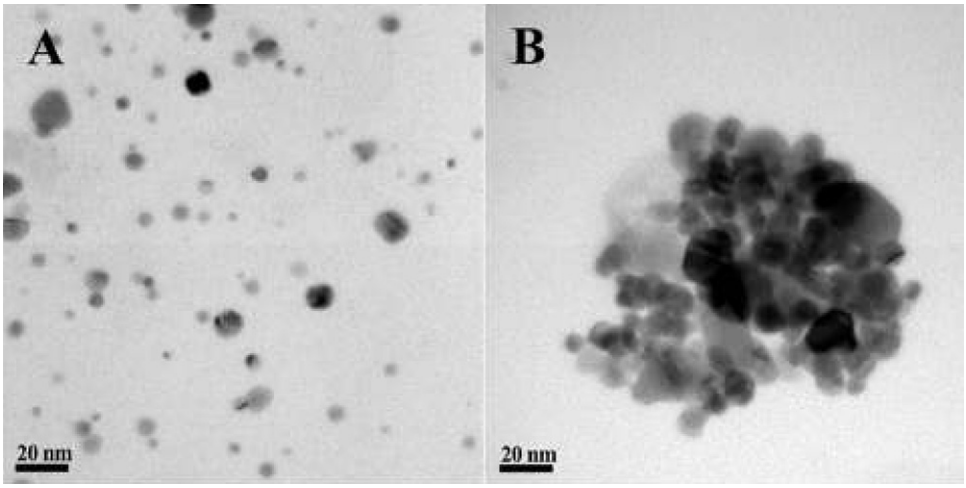

Fig. 3. TEM images of 10 nm AgNPs after incubation for 1 min in (A) the absence or (B) the presence of 10 μM Hcy.

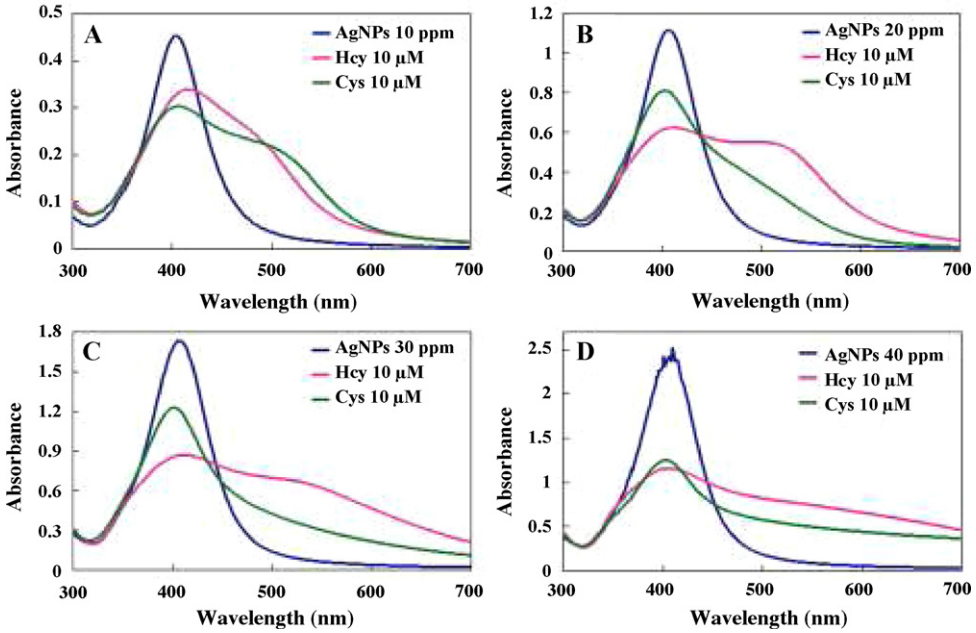

Fig. 4. UV–vis spectra of 10 nm average diameter AgNPs (yellow suspension), at AgNP concentrations of (A) 10 ppm, (B) 20 ppm, (C) 30 ppm and (D) 40 ppm, after a 1 min incubation with or without 10 μM of either Hcy or Cys.

in the selective determination of Hcy concentrations. The absorption spectra of the 10 nm average diameter AgNPs (20 ppm) in the presence of 10 μM Hcy or Cys revealed an increased absorbance at 525 nm over time, with a decreased absorbance at 400 nm over the same time scale. The aggregation of AgNPs induced by Hcy showed the new absorption peak at 525 nm after only 1 min of incubation, in contrast to that with Cys (Fig. 5). In other words, no absorption peak at 525 nm appeared after the addition of Cys to AgNPs at 1 min. The inset in Fig. 4 shows the absorbance at 525 nm of AgNPs in the presence of Hcy and Cys as a function of the incubation time, which clearly shows that the potential selectivity for the detection of Hcy over Cys is optimal at an incubation time of 1 min with AgNPs and decreases thereafter.

To further investigate the apparent selectivity of AgNPs for Hcy, four other amino acids (Met, Ala, GSH and His), along with Cys, were evaluated by monitoring the absorbance at 525 nm in the presence of 10 nm average diameter AgNPs (20 ppm). The concentration of Hcy was 10 μM, and the concentration of the other five amino acids was 10 times greater than that of Hcy (100 μM), which is far greater than the levels found in biological samples such as plasma. Upon the addition of the different amino acids to AgNPs, an absorbance shift to 525 nm occurred, but the shift for Hcy was much higher than that of the other amino acids (Fig. 6) despite the great excess molar levels of these other amino acids relative to that found in human plasma samples. This was especially relevant for Cys, as Cys is structurally similar to Hcy. Therefore, Hcy could be distinguished from the other amino acids including Cys using this method.

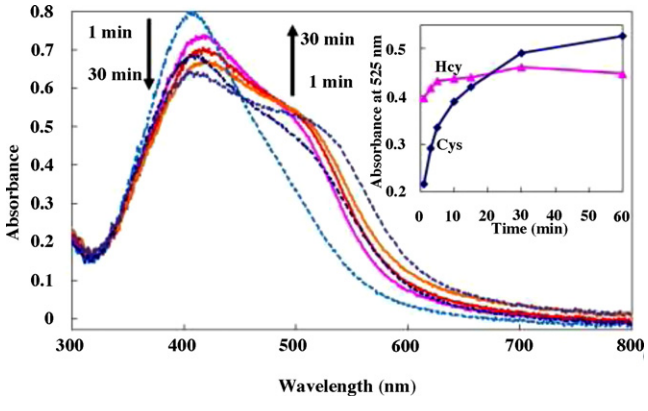

Fig. 5. UV–vis spectra of 10 nm average diameter AgNPs (20 ppm), with or without the addition of 10 μM of either Hcy (solid line) or Cys (dash line) and incubated for the indicated times (1, 10 and 30 min). Inset: the kinetics for Hcy-AgNPs and Cys-AgNPs obtained by monitoring the absorption peak at 525 nm at 1, 3, 5, 10, 15, 30 and 60 min.

3.3. Analytical performance

As the previous results suggested that Hcy could induce the aggregation of 10 nm average diameter AgNPs, we expected that AgNPs could be used to quantitatively determine Hcy levels, which is a biologically important analyte. The UV–vis spectra of AgNPs with different concentrations of Hcy were monitored under the optimal conditions (see above) of 1 min incubation time and 20 ppm of 10 nm average diameter AgNPs. After the addition of different amounts of Hcy (2–12 μM) to the solution of AgNPs, the absorbance at 525 nm was plotted against the concentration of Hcy. This plot was found to be linear within the range of 2–12 μM

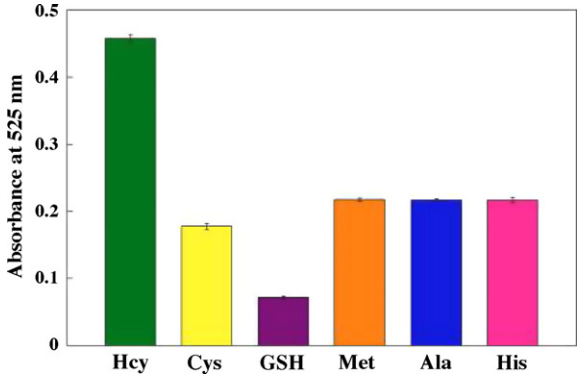

Fig. 6. Selectivity of 10 nm average diameter AgNPs (20 ppm) for 10 μM Hcy and five other amino acids, each at 100 μM. Data are shown as the mean ± SD and were derived from three replicates.

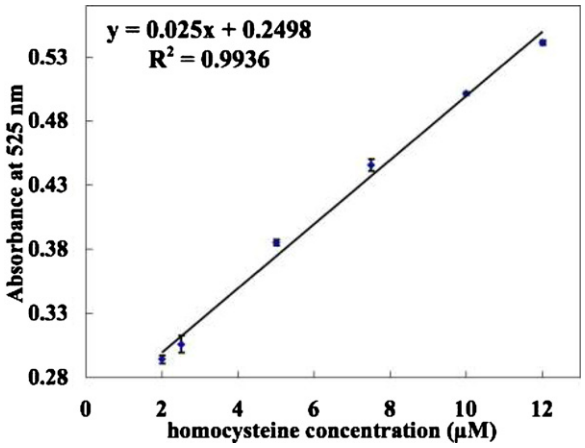

Fig. 7. The calibration curve between the absorbance at 525 nm and the concentration of Hcy. Ten nanometer average diameter AgNPs (20 ppm) were incubated for 1 min with Hcy at the indicated final concentrations before colorimetric detection. The data are shown as the mean ± SD and were derived from three replicates. The equation for the best fit linear line and the regression coefficient are shown.

(Fig. 7), with a correlation coefficient (R^2) of 0.9936. The detection and quantification limit (LOD and LOQ), that is the levels which produced the signal at three and ten times, respectively, of the standard deviation of a blank signal ($n = 10$), were 0.5 μM and 1.7 μM, respectively. Although both the LOD and LOQ values of this method reported here are slightly higher than those of the previously reported methods based on NP aggregation, the linear range of Hcy using this AgNP-based approach was within the range of normal levels of Hcy found in plasma (5–15 μM). Furthermore, an important advantage of the system presented herein over the previously reported methods is the selective detection of Hcy without the need for complicated surface modification of the NPs. Therefore, this method should be comprehensive for the routine determination of Hcy levels in plasma samples.

3.4. Analytical application in real sample

Having evaluated the selectivity and potential application of this method with pure single analyte solutions, the proposed method was evaluated using a real biological sample, that is, in the presence of other potentially interfering components. Human plasma samples were used for this purpose. The main form of Hcy in plasma is the disulfide form bound to proteins or other thiols [1,2,44], which requires reduction prior to detection. After reduction with TCEP, Hcy is free in the plasma. To minimize the effect of proteins and other substances, deproteination was then performed by standard TCA precipitation as previously described. The unknown amounts of Hcy in three different plasma samples were then determined by both the AgNP-based method reported herein and the standard CMIA method (Table 1). The data were then compared by a paired *t*-test. The levels obtained using the method described in this report were in good agreement with those from the CMIA method, falling within the 95% confidence level.

Table 1
Determination of Hcy levels in human plasma samples using 10 nm diameter AgNPs at 20 ppm.

Sample	Concentration of Hcy (μM ± SD, $n = 3$)		Paired *t*-test
	CMIA method	Our method	
1	10.10 ± 0.40	10.15 ± 0.01	
2	10.40 ± 0.42	11.22 ± 0.01	−1.01
3	15.40 ± 0.62	15.36 ± 0.01	

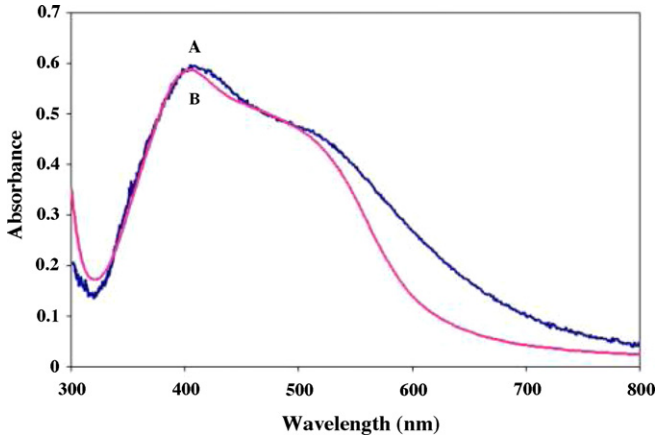

Fig. 8. UV–vis spectra of 10 nm average diameter AgNPs (20 ppm) with the addition of (A) standard 10 μM Hcy and (B) 10 μM Hcy in a normal human plasma sample.

Fig. 8 displays the obtained UV–vis spectra of the standard Hcy and the Hcy in human plasma at a similar concentration. The absorption spectra of Hcy in the human plasma sample was almost identical to that of the standard Hcy, showing no significant evidence of any interference from the other components in the biological plasma sample. Therefore, the AgNP-based method outlined here is potentially feasible for use in the reliable determination of Hcy in real biological samples.

4. Conclusions

A new straightforward approach for the determination of Hcy using AgNPs has been developed. The detection is based on the aggregation of AgNPs, which leads to a shift in the absorption spectrum. Hcy was clearly distinguishable from the other amino acids, including Cys, under the optimum conditions without the need for surface modification of the NPs, attaining good linear detection in the range of 2–12 μM. Furthermore, this method was successfully used for the determination of Hcy in human plasma samples and was validated using the CMIA method. This method has great potential for the inexpensive, rapid and simple determination of Hcy concentrations in real biological samples.

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