Talanta 91 (2012) 103–109

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Gold nanoparticle extraction followed by *o*-phthaldialdehyde derivatization for fluorescence sensing of different forms of homocysteine in plasma

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ARTICLE INFO

Article history:
Received 1 December 2011
Received in revised form 9 January 2012
Accepted 12 January 2012
Available online 21 January 2012

Keywords:
Gold nanoparticles
o-Phthaldialdehyde
Fluorescence
Homocystine
Plasma

ABSTRACT

This study reports a selective and sensitive method for fluorescent detection of total, protein-bound, free, and free oxidized homocysteine (HCys) using tris(2-carboxyethyl)phosphine (TCEP) as a reducing agent, fluorosurfactant-capped gold nanoparticles (FSN–AuNP) as a preconcentrating probe, and *o*-phthaldialdehyde (OPA) as a derivatizing agent. TCEP was used to reduce the disulfide bonds of protein-bound and free oxidized HCys. FSN–AuNPs can extract HCys from a complicated complex because the FSN capping layer stabilizes the AuNPs in a high-salt solution and inhibits non-specific adsorption. HCys was selectively derivatized with OPA in the absence of a nucleophile. By taking advantage of these features, the selectivity of the proposed system is greater than 100-fold for HCys and homocystine (HCys–HCys disulfide; diHCys) compared to any aminothiols. The limits of detection for HCys and diHCys were 4.4 and 4.6 nM, respectively. Different forms of plasma HCys were determined by varying the order of disulfide reduction with TCEP.

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1. Introduction

Total homocysteine (HCys) in plasma consists of the major protein-bound HCys, homocystine (HCys–HCys disulfide; diHCys), mixed disulfides containing a HCys residue, and trace amounts of reduced HCys. The normal concentrations of total HCys in plasma range from 5 to 15 μM, while an increased level of total HCys (>15 μM) in plasma are associated with numerous cardiovascular, metabolic, and neurodegenerative disorders [1]. Protein-bound HCys is the primary form of circulating HCys in homocystinuria [2], end-stage renal disease [3], and cobalamin deficiency [4]. The concentration of free HCys (free oxidized and free reduced HCys) larger than 4.11 μM is an independent risk factor for recurrent cardiovascular events in patients hospitalized for acute coronary syndrome [5]. The level of reduced HCys may not only be a more relevant marker of cardiovascular risk than the level of total HCys [6], but is also implicated in vascular endothelial dysfunction during hyperhomocysteinemia in humans [7]. Due to their biological and clinical significance, a convenient, selective, and sensitive method is required for the measurement of different forms of HCys in plasma.To date, significant attention has been given to the analysis of total HCys in plasma using high performance liquid chromatography [8,9], and capillary electrophoresis [10,11]. These separation methods are commonly combined with fluorescence, mass spectrometry, UV absorption, or electrochemical detection. Although a more differentiated picture may be obtained from the analysis of reduced, oxidized, and protein-bound forms, very little research has been devoted to determining different forms of plasma HCys using the above-mentioned separation methods [12–16].

Alternatively, massive sensors have been devised for sensing of HCys, based on organic dyes [17–24], silver nanoclusters [25], and semiconductor quantum dots [26]. For example, a series of organic dyes with aldehyde functionality were utilized for sensing HCys through the formation of a 6- or 5-membered ring [18–21]. This cyclization reaction leads to a decrease/increase in fluorescence intensity, the enhancement in phosphorescence intensity, and a shift in absorption wavelength. Additionally, the recognition of HCys in living cells has been successfully achieved using a highly electron-deficient system as a turn-on fluorescence sensor [22]. However, the limitations of these probes include poor aqueous solubility, complex synthesis, cross-sensitivity toward cysteine (Cys), and/or poor sensitivity. Recently, citrate-capped gold nanoparticles (AuNPs) modified with nonionic fluorosurfactant (FSN) have become another emerging material for colorimetric sensing of HCys because their surface plasmon resonance (SPR) band is highly sensitive to the distance between each NP [27–29]. When HCys binds onto the Au surface through the formation of an Au—S bond, the assembly of HCys-attached AuNPs occurs by electrostatic interaction and hydrogen bonding. This aggregation results in a SPR

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doi:10.1016/j.talanta.2012.01.026

peak decrease at approximately 520 nm and the formation of a new SPR peak at a longer wavelength. However, the selectivity of FSN-capped AuNPs (FSN–AuNPs) toward HCys suffers interference from Cys. When the particle size of FSN–AuNPs increased from 12 to 40 nm, the aggregation rate of HCy-attached AuNPs was faster than that of Cys-attached AuNPs [30]. Based on this phenomenon, selective detection of HCys can be successfully achieved by optimizing aggregation kinetics. Additionally, previous studies have demonstrated that *o*-phthaldialdehyde (OPA) containing aldehyde moiety can react with HCys to form highly fluorescent derivative in the absence of nucleophile [31–35]. This feature is helpful to prevent the interference of Cys as it is the most abundant aminothiol in plasma. Notably, the reaction between OPA and primary amine requires the presence of nucleophile to generate a fluorescent derivative [36]. Therefore, the combination of FSN–AuNP extraction and OPA derivatization can be utilized for selective and sensitive detection of HCys [37]. Although the above-mentioned sensors provided high sensitivity and selectivity toward HCys, they have not yet been utilized for sensing HCys in plasma. Moreover, no study has reported that different forms of HCys can be detected using a sensor.

The aim of this study is to develop a convenient and sensitive method for the quantification of total, protein-bound, free, and free oxidized HCys in plasma without chromatographic separation. Scheme 1 illustrates the quantification of different forms of HCys in plasma: (a) separation of protein-bound and free HCys using a centrifugal device; (b) reduction of the disulfide bonds of protein-bound or oxidized HCys with tris(2-carboxyethyl)phosphine (TCEP); (c) extraction of TCEP-reduced HCys with FSN–AuNPs; (d) liberation of HCys from the Au surface through a ligand exchange reaction with 2-mercaptoethanol (2-ME); (e) derivatization of HCys with OPA. This study evaluated the effects of reducing agents, AuNP concentration, and incubation time on the fluorescent sensing of HCys and diHCys.

2. Experimental

2.1. Chemicals

HCys, diHCys, Cys, glutathione, γ-glutamylcysteine, cysteinylglycine, $HAuCl_4$, trisodium citrate, Na_3PO_4, sodium hydroxide, 2-ME, OPA, Zonyl FSN-100, dithiothreitol (DTT), tributylphosphine (TBP), and TCEP were obtained from Sigma–Aldrich (St. Louis, MO, USA). The molecular formula of Zonyl FSN-100 is $F(CF_2CF_2)_{3-8}CH_2CH_2O(CH_2CH_2O)_xH$. Water used in all experiments was doubly distilled and purified by a Milli-Q system (Millipore, Milford, MA, USA).

2.2. Synthesis of FSN–AuNPs

To prepare citrate-capped AuNPs, we rapidly added 38.8 mM of trisodium citrate (25 mL) to a solution of 1-mM $HAuCl_4$ (250 mL) that was heated under reflux. This heating continued for an additional 15 min, during which time the color of the solution changed to a deep red. A H7100 transmission electron microscopy (TEM) (Hitachi High-Technologies Corp., Tokyo, Japan) operating at 75 keV was used to collect TEM images of citrate-capped AuNPs. TEM images (Fig. S1, Supplementary material) confirmed that the diameter of the AuNPs is 13 ± 1 nm. The concentration of spherical AuNPs was estimated using the following equation:

$$n = \frac{3m}{4\pi r^3 s}$$

where n is the number of AuNPs, m is the molar mass of Au in the substance, r is the radius of the AuNPs, and s is the specific gravity of AuNPs (19.3 g/cm^3). The values of m and r were obtained by conducting inductively coupled plasma mass spectroscopy (Perkin Elmer-SCIEX, Thornhill, ON, Canada) and TEM measurements, respectively. The concentration of the particles was calculated to be 2.5 nM (1.55×10^{12} particles/mL). A solution of FSN–AuNPs was prepared by adding FSN (10%, 240 μL) to citrate-capped AuNPs (2.5 nM, 60 mL). The resulting mixture was stored at 4 °C until further use. The extinction spectra of FSN–AuNPs were recorded using a double-beam UV-vis spectrophotometer (Cintra 10e; GBC, Victoria, Australia). Fig. S2 (Supplementary material) shows the extinction spectrum of FSN–AuNPs.

2.3. Extraction procedure

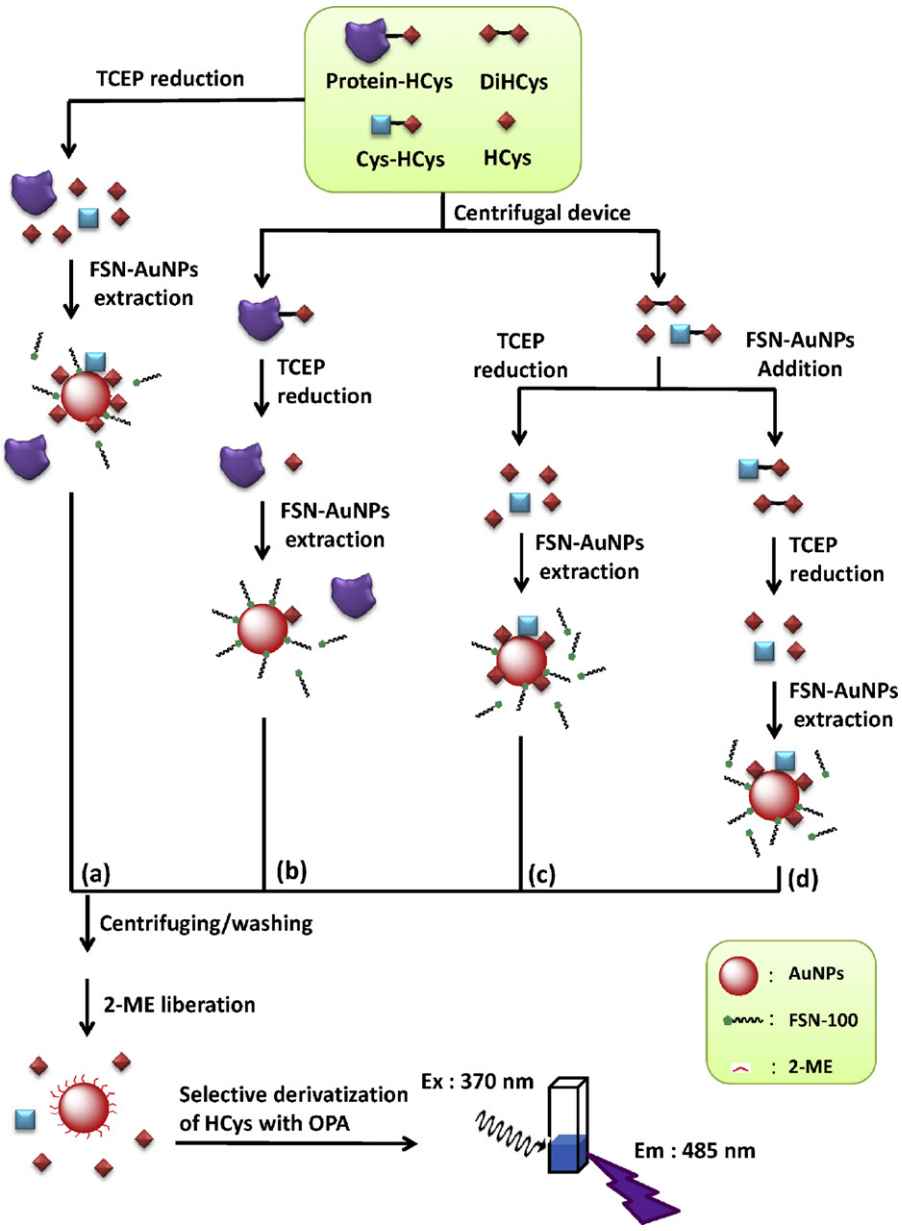
A stock solution of diHCys (1 mM) was prepared in deionized water, while FSN–AuNPs were prepared in 40 mM phosphate at pH 13. We added TCEP (1 M, 27 μL) to a solution of diHCys (10–5000 nM; 3 mL) and equilibrated the mixture at ambient temperature for 0–40 min. TCEP-treated diHCys was incubated with a solution of FSN–AuNPs (0–8.3 nM; 100 μL) for 30 min. The resulting solutions were centrifuged at 17,000 rpm for 20 min. The obtained precipitates were washed with deionized water. Following two centrifugation/washing cycles, the supernatant was carefully removed up to a residual volume of 5 μL. The precipitates were resuspended in a freshly prepared solution of 2-ME (1 M; 30 μL) to release the extracted aminothiols from the Au surface. After 30 (0–40) min, the released aminothiols were isolated from the precipitates by centrifugation at 17,000 rpm for 10 min. The obtained supernatants were derivatized with a solution containing 1 mM OPA and 0.6 M NaOH. After 2 min, the fluorescence spectra of OPA-derivatized HCys (300 μL) were collected by operating a Hitachi F-7000 fluorometer (Hitachi, Tokyo, Japan) at an excitation wavelength of 370 nm.

To test the effect of reducing agent type on the proposed method, this study replaced TCEP with DTT and TBP, one at a time. Additionally, to test the selectivity of the proposed method toward diHCys, this study replaced diHCys with HCys, Cys, glutathione, γ-glutamylcysteine, and cysteinylglycine, one at a time.

In the quantitative analysis, we separately mixed HCys (3 mL, 10–5000 nM) and diHCys (3 mL, 10–5000 nM) with TCEP (27 μL, 1 M) and equilibrated the resulting solution at ambient temperature for 20 min. TCEP-treated HCys and diHCys were separately added to a solution of FSN–AuNPs (100 nM; 100 μL). The following steps, including centrifuging, washing, 2-ME-released HCys, and OPA derivatization, were the same as those used in the extraction procedure. The total analysis time for quantification of HCys and diHCys was 2 h.

2.4. Determination of total, protein-bound, free oxidized, and reduced HCys in plasma

Whole blood samples from a healthy adult female were collected into evacuated blood-collecting tubes containing ethylenediaminetetraacetic acid. To obtain plasma samples, whole blood samples were immediately centrifuged at 3000 rpm for 10 min at 4 °C. (a) *Total*, *Protein-bound*, *and Free HCys Determination*. To obtain total HCys, plasma samples (30 μL) were added to a solution containing TCEP (1 M, 27 μL) and deionized water (2343 μL) for 20 min. TCEP was used to reduce protein-bound and free oxidized HCys. To quantify protein-bound HCys, plasma samples were filtered using the 3 kDa Nanosep centrifugal device (Pall Co., East Hills, NY) at 12,000 rpm with 20 min to obtain plasma proteins. The pore size of centrifugal ultrafiltration (3 kDa) is smaller than the size of proteins. The obtained proteins (30 μL) were incubated with TCEP (1 M, 27 μL), and deionized water (2343 μL) for 20 min. For sensing free HCys, plasma proteins were removed using the 3 kDa Nanosep centrifugal device (12,000 rpm, 20 min). The



Scheme 1. Determination of (a) total, (b) protein-bound, (c) free, and (d) free oxidized HCys in plasma by combining TCEP reduction, FSN–AuNPs extraction, and OPA derivatization.

protein-free solutions (300 μL) containing free oxidized and free reduced HCys were immediately mixed with TCEP (1 M, 27 μL) and deionized water (2073 μL) for 20 min. Note that free reduced HCys was not oxidized in the presence of TCEP. To determine the concentrations of total, protein-bound, and free HCys, this study added a series of samples spiked with solutions of standard HCys (0–5000 nM, 300 μL), FSN–AuNPs (100 nM, 100 μL) and sodium phosphate (300 μL, 400 mM; pH 13.0). The following steps, including incubation time, centrifuging, washing, 2-ME-released HCys, and OPA derivatization, were the same as those used in the quantification of HCys. (b) *Free oxidized HCys Determination*. Plasma samples were filtered using the 3 kDa Nanosep centrifugal device (12,000 rpm, 20 min) to remove plasma proteins. To remove reduced HCys, the obtained solutions (870 μL) were directly mixed with FSN–AuNPs (100 nM, 50 μL) and sodium phosphate (80 μL, 500 mM; pH 13.0). Following 30-min incubation and centrifugation (17,000 rpm, 20 min), free oxidized HCys was obtained. A portion of the samples (300 μL) were incubated with TCEP (1 M, 27 μL) and deionized water (2073 μL) for 20 min. This study added a series of samples spiked with solutions of standard HCys (0–5000 nM, 300 μL), FSN–AuNPs (100 nM, 100 μL), and sodium phosphate (300 μL, 400 mM; pH 13.0). The following steps, including incubation time, centrifuging, washing, 2-ME-released HCys, and OPA derivatization, were the same as those used in the quantification of HCys.

2.5. Fluorescence polarization immunoassay

The concentration of HCys in plasma was determined by Lezen reference laboratory center in Kaohsiung. This assay involves dithiothreitol reduction of mixed disulfides, diHCys, and protein-bound HCys to HCys, followed by enzyme conversion HCys to S-adenosyl-L-homocysteine. After mouse monoclonal S-adenosyl-L-homocysteine antibody was added to the resulting

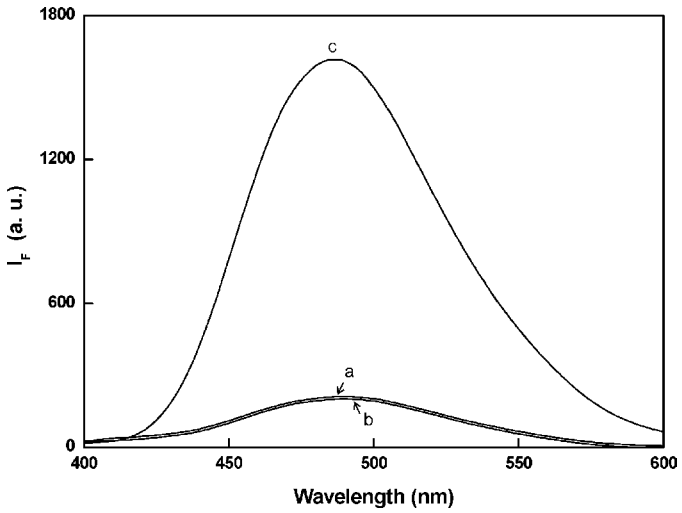


Fig. 1. Fluorescent detection of diHCys through the combination of (a) TCEP reduction and OPA derivatization and (b and c) TCEP reduction, FSN–AuNP extraction, and OPA derivatization. TCEP (1 M, 27 μL) was added to (a and b) 300 μL and (c) 3 mL of 5 μM diHCys. (a) After 20 min, TCEP-treated diHCys was derivatized with a solution containing 0.1 mM OPA and 0.1 M NaOH for 2 min. (b and c) After 20 min, TCEP-treated diHCys was extracted with FSN–AuNPs (100 nM, 100 μL). The resulting solution was incubated with 20 min and centrifuged to obtain the precipitate. The precipitate was resuspended in a solution of 2-ME (1 M, 30 μL). See text for more detailed information.

solution, S-adenosyl-L-cysteine fluorescein tracer is added. Finally, total HCys was quantified by Abbott fluorescence polarization immunoassay (Abbott Laboratories).

3. Results and discussion

3.1. Extraction of diHCys by FSN–AuNPs

Plasma HCys includes protein-bound, free oxidized, and free reduced forms. The reduction of disulfide bonds is a crucial step to determine different forms of plasma HCys prior to FSN–AuNP extraction and OPA derivatization. diHCys was chosen as the model to test whether this proposed method can be used to detect different forms of HCys. diHCys was reduced to HCys with TCEP. TCEP was selected as a reducing agent because of its good water solubility (310 mg/mL), high stability against pH change, fast reduction, and odorless composition [38]. The produced HCys was derivatized with OPA in the absence of a nucleophile. Curve *a* in Fig. 1 shows that the fluorescence band of OPA derivatives was centered at 485 nm when they were excited at 370 nm. The fluorescence profile of the OPA derivatization of TCEP-treated diHCys resembled that of standard HCys (Fig. S3, Supplementary material). This result demonstrates that TCEP does not interfere with the derivatization reaction. Moreover, TCEP is efficient to reduce diHCys. The reduction of diHCys with 9 mM TCEP was completed after 20 min (Fig. S4, Supplementary material). FSN–AuNPs (100 nM, 100 μL) were adopted for the extraction of TCEP-treated diHCys (300 μL, 5 μM). Following the addition of 2-ME, HCys was liberated from the NP surface. The released HCys was labeled with OPA. When compared to curve *a* in Fig. 1, the OPA derivatization of released HCys has a similar fluorescence feature (curve *b* in Fig. 1). Evidently, the combination of TCEP reduction, OPA derivatization, and FSN–AuNP extraction is a useful strategy to selectively detect diHCys. At a fixed concentration of diHCys, the number of diHCys molecules (mole) increased with increasing sample volume. Thus, when the sample volume becomes larger, the more number of diHCys molecules can be extracted with FSN–AuNPs. Compared to the extraction of 300 μL diHCys with FSN–AuNPs, the extraction of 3 mL with FSN–AuNPs resulted in a remarkable increase (9-fold) in fluorescence intensity at 485 nm (curve *c* in Fig. 1). This result indicates that the sensitivity of the proposed method toward diHCys can be greatly improved by increasing sample volume.

3.2. Effects of reducing agents, AuNP concentration, and incubation time

This study investigated how various reductant types influenced the reduction of diHCys to HCys at a fixed sample volume (3 mL). The incubation time between the reductant and diHCys was set to 20 min. Compared to DTT and TBP, the use of TCEP as a reducing agent provided relatively high fluorescence intensity (Fig. 2A). Because thiol groups of DTT molecules strongly bind to the surface of the AuNPs [15], they can occupy the binding sites of FSN–AuNPs. Therefore, the extraction of TCEP-treated diHCys with FSN–AuNPs was unsuccessful in the presence of using DTT as a reducing agent. Moreover, TCEP was found to be more efficient in the reduction of diHCys to HCys than TBP. The loading of analytes on FSN–AuNPs relies on the NP concentration and the incubation time between analytes and FSN–AuNPs [15,37]; thus, we next examined these parameters. As indicated in Fig. 2B, the extraction efficiency of diHCys (1 μM) gradually increased with an increased concentration of FSN–AuNPs and reached a plateau at 3.3 nM. The loading of diHCys on a single 13 nm AuNPs was estimated to be 303 molecules. In contrast, the loading of HCys on the same particle was 597 molecules (estimated from Fig. S5, Supplementary material). Because one diHCys corresponds to two HCys residues linked by a disulfide bond, the concentration of FSN–AuNPs for complete extraction of diHCys is approximately 2-fold higher than that of HCys. Fig. 2C reveals that the loading of diHCys on FSN–AuNPs was increased with the incubation time between TCEP-treated diHCys and FSN–AuNPs. The saturation loading of diHCys was determined to be 20 min. The liberation of analytes on the NP surface is greatly sensitive to the concentration of releasing agents and the incubation time between the releasing agents and analyte-adsorbed AuNPs. When the excess of 2-ME (1 M) was used to liberate HCys, the complete liberation of HCys on the NP surface was found to be above 30 min (Fig. 2D). The total time of the proposed method for determining the total HCys is approximately 120 min.

3.3. Sensitivity and selectivity

The selectivity of the proposed method toward diHCys was evaluated under the optimal conditions mentioned above. Fig. 3 displays the changes in the fluorescence intensity at 485 nm of OPA-derivatized aminothiols, which have been treated with TCEP. The combination of TCEP reduction, FSN–AuNP extraction, and OPA derivatization provided a higher selectivity for diHCys and HCys over other aminothiols. The fluorescence intensity obtained from the analysis of diHCys is reasonably 2-fold higher than that obtained from the analysis of HCys. This study suggests that the selectivity of the present method is 100-fold greater for diHCys and HCys than any aminothiols. The sensitivity of the proposed method toward diHCys was next tested. The fluorescence spectra of the OPA derivatization of TCEP-treated diHCys displayed a gradual increase in fluorescence at 485 nm when the concentrations of diHCys were increased from 10 to 5000 nM (Fig. 4). A linear relationship of the value of fluorescence intensity at 485 nm versus diHCys concentration, shown in the inset of Fig. 4, was from 10 to 1000 nM (R^2 = 0.9945). The limit of detection (LOD) at a signal-to-noise ratio of 3 for diHCys was 4.6 nM, significantly lower than the maximum permissible limit of total HCys in a healthy individual [39]. Few studies analyze diHCys. To compare the sensitivity of the proposed method with other reported methods, this study used HCys instead of diHCys under identical conditions. The linear range for the

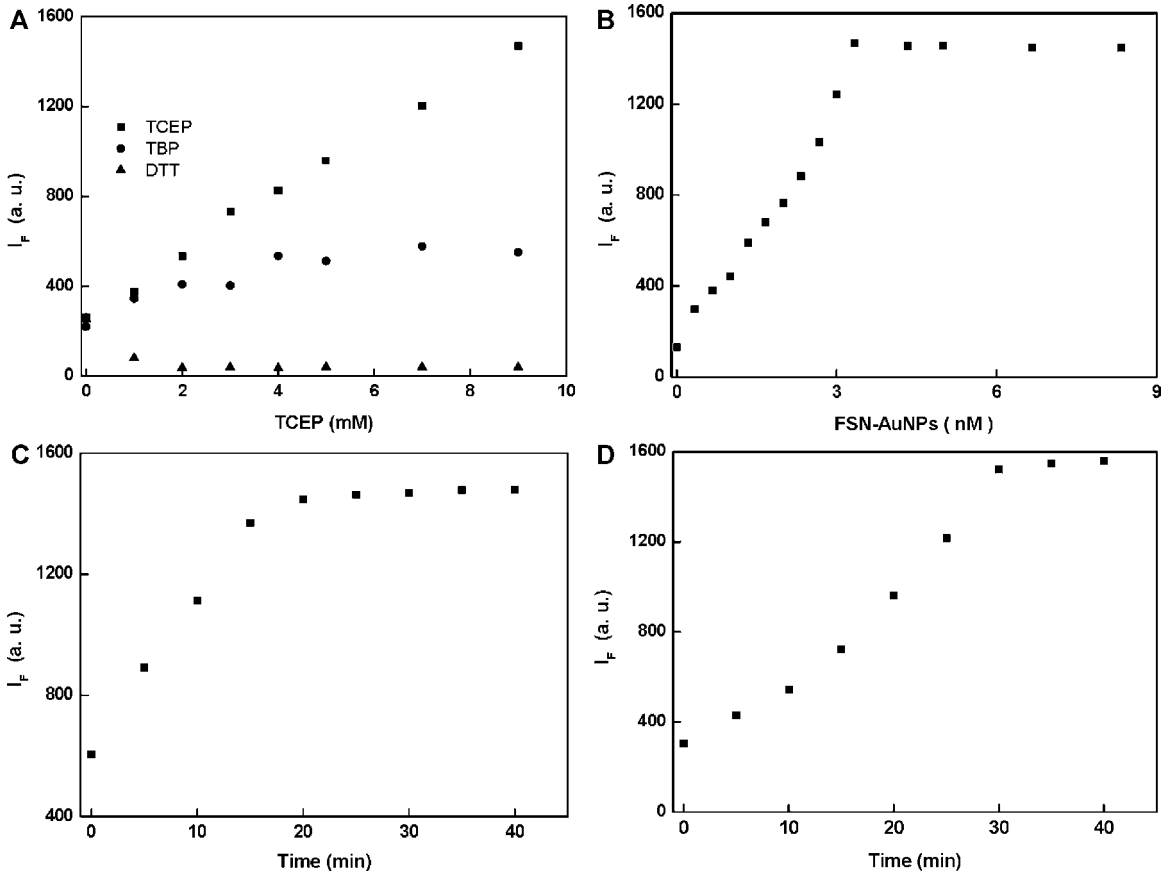


Fig. 2. Effects of (A) the type of reducing agent, (B) the concentration of FSN–AuNPs, (C) the incubation time between TCEP-treated diHCys and FSN–AuNPs, and (D) the incubation time between 2-ME and HCys-adsorbed AuNPs on the fluorescent sensing of diHCys through the combination of TCEP reduction, FSN–AuNP extraction, and OPA derivatization. (A) Reducing agents (1–9 mM) and (B–D) TCEP (1 M, 27 μL) was incubated with diHCys (3 mL, 1 μM) for 20 min. TCEP-treated diHCys was extracted with (A, C, D) 3.3 nM and (B) 0–8.3 nM FSN–AuNPs. After (A, B, D) 20 min and (C) 0–40 min, the precipitate was collected by the centrifugation of the resulting solution. The precipitate was incubated with a solution of 2-ME (1 M, 30 μL) for (A–C) 30 min and (D) 0–40 min. See text for more detailed information.

quantification of HCys was observed from 10 to 1000 nM, while its LOD was estimated to be 4.4 nM (Fig. S6, Supplementary material). Compared to organic dyes, Ag nanoclusters, semiconductor quantum dots, and AuNP-based sensors [18–26,28,30,37,40], the proposed method provides a 3–300-fold improvement in HCys fluorescence detection sensitivity (Table 1). Importantly, the proposed method is capable of sensing HCys without the interference of Cys.

3.4. Quantification of different forms of HCys in plasma

To evaluate the practicality of the proposed method, FSN–AuNPs were utilized to extract and enrich total HCys from the TCEP-treated plasma. Protein-bound HCys and free oxidized HCys were reduced to HCys with TCEP. Although plasma samples also contain other aminothiols, FSN–AuNPs can selectively extract Cys and HCys. The

Table 1
Comparison of the linear range, LOD, and selectivity of different probes for the determination of HCys.

Sensor	Detection	Dynamic range (μM)	LOD (S/N = 3) (nM)	Selectivity[a]	Reference
Dialdehyde-functionalized fluorescein	Fluorescence	2.9–2500	Not given	Cys and HCys	[18]
Ruthenium(II) complex	Phosphorescence	15–180	1190	Cys and HCys	[19]
Aldehyde-functionalized fluorescein	Fluorescence	Not given	100	Cys, HCys, and GSH	[20]
Azo derivative	Colorimetry	5–120	Not given	Cys and HCys	[21]
8-Oxo-8H-acenaphtho[1,2-b]pyrrole-9-carbonitrile	Fluorescence	5–200	Not given	Cys and HCys	[22]
2,2′,2″-Trisindolylmethene	Colorimetry	14–3000	1400	Cys and HCys	[23]
Organic dye consisting of diarylazo quencher and a fluorescein fluorophore	Fluorescence	10–300	Not given	Cys and HCys	[24]
Silver nanocluster	Fluorescence	0.6–2	200	Cys, HCys, and GSH	[25]
CdTe/CdSe quantum dot	Fluorescence	0.2–100	26	Cys, HCys, and GSH	[26]
FSN–AuNPs (13 nm)	Colorimetry	0.1–0.5	Not given	Cys and HCys	[28]
FSN–AuNPs (40 nm)	Colorimetry	0.5–2	200	HCys	[30]
Extraction of HCys with FSN–AuNPs followed by OPA derivatization	Fluorescence	0.6–40	180	HCys	[37]
Nile red-modified AuNPs	Fluorescence	0.05–5	10.9	Cys, HCys, and GSH	[40]
Enrichment and Extraction of HCys with FSN–AuNPs followed by OPA derivatization	Fluorescence	0.01–1	4.4	HCys	This study

[a] GSH, glutathione.

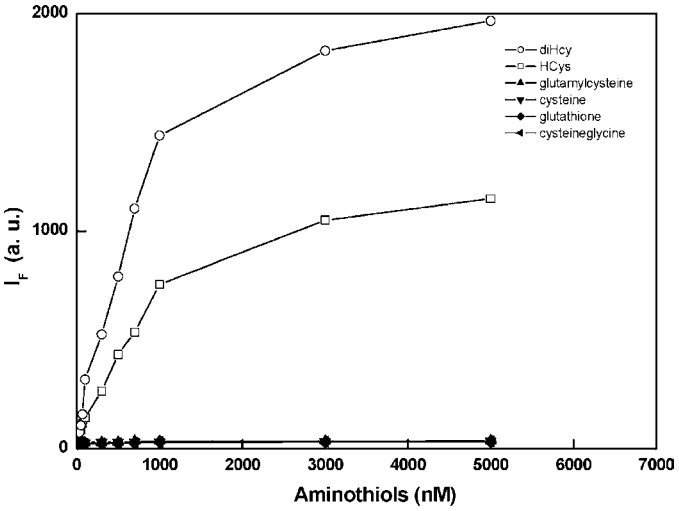


Fig. 3. Selectivity of the combination of FSN–AuNPs (13 nm) extraction and OPA derivatization. TCEP (1 M, 27 μL) was incubated with 3 mL of 0–5000 nM analytes for 20 min. TCEP-treated analyte was extracted with 50 μL of 100 nM FSN–AuNPs. The resulting solution was incubated with 20 min and centrifuged to obtain the precipitate. The precipitate was resuspended in a solution of 2-ME (1 M, 30 μL). See text for more detailed information.

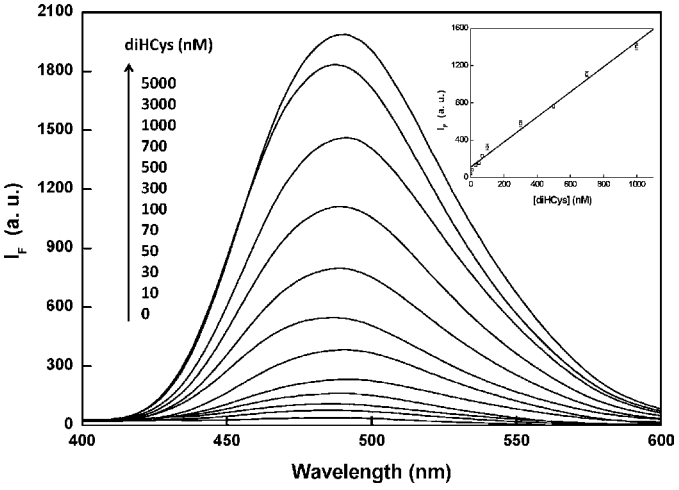


Fig. 4. Quantification of diHCys by combining FSN–AuNPs extraction with OPA derivatization. TCEP (1 M, 27 μL) was incubated with 3 mL of 0–5000 nM diHCys for 20 min. TCEP-treated diHCys was extracted with 100 μL of 100 nM FSN–AuNPs. The following steps, including centrifuging, incubation time, 2-ME-released HCys, and OPA derivatization are the same as those in Fig. 3. Inset: a plot of fluorescence intensity at 485 nm versus the concentration of diHCys. The error bars represent standard deviations based on five independent measurements.

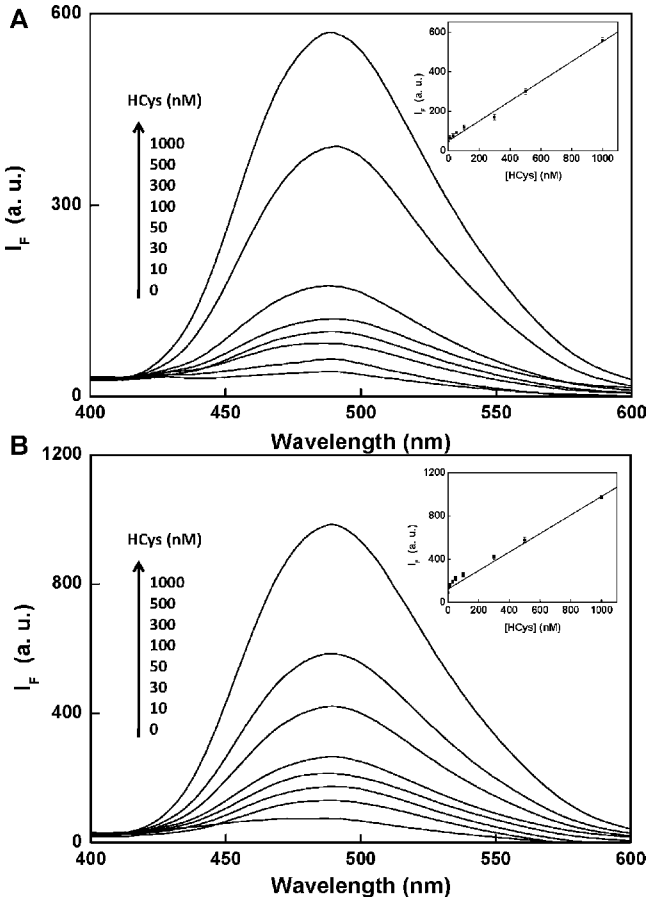


Fig. 5. Quantification of (A) total HCys and (B) free HCys in plasma by combining FSN–AuNPs extraction and OPA derivatization. (A) Plasma samples (30 μL) were treated with TCEP for 20 min. TCEP-reduced plasma samples were spiked with standard solutions of HCys (0–1000 nM). (B) Plasma proteins were removed using a centrifuge filter device. Protein-free solutions (300 μL) were treated with TCEP and then spiked with standard solution of HCys (0–1000 nM). (A, B) TCEP-treated samples were extracted with 100 μL of 100 nM FSN–AuNPs. The final sample volume was 3 mL. The following steps, including centrifuging, incubation time, 2-ME-released HCys, and OPA derivatization are the same as those in Fig. 4. Inset: a plot of fluorescence intensity at 485 nm as a function of the HCys concentration. The error bars represent standard deviations based on five independent measurements.

normal concentrations of total Cys and HCys in human plasma are 150 ± 50 and 11 ± 4 μM, respectively [41]. Plasma samples (30 μL) were diluted to 100-folded prior to the analysis of total HCys. Because 100 μL of 100 nM FSN–AuNPs was capable of completely extract 3 mL of 1 μM diHCys (Fig. 2B), we suggest that the optimized extraction conditions were sufficient to capture total Cys and HCys in diluted plasma samples (3 mL). Samples of plasma were spiked with standard solutions containing different concentration of HCys. The present method for the determination of these spiked samples resulted in a gradual increase in the fluorescence intensity at 485 nm (Fig. 5A). A linear ($R^2 = 0.9911$) calibration curve was obtained by plotting the value of fluorescence intensity at 485 nm against the concentration of spiked HCys (inset in Fig. 5A). By applying standard addition, the concentration of total HCys was calculated to be 11.86 ± 0.35 μM ($n = 5$). This result is consistent with the concentration (11.36 ± 0.20 μM, $n = 5$) of total HCys in plasma determined through fluorescence polarization immunoassay [42]. Based on a *t*-test (95% confidence level, 4 degrees of freedom) and *F*-test (95% confidence level), the result obtained from the proposed method was shown to be in accordance with that obtained through fluorescence polarization immunoassay. Plasma proteins were collected using a centrifugal device. Following the addition of TCEP, HCys was liberated from the collected plasma proteins. To obtain free HCys (free oxidized and free reduced HCys), plasma proteins were removed by a centrifugal device. TCEP was used to reduce the disulfide bonds between HCys and other thiols in a plasma-free solution. Fig. S7 (Supplementary material) and Fig. 5B show the analysis results for protein-bound HCys spiked with standard HCys (0–1000 nM) and free HCys spiked with standard HCys (0–1000 nM), respectively. The concentrations of protein-bound HCys and free HCys were measured to be 9.89 ± 1.4 μM ($n = 5$) and 1.84 ± 0.1 μM ($n = 5$), respectively. The sum of the concentrations of protein-bound HCys and free HCys is compatible with the concentration of total HCys, suggesting that the proposed method is reliable for determining different forms of plasma HCys. To determine free oxidized HCys, 2 nM FSN–AuNPs was added to a plasma-free solution. Free reduced HCys can bind to the surface of FSN–AuNPs through the formation of Au—S bonds, but not

in the case of free oxidized HCys. Following centrifugation of a plasma-free solution containing FSN–AuNPs, the obtained supernatant corresponding to free oxidized HCys was reduced with TCEP. Standard additions show that the concentration of free oxidized HCys was $1.76 \pm 0.3\ \mu M$ ($n = 5$) (Fig. S8, Supplementary material). Free reduced HCys was calculated as the concentration of free HCys minus free oxidized HCys. The concentration of free reduced HCys was estimated to be $0.08\ \mu M$. These results suggest that plasma total HCys consists of large quantities of protein-bound HCys, small amounts of free oxidized HCys, and trace amounts of free reduced HCys, indicating that HCys in blood is rapidly oxidized.

4. Conclusions

This study demonstrates that the combination of TCEP reduction, FSN–AuNP extraction, and OPA derivatization is a reliable method of analyzing total, protein-bound, free, and free oxidized HCys. diHCys with a disulfide bond was used to optimize the reduction and extraction conditions. The proposed method provided a number of distinct advantages. Firstly, this method provided excellent selectivity toward HCys and the lowest LOD value for HCys compared to organic dyes, Ag nanoclusters, semiconductor quantum dots, and AuNP-based sensors. Secondly, to the best of our knowledge, this is first example of a successful determination of total, protein-bound, free, and free oxidized HCys in plasma without chromatographic separation. Lastly, only a small sample volume ($30\ \mu L$) is required to determine total and protein-bound HCys. Since *S*-adenosylhomocysteine hydrolase can catalyze the hydrolysis of *S*-adenosylhomocysteine to HCys [29], the proposed method could be further applied to the detection of *S*-adenosylhomocysteine through the assistance of *S*-adenosylhomocysteine hydrolase.

Acknowledgments

We would like to thank National Science Council (NSC 98-2113-M-110-009-MY3) for the financial support of this study. We also thank National Sun Yat-sen University and Center for Nanoscience & Nanotechnology for the measurement of fluorescence spectrum.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2012.01.026.

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