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Review

Molecular and nanoparticle postcolumn reagents for assay of low-molecular-mass biothiols using high-performance liquid chromatography☆

Yanbing Zu*

Institute of Bioengineering and Nanotechnology, 31 Biopolis Way, Singapore 138669, Singapore

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ABSTRACT

Determination of low-molecular-mass (LMM) biothiols in biological matrixes is of importance in the studies of their related bio-processes and for the clinical diagnostics of a variety of diseases. Standard method for the assay of the small biothiols is in demand. Postcolumn techniques used in high-performance liquid chromatography (HPLC) allow automation of the derivatization step and, therefore, are suitable for standardization of HPLC analysis. This paper gives an overview of the existing reaction systems useful for the postcolumn assay of the LMM biothiol molecules in conjunction with HPLC. The postcolumn reagents are classified by the types of their reactions with thiol-containing compounds. The chemical reactivity and selectivity as well as the spectroscopic characteristics of the postcolumn reagents have been addressed. The emerging strategies of using nanoparticles as thiol-reactive reagents and their applications in postcolumn detection of the LMM biothiols have also been discussed in detail.

Contents

1. Introduction

Low-molecular-mass (LMM) biothiols play important roles in biological systems [1–4]. They include sulfhydryl-containing amino acids such as cysteine (Cys) and homocysteine (Hcy), and small peptides such as cysteinylglycine (Cys-Gly) and glutathione (GSH). Abnormal levels of these molecules in biological fluids may be risk factors for a variety of diseases [5–7]. Therefore, their determination has been intensively studied [8,9]. The LMM biothiols could exist in their oxidation forms that contain disulfide groups. For example, the majority of plasma Hcy is protein-bound (~70%); the free Hcy is mainly oxidized to homocystine or Hcy-Cys mixed disulfide, and only ~2–3% remains as free reduced Hcy. In the assay of total plasma Hcy, dithiothreitol (DTT) or *n*-tributylphosphine can be used to reduce the disulfide to give the corresponding thiol compounds.

Because of the component complexity of the biological fluids, specific detection of the small biothiols is usually based on high-performance liquid chromatography (HPLC). The fluorescence polarization immunoassay, run on Abbott's IMx and AxSYM plat-

Abbreviations: Cys, cysteine; Cys-Gly, cysteinylglycine; DTNA, 6,6′-dithiodinicotinic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; GSH, glutathione; γ-Glu-Cys, γ-glutamylcysteine; Hcy, homocysteine; HPLC, high-performance liquid chromatography; LMM, low-molecular-mass; NAM, *N*-(9-acridinyl)maleimide; NP, nanoparticle; NPM, *N*-(1-pyrenyl)maleimide; OPA, *ortho*-phthalaldehyde; PCs, phytochelatins; SPR, surface plasmon resonance.

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* Fax: +65 6478 9085.
E-mail address: ybzu@ibn.a-star.edu.sg.

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forms, is also widely used for the assay of Hcy [8]. Compared to immunoassay, HPLC technique needs no relatively expensive biological reagents, and can work in a wide analytical range and allow for simultaneous determination of a number of related compounds. These advantages make HPLC a very useful method for the analysis of the LMM biothiols. Following HPLC separation, colorimetric or fluorometric detection techniques are commonly used for quantification of the analytes. Since the LMM biothiols generally show very weak absorbance and only at short wavelengths, and exhibit no native fluorescence, it is necessary to modify these molecules with reagents exhibiting high extinction coefficients or high fluorescence yields [9,10]. Most of these reagents bear electrophilic alkylating moieties which can provide linkages after reaction with sulfhydryls.

The derivatization reactions can be carried out either prior to or following HPLC separation. Postcolumn technique (Fig. 1) allows automation of the derivatization step and has several advantages over precolumn method [11]. The sample treatment prior to assay is minimized by employing postcolumn reactions, avoiding the changes of the chromatographic properties of the analytes. In addition, the influences of performance variation of the assay are generally less for an automatic postcolumn detection. Therefore, postcolumn techniques are more suitable for standardization of assays of the LMM biothiols. However, the requirements for a successful postcolumn reaction system are more rigid. First, the kinetics of a postcolumn derivatization reaction must be fairly fast. Otherwise, a reactor with larger volume will be needed which leads to a significant peak-broadening and a loss in resolution. Secondly, interfering signals from the derivatizing reagents and solvents must be as low as possible, so that a high precision and a low detection limit can be achieved. Because of these limitations, the postcolumn reagents reported in the literature for the determination of the LMM biothiols are less numerous than the precolumn ones. There is thus great interest in developing new postcolumn reaction systems for HPLC assay of the LMM biothiols. Encouragingly, recent studies revealed that nanoparticles (NPs) can serve as novel postcolumn reagents and the reaction selectivity towards the LMM biothiols can be tuned by capping ligands of the NPs [12,13].

Here, I present a brief overview of existing postcolumn reaction systems for detection of the LMM biothiols with emphases on commonly used molecular reagents and the emerging nanoparticle reagents. Their reactivity, selectivity and spectroscopic characteristics have been addressed.

2. Postcolumn detection systems for HPLC assay of low-molecular-mass biothiols

In this section, the postcolumn systems are organized according to their chemical features and summarized in Table 1.

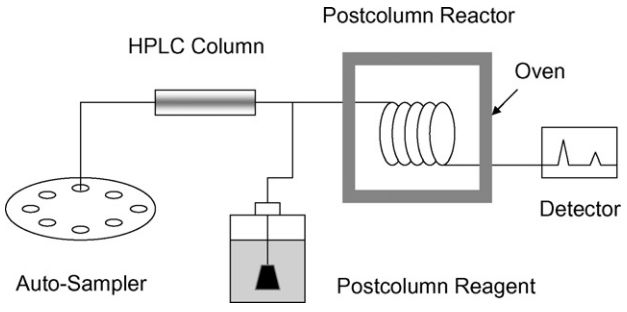

Fig. 1. Schematic drawing of the automatic HPLC system in conjunction with postcolumn derivatizaton.

2.1. ortho-Phthalaldehyde labeling

ortho-Phthalaldehyde (OPA) is one of the most popular derivatization reagents for postcolumn detection of the LMM biothiols [14–23]. OPA exhibits no native fluorescence, but the reaction of OPA with a thiol- and a primary amino-containing molecule generates a thiol-2-alkyl-substituted isoindole which is highly fluorescent and is monitored with an excitation wavelength of ~348 nm and an emission wavelength of ~450 nm. This reaction system has been developed for the determination of amino acids and polyamines, but it has also been applied to derivatize the LMM biothiols in either precolumn or postcolumn mode.

Fig. 2 shows the thiol-selective (Fig. 2a) and the heterobifunctional (Fig. 2b) OPA reactions. These reactions can proceed rapidly at ambient temperature. In the thiol-selective OPA reaction (Fig. 2a), the presence of an amine as a co-reagent is needed for the selective derivatization of a thiol molecule. Glycine is commonly used as the co-reagent [19]. Because chemical interaction is possible between OPA and the amino compound, the co-reagent needs to be mixed just prior to the derivatization reaction. This can be achieved either by using a two-way postcolumn device or simply by addition of the co-reagent to the mobile phase [19]. Typically, the mobile phase (flow rate, 1.2 mL min^{-1}) consists of a mixture of acetonitrile (5%, v/v) and 10 mM phosphate buffer (pH 2.5) containing 0.1 mM disodium EDTA, 0.5 mM sodium *n*-decylsulfate, and 0.5 mM glycine; the postcolumn reagent (flow rate, 0.3 mL min^{-1}) is a 0.8 g L^{-1} OPA solution prepared in 0.35 M borate buffer (pH 10.5) containing 0.4% (v/v) Brij-35. It has been found that the fluorescence yield of isoindole adduct of a thiol is very sensitive to its structure environment, especially to carbonyl and amino groups present in the lateral chains close to the fluorophore. The isoindole adduct of Cys exhibits relatively weak fluorescence, resulting in lower detection sensitivity as compared to other LMM biothiols [19].

As to the compounds containing both thiol and primary amine moieties, such as GSH and γ-Glu-Cys, they may react directly with OPA, which is also known as heterobifunctional OPA reaction (Fig. 2b). This type of reaction is quite rare because rigid requirements on the steric hindrance and the distance between the amino and thiol groups need to be fulfilled in order to attain a highly fluorescent adduct. Therefore, the OPA postcolumn system containing no amino co-reagent is highly specific for GSH and γ-Glu-Cys at pH 10.5. Parmentiar et al. used the HPLC postcolumn reaction to determine GSH-related enzyme activities [19]. Although the derivatization of Hcy with OPA is not efficient under conditions of about pH 10, the heterobifunctional OPA reaction of Hcy can proceed rapidly at pH 13 and produces adduct with high fluorescence [20]. This reaction has been used to detect Hcy and Hcy thiolactone in cell cultures and human urine and plasma samples [20–22]. The postcolumn reagent usually consists of several millimolar OPA and 0.5 M NaOH. Note that, under alkaline conditions, Hcy thiolactone is almost instantly hydrolyzed to Hcy. Protein N-linked Hcy was also quantified after being liberated from proteins by acid hydrolysis and converted to Hcy thiolactone [23].

The major drawbacks of the OPA labeling method include that (1) the reaction is highly pH sensitive and (2) the fluorescent thiol adducts are somewhat unstable in aqueous solution because of hydrolysis and attack by excess OPA. To improve the method, cyclodextrins have been used to protect the isoindole derivatives. Inclusion of the isoindole derivative inside the cyclodextrin cavity not only stabilizes the fluorophore but also enhances the fluorescence. Wagner et al. found that a modified cyclodextrin, i.e., 2-hydroxypropyl-b-cyclodextrin, is more effective than the parent compound [24].

Table 1
Postcolumn reagents for use in HPLC assay of LMM biothiols.

Reagent	Reaction	Detection	Analytes	Reference
OPA	*o*-Phthalaldehyde labeling	Fluorescence Ex ~348 nm Em ~450 nm	Common LMM biothiols Could be specific towards Hcy, Glu and Glu-Cys	[14–23]
Ninhydrin	Thiazolidine reaction	UV–vis ~560 nm	Common LMM biothiols	[25–29]
Fluorescein dialdehyde	Thiazolidine reaction	UV–vis ~480, 505 nm or Fluorescence Ex ~460 nm Em ~520 nm	Specific towards Cys and Hcy	[10]
DTNB	Sulfhydryl/disulfide exchange reaction	UV–vis ~412 nm	Common LMM biothiols	[30–37]
DTNA	Sulfhydryl/disulfide exchange reaction	UV–vis ~344 nm	Common LMM biothiols	[38]
4,4′-Dithiodipyridine	Sulfhydryl/disulfide exchange reaction	UV–vis ~324 nm	Common LMM biothiols	[39]
NAM	Maleimide labeling	Fluorescence Ex ~365 nm Em ~435 nm	Common LMM biothiols	[41]
NPM	Maleimide labeling	Fluorescence Ex ~342 nm Em ~390 nm	Common LMM biothiols	[19,40,42]
Hexaiodoplatinate	Ligand substitution reaction	UV–vis ~500 nm	Common LMM biothiols	[45–47]
Palladium(II)–calcein	Ligand substitution reaction	Fluorescence Ex ~492 nm Em ~512 nm	Common LMM biothiols	[48]
Methyl viologen	Electron-transfer reaction	UV–vis ~398, 605 nm	Specific towards Cys and Hcy	[10]
Fluorone black	Electron-transfer reaction	UV–vis ~505 nm	Specific towards Cys and Hcy	[10]
Brij® 35-capped gold NPs	Analyte-induced aggregation	UV–vis ~680 nm	Common LMM biothiols	[12]
Zonyl® FSN-capped gold NPs	Analyte-induced aggregation	UV–vis ~680 nm	Specific towards Cys and Hcy	[13]

2.2. Thiazolidine reaction

Ninhydrin (triketohydrindene hydrate), a primary amino-active reagent known since 1910, has been extensively used for the determination of α-amino acids [25,26]. The reaction product of a common α-amino acid with ninhydrin is a purple colored complex named Ruhemann's Purple, whose color intensity can be monitored for most amino acids at 570 nm. However, for the biothiol molecules containing both of amine and thiol groups, the proximity of the amine and thiol nucleophiles allows for a peculiar chemical reactivity between these species with ninhydrin, resulting in the formation of a thiazolidine compound (see Fig. 3a) [25,26]. In acid solution, the reaction products are pink in color with maximum absorption at ~560 nm. A progressive increase of color factor (color yield relative to leucine) has been obtained for the ninhydrin derivatization of L-Cys (0.12), the dipeptide γ-Glu-Cys (0.15), and

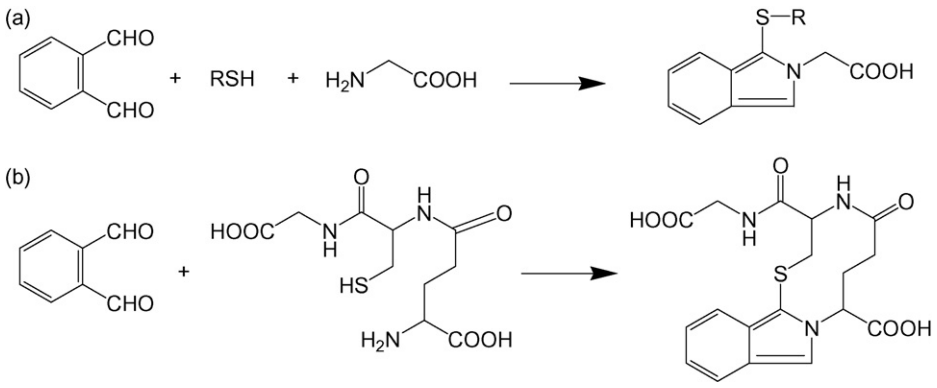

Fig. 2. OPA labeling of thiol-containing compounds: (a) thiol-selective reaction and (b) heterobifunctional reaction of GSH.

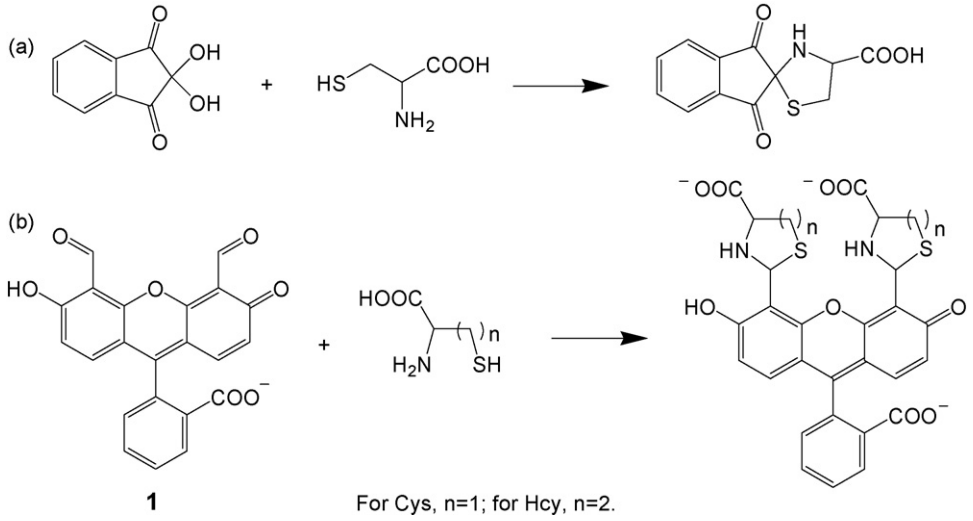

Fig. 3. Labeling of the LMM biothiols via thiazolidine reactions: (a) reaction of ninhydrin with Cys and (b) reaction of fluorescein dialdehyde with Cys or Hcy.

the tripeptide GSH (0.72) [27]. It was suggested that the increase in these values is probably because of the increase in distance between the NH_2 and the SH groups in the compounds which minimizes the extent of the thiazane ring formation in the higher homologs.

Ion-exchange chromatography with ninhydrin postcolumn derivatization is widely used for the assay of Cys and Hcy in studies of diagnosis and management of inborn errors of transsulfuration pathways [28,29]. The automated method can be employed to quantify total Hcy level in human plasma as an indicator of inherited defects of Hcy metabolism as well as a marker of increased risk of cardiovascular disease. In these studies, conventional amino acid analyzers were used, which also enabled the routine measurement of all other commonly occurring amino acids.

The major drawback of ninhydrin reaction system is the low selectivity towards the LMM biothiols. There are a number of ninhydrin-positive compounds, such as various free amino acids, present in physiological fluids (e.g., urine, plasma). The broad cross-reactivity with interferences may lead to complex chromatograms [25,26].

The reaction of aldehyde with Cys or Hcy also produces thiazolidine. Strongin's group [10] studied the interactions between a new fluorescein dialdehyde compound (Fig. 3b, compound 1) and a variety of biological molecules, and found that the UV–vis absorbance and fluorescence changes are highly selective towards Cys and Hcy (see Fig. 3b). Following the addition of Cys or Hcy to a solution of the fluorescein dialdehyde, solution color changes from bright yellow to brownish-orange, and the fluorescence is quenched. However, other sulfur-containing compounds (such as methionine and GSH) and other amino acids would induce no optical spectral changes. The UV–vis absorbance changes and the fluorescence quenching effect have also been observed upon addition of Cys and Hcy to human plasma samples containing the fluorescein dialdehyde. It was demonstrated that the fluorescein dialdehyde could serve as a postcolumn reagent for HPLC determination of Cys and Hcy [10]. The reagent was made of 6.4 μM fluorescein dialdehyde in 0.125 M carbonate buffer (pH 9.5), and the reactor temperature maintained at 80 °C. UV–vis detector wavelength used in this study is 510 nm.

2.3. Sulfhydryl/disulfide exchange reaction

The sulfhydryl/disulfide exchange reaction is highly specific for the detection of thiols. A well-known example is DTNB [5,5′-dithiobis(2-nitrobenzoic acid)], also called Ellman's reagent [30], that responds rapidly towards a thiol molecule via the exchange reaction, as shown in Fig. 4a. Half of the DTNB molecule, TNB anion, is attached to the thiol. The remaining free 5-thio-(2-nitrobenzoic acid) shows absorption at ~412 nm with a large extinction coefficient of $\sim 1.36 \times 10^4\ M^{-1}\ cm^{-1}$ and can be detected by UV–vis spectroscopy. DTNB is widely used for the postcolumn determination of thiol-containing compounds [31,32]. It has been a routine procedure to estimate free thiols in protein by using DTNB [31]. Typical postcolumn reagent is made of millimolar DTNB in 0.3 M phosphate buffer (pH ~8) containing 15 mM EDTA.

Phytochelatins (PCs), a family of small thiol-rich peptides, are produced by plants to protect against toxicity in response to excessive uptake of heavy metals. PCs have the common structure $(\gamma\text{-Glu-Cys})_{2-11}$-Gly, and can be determined by using HPLC with postcolumn DTNB derivatization [33–36]. It was found that the derivatization efficiency decreased with increasing chain length of PCs because of steric hindrance [34]. Yamato et al. reported a method for coenzyme A detection by using Ellman's reagent to quantify thiol-coenzyme A liberated from acetyl-coenzyme A via the phosphotransacetylase reaction [37].

However, the reactions of thiols with DTNB require mild alkaline conditions. Nishiyama and Kuninori [38] reported a similar postcolumn reaction with 6,6′-dithiodinicotinic acid (DTNA), as shown in Fig. 4b. The reaction produces 6-mercaptonicotinic acid that shows absorption at 344 nm. Although the molecular extinction coefficient of 6-mercaptonicotinic acid at 344 nm ($\sim 1.0 \times 10^4\ M^{-1}\ cm^{-1}$) is a bit lower than that of TNB at 412 nm, the postcolumn reaction with DTNA has advantage of generating stable product over a wider range of physiological pH. It was noted that this property is valuable when a mobile phase of low pH is used to increase the retention volume of polar thiols and to protect against oxidation of thiols during HPLC [38].

4,4′-Dithiodipyridine has also been used as a postcolumn reagent for the detection of the LMM biothiols (see Fig. 4c) [39]. A thiol reacts with 4,4′-dithiodipyridine to form a mixed disulfide and 4-thiopyridone. Because of the stabilizing effect exerted by the thione form, the reaction equilibrium favors the formation of 4-thiopyridone. The reaction product has a larger extinction coefficient ($\sim 1.95 \times 10^4\ M^{-1} \times cm^{-1}$) than that of DTNB. Total, free, and reduced forms of Hcy and other small biothiols in human plasma have been quantified by using this postcolumn reagent [39]. The

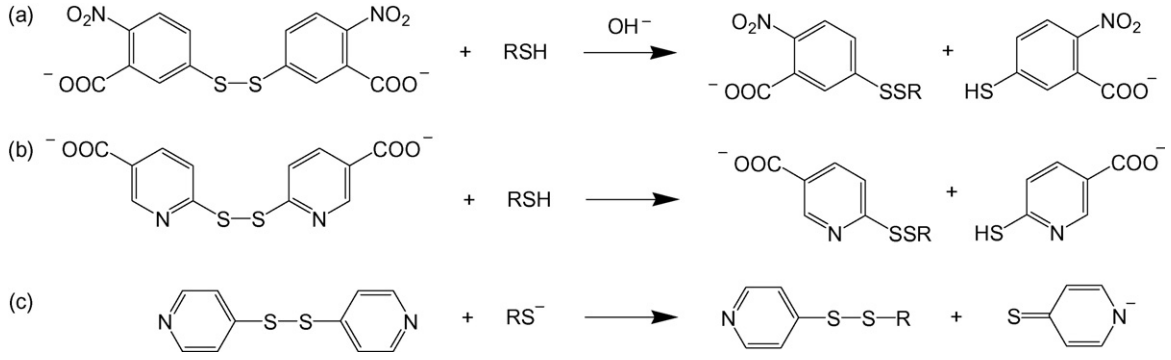

Fig. 4. Sulfhydryl/disulfide exchange reactions of thiol-containing compounds with (a) DTNB; (b) DTNA; and (c) dithiodipyridine.

colorimetric detection was conducted at 324 nm. It was found that the reaction rates for Hcy, Cys, GSH, and Cys-Gly were similar, but γ-Glu-Cys exhibited a slower reaction rate. The detection limit for Hcy was ~50 nM.

2.4. Maleimide labeling

N-Substituted maleimide can be attached to a thiol molecule facilely (Fig. 5). The derivatization is based on a nucleophilic addition of the thiol group to the carbon–carbon double bond of the maleimide reagent. The reaction rate depends on the pK_a value of the thiol [40]. Many of the maleimide reagents exhibit weak fluorescence, but their reaction products with thiols are highly fluorescent. The maleimide/thiol conjugates are unstable and may undergo further rearrangement, leading to the formation of two ring-cleaved products, as shown in Fig. 5a. Multiple chromatographic peaks could appear [9]. The selectivity of maleimide labeling towards the LMM biothiols is generally low because of the cross-linking to

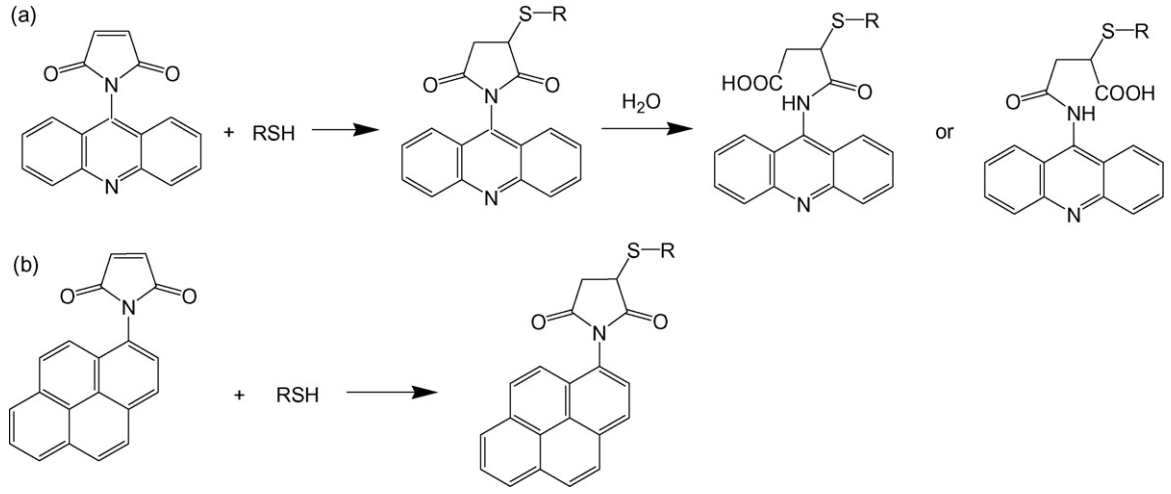

Fig. 5. Reactions between N-substituted maleimides and thiol-containing compounds: (a) NAM reaction and (b) NPM reaction.

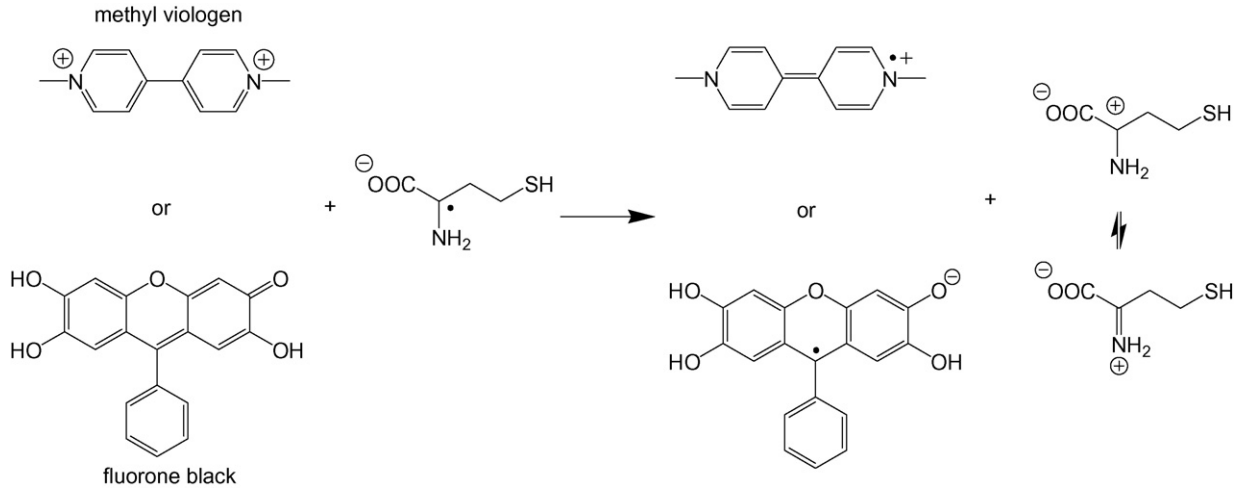

Fig. 6. Electron transfer from Hcy carbon-centered radical to methyl viologen or fluorone black.

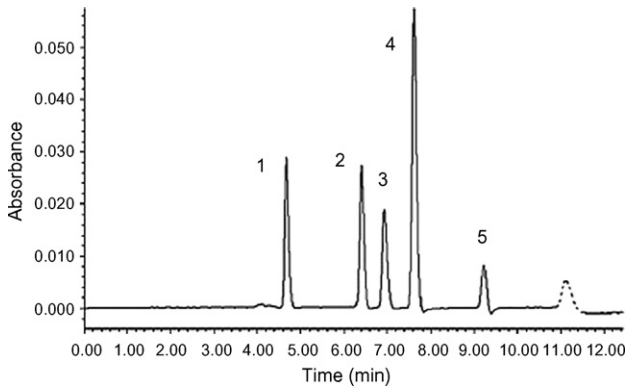

Fig. 7. Typical HPLC chromatogram of a mixture (6 μM each thiol) of Cys (1), Cys-Gly (2), Hcy (3), γ-Glu-Cys (4), and GSH (5). The peak presented in dotted line was produced by 100 μM methionine. HPLC conditions: column, SunFire C18 (4.6 mm × 150 mm, 3.5 μm particle size, Waters); sample injection volume, 20 μL; mobile phase, 9% methanol and 91% aqueous solution (containing 0.05% TFA and 50 μM 1-octanesulfonate sodium salt), 0.7 mL/min; postcolumn reagent, colloidal solution of Brij 35-capped gold NPs containing 60 mM NaCl and 0.07% TFA, 0.35 mL/min; postcolumn reaction temperature, 60 °C; detection wavelength, 680 nm. Reprinted with permission from Ref. [12]. Copyright 2007 Elsevier.

amines. These labeling reaction systems are usually used in precolumn derivatization. *N*-(9-acridinyl)maleimide (NAM) has been employed as postcolumn reagent, but high background level was observed [41].

The use of another maleimide, *N*-(1-pyrenyl)maleimide (NPM), in the postcolumn HPLC detection of the LMM biothiols (Fig. 5b) was also reported [19,40,42]. Because NPM is unstable in aqueous solution, the postcolumn reagent has been prepared in a water miscible organic solvent (i.e., acetonitrile). The NPM postcolumn system was found to allow detection of a variety of LMM biothiols with a similar detection limit and was used to identify and quantify the intracellular thiols in different cell lines [19]. In this study, the NPM reaction was carried out by using a two-way postcolumn device and using two co-solutions: 2% (v/v) triethylamine-1% (v/v) Brij-35 in CH_3CN–water (30:70, v/v) and 0.015 g L^{-1} NPM in acetonitrile. Fluorescence detection was operated at respective excitation and emission wavelengths of 342 and 389 nm. One advantage of the NPM labeling is the low response for DTT that is often used in excess as a reducing reagent for thiol studies [19].

2.5. Ligand substitution reaction

Hexaiodoplatinate is known to be decolorized by organic sulfides via ligand exchange reactions, resulting in absorbance decrease at 500 nm (reactions 1 and 2) [43,44]. HPLC analysis of the LMM biothiols based on the optical changes following the substitution of the coordination bonds has been studied [45–47]. Hexaiodoplatinate exhibits a broad reactivity. For the detection of the LMM biothiols, interferences from thioethers, thiazolidines and ascorbic acids have been reported [45]. However, Harada et al. found that these interferences could be suppressed under certain experimental conditions [47]. The reaction medium used in their study is 0.1 M sodium phosphate buffer at pH 2.2 containing 100 mM $H_2PtCl_6{\cdot}6H_2O$ and 10 mM potassium iodide. The derivatization was performed in a polyether ether ketone tube which was maintained at 40 °C. The method was used to determine three concentrations (reduced, protein-unbound, and total) of *N*-acetyl-L-cysteine and Cys in rat plasma with the detection limit of ~20 pmol [47]. It was found that the reaction between hexaiodoplatinate and DTT leads to the formation of a contaminating substance that may be adsorbed to the inner walls of the HPLC pipeline and induce peak-broadening and tailing [47]. Therefore, it was recommended to minimize the amount of DTT used in the sample pretreatment and to employ a non-metallic pipeline for this postcolumn system.

$$[PtI_6]^{2-} + 4R_2S \rightarrow [Pt(R_2S)_4]^{2+} + I_2 + 4I^- \quad (1)$$

$$[PtI_6]^{2-} + 2R_2S \rightarrow [Pt(R_2S)_2I_2] + I_2 + 2I^- \quad (2)$$

Palladium(II)–calcein is another postcolumn reagent reported for the detection of sulfur-containing compounds based on a ligand-exchange reaction [48]. The non-fluorescent reagent releases free calcein when reacted with organosulfur compounds, providing an indirect measure of the compounds via fluorescence detection. Obviously, the reaction is not specific for thiols.

2.6. Electron-transfer reaction

It is well known that the oxidation of the LMM biothiols in nature leads to the formation of sulfenyl radicals. Through an intramolecular hydrogen abstraction, a reducing α-amino carbon-centered radical can be generated. It was reported that the reaction is particularly favorable for Hcy as compared to Cys and GSH molecules because of a favored 5-membered ring transition state [10]. Fig. 6 shows the electron-transfer reactions of the Hcy carbon-centered radicals with methyl viologen and fluorone black. The dication methyl viologen responds to GSH, Cys and Hcy under basic conditions (pH 10.5). The formation of the methyl viologen radical cation can be monitored via changes in the UV–vis absorbance spectra. At neutral pH, the reaction is highly selective for Hcy [10]. In the case of fluorone black, the formation of semi-quinone radical of the reagent molecule leads to the absorbance increase. Strongin's group conducted initial trials of using these electron-transfer reactions for HPLC postcolumn determination of Cys and Hcy [10]. In these

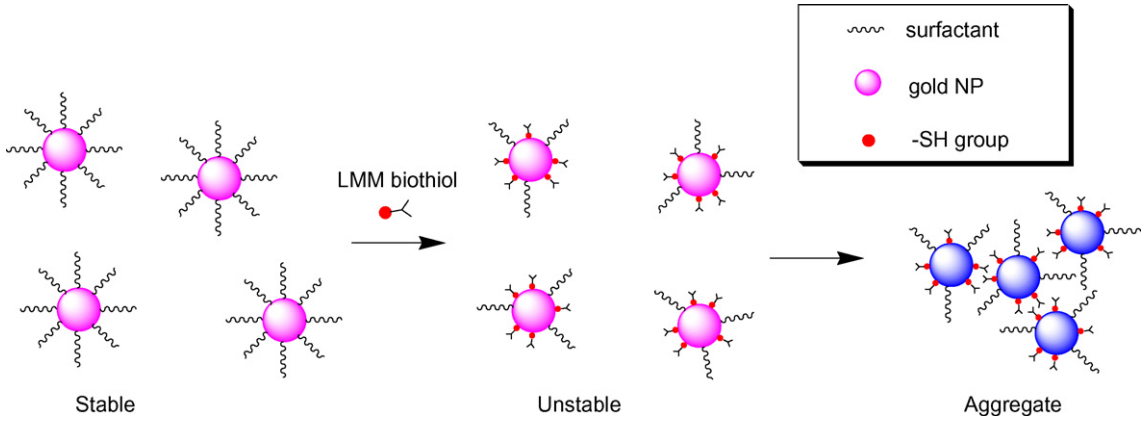

Fig. 8. LMM biothiol-induced aggregation of surfactant-capped gold NPs.

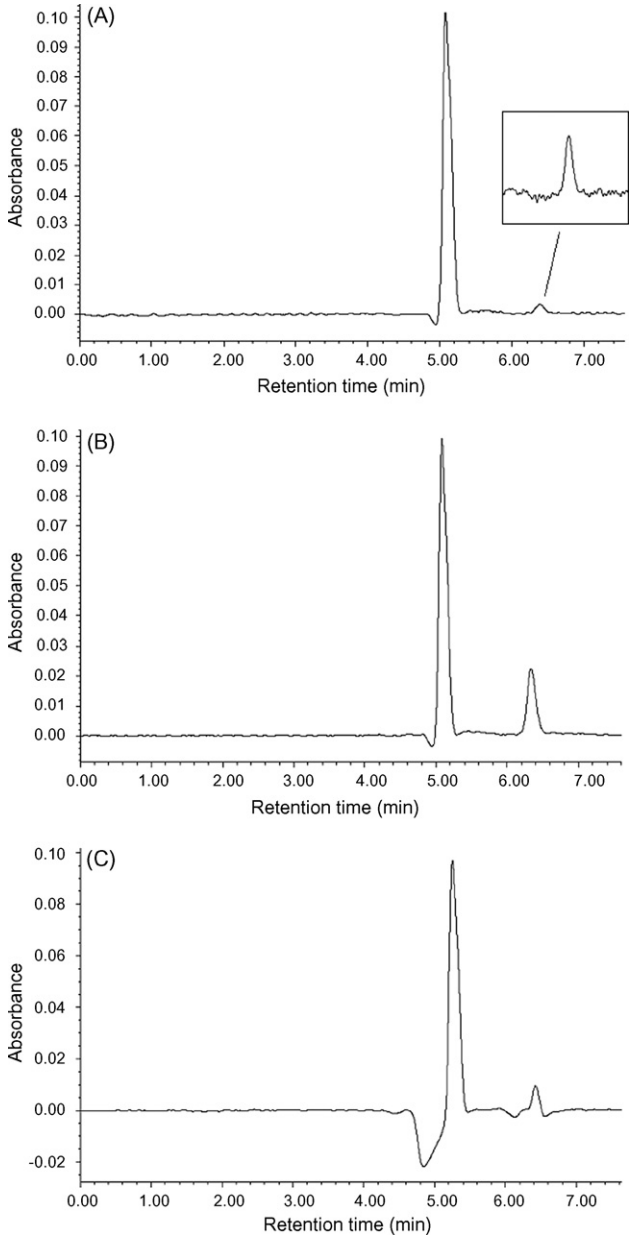

Fig. 9. (A) HPLC chromatogram of a human urine sample. The pretreated urine sample solution was diluted three times with water before injection. (B) HPLC chromatogram of a human urine sample spiked with 8.0 μM Hcy. (C) HPLC chromatogram of a commercial human plasma sample spiked with 5.0 μM Hcy. HPLC conditions: column, SunFire C18 (4.6 mm × 150 mm, 5 μm particle size, Waters); sample injection volume, 20 μL; mobile phase, 0.05% TFA, 0.5 mL/min; postcolumn reagent, colloidal solution of FSN-capped gold NPs (4.9 nM containing 100 mM phosphate, pH 6), 0.5 mL/min; postcolumn reaction temperature, 70 °C; detection wavelength, 680 nm. Reprinted with permission from Ref. [13]. Copyright 2007 American Chemical Society.

experiments, 10 mM methyl viologen in 0.25 M carbonate buffer (pH 9.5) or 12.5 μM fluorone black in 50/50 (v/v) mixture of MeOH and aqueous carbonate buffer (0.25 M, pH 9.5) were used as the postcolumn reagents, and reactor temperature was maintained at 80 °C. The wavelength of the UV–vis detector is 610 nm or 505 nm for the detection using methyl viologen or fluorone black, respectively. Highly selective responses towards Cys and Hcy have been observed, although the sensitivity is relatively low (with the detection limit of around several hundred pmol). Further optimization of the sensing systems is required.

2.7. Analyte-induced aggregation of gold nanoparticles

Over the past decade, the studies of gold NPs as novel reagents in bioanalysis have drawn great interest [49–53]. Gold NPs possess strongly distance-dependent optical properties and large surface areas. The extinction coefficients of the colloid solutions of gold NPs could be ~3 orders of magnitude larger than those of organic dyes. The colloids are generally stabilized by NP surface charges. The interactions of gold NPs with some compounds may destabilize the colloids and lead to aggregation of the NPs. The analyte-induced aggregation shifts the surface plasmon resonance (SPR) absorption peak of gold NPs towards longer wavelength. The change of the optical property can serve as a colorimetric measurement of the analyte. For example, the stable gold NPs with an average diameter of 12 nm exhibit a strong SPR absorption peak at ~523 nm, and the height of the peak decreases as the NPs become unstable and aggregate; simultaneously, another SPR absorption wave at a longer wavelength (>600 nm) begins to appear and its height increases along with the NP aggregation process. Therefore, by measuring the changes of absorbance at 523 nm or at the longer wavelength, the analyte is quantified. On the basis of this mechanism, colorimetric sensing of ssDNA, proteins, and metal ions has been achieved [54–58]. It has also been discovered that amino acids possessing additional (besides the α-amine) functional groups such as amine, imidazole, thioether, or thiol can be attached on gold NPs through N–Au or S–Au bonds, and the NP-bound amino acid molecules may serve as cross-linkers, inducing the aggregation of the gold NPs [59–64]. Under certain conditions, selective responses of gold NPs toward the LMM biothiols have been observed [65,66]. These findings indicate that gold NPs can be used as novel thiol-sensing reagents. However, for the specific determination of the LMM biothiols in complex biological matrixes, separation techniques are required prior to the sensing step. It was found in the above studies that the response of gold NPs towards the small thiols generally takes several to tens of minutes. Therefore, in order to be conjugated with HPLC as useful postcolumn detection systems, the reactions between gold NPs and the LMM biothiols must be facilitated.

In our recent study [12], gold NPs of ~12 nm in diameter were stabilized with Brij® 35, a type of nonionic surfactant. We found that the Brij® 35-capped gold NPs were stable in aqueous solutions over a wide pH range (from 1 to 13) and in the presence of salt (up to 200 mM NaCl). Upon the addition of the LMM biothiols, rapid aggregation of the gold NPs was induced, leading to the appearance of a new SPR absorption peak at ~680 nm. The height of the new absorption peak is proportional to the concentration of the LMM biothiols. The solution color change could be significantly accelerated by higher solution ionic strength and elevated temperature. Under optimal conditions, the reaction could be completed within one minute. Therefore, the gold NPs fulfill the requirements for an ideal postcolumn system. Fig. 7 shows a typical chromatogram of a mixture of the LMM biothiols (6 μM each). The five sharp peaks corresponds to Cys (~4.6 min), Cys-Gly (~6.4 min), Hcy (~6.9 min), γ-Glu-Cys (~7.6 min), and GSH (~9.2 min), respectively. The colorimetric response is highly selective towards the LMM biothiols. The interactions between the NP system and other biomolecules of much higher concentrations (50 μM each), including glucose, ascorbate, urate, and standard amino acids other than Cys, have also been examined, and only a chromatographic peak corresponding to methionine was observed with a retention time of ~11.1 min.

The reaction process may comprise an initial attachment of the LMM biothiol species on the NPs and a following aggregation of the NPs (see Fig. 8). The displacement of the adsorbed surfactant molecules by the LMM biothiols destabilizes the colloidal solution, and the aggregation is driven by the London–van der Waals attraction force. The capping of gold NPs with surfactant species is crucial for the rapid and selective responses of the colloids towards the

LMM biothiols. First, Brij® 35 molecules physically adsorbed on the NPs are readily displaced by the LMM biothiols, and the analyte-induced aggregation of the NPs can be remarkably accelerated by the salt. The gold NPs capped with surfactants exhibit good stability under this solution condition, making the rapid response possible. Secondly, the surfactant capping layers greatly suppress the interactions between the NPs and non-thiol molecules, leading to selective responses towards the thiol-containing molecules.

It has also been found that the kinetics of the biothiol-induced aggregation is sensitive to gold NP size [67]. Much slower response kinetics has been observed when the NPs with a larger average diameter (e.g., ~40 nm) were employed. Therefore, these larger NPs are not suitable for the postcolumn sensing reaction.

Highly selective responses of gold NPs towards Cys and Hcy have been achieved by changing the capping ligand with a type of commercially available nonionic fluorosurfactant, i.e., Zonyl® FSN [13]. Probably because of the steric effect, the FSN-capped gold NPs do not respond rapidly to other sulfur-containing molecules, such as Cys-Gly and GSH. Therefore, the colloid can be used as a specific postcolumn reagent for HPLC assay of Cys and Hcy. Fig. 9 shows a chromatogram obtained in the analysis of human urine and plasma samples. The background noises are relatively low, and the interferences from other species are greatly diminished, demonstrating the high selectivity of the method.

3. Conclusions

The determination of the LMM biothiols by using HPLC in conjunction with postcolumn derivatization has been studied intensively over past several decades. Traditional derivatization reactions generally produce spectroscopic measurable compounds by labeling the biothiols with molecular chromophores or fluorophors. Typical examples are OPA labeling, ninhydrin labeling via thiazolidine reaction, and maleimide labeling. These derivatization reactions can be used in either precolumn or postcolumn mode. Some other reagents, such as DTNB, generate free chromophores upon reactions with the LMM biothiols via sulfhydryl/disulfide exchange mechanism. They are used specifically for postcolumn detection of thiol-containing molecules. The development of new molecular probes for the LMM biothiols has been the interest of a number of studies in the literature [10 and refs therein]. By using new reaction systems, the improvement of detection sensitivity and selectivity as well as reagent stability is expected.

Recently, intensive studies on NPs revealed that gold NPs could serve as new sensing reagents for the LMM biothiols. The aggregation of gold NPs induced by the LMM biothiols can be accelerated in the presence of high concentration of salt and at high temperature. The rapid reaction kinetics allows the application of the NPs in HPLC postcolumn detection. Interestingly, the selectivity of the analyte-induced aggregation of gold NPs can be tuned by the capping ligands. Further optimization of the reaction conditions to attain higher sensitivity is in progress. Other NPs, such as fluorescent semiconductor quantum dots, may also respond to thiol-containing compounds. Their potential applications as postcolumn reagents are subject to evaluation.

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