Detection of aminothiols through surface-assisted laser desorption/ionization mass spectrometry using mixed gold nanoparticles

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We have employed mixtures of two differently sized (average diameters: 3.5 and 14 nm) gold nanoparticles (Au NPs) as selective probes and matrices for the determination of aminothiols using surface-assisted laser desorption/ionization mass spectrometry (SALDI-MS). When using 38 and 150 pM solutions of the 3.5- and 14-nm Au NPs, respectively, as the probe and matrix, SALDI-MS provided limits of detection (signal-to-noise ratio = 3) of 2, 20, and 44 nM for 1.0 mL solutions of glutathione (GSH), cysteine (Cys), and homocysteine, respectively. The signal intensities of these analytes varied by less than 20% for SALDI-MS analyses recorded over 50 sample spots; in contrast, they varied by as much as 60% when using a conventional matrix (2,5-dihydroxybenzoic acid). We validated the practicality of this approach – with its advantages of sensitivity, reproducibility, rapidity, and simplicity – through the analysis of GSH in MCF-7 cell lysates and Cys in plasma.

Increases in the concentrations of the thiol compounds glutathione (GSH), cysteine (Cys), and homocysteine (Hcys) are correlated with several diseases, including cardiovascular, atherosclerotic vascular, and chronic kidney disease.1 Because these aminothiols possess anti-oxidative and antioxidative properties, they perform several vital functions in cells.2 For example, the reduced form of GSH – the most abundant intracellular low-molecular-weight thiol in cells – plays an essential role in protecting cells from toxic species.3 Cys and Hcys can promote atherogenesis through their effects on endothelial function, vascular smooth muscle cell activation, and hemostatic activation.4

Because of their important biological roles, many separation techniques, including gas chromatography, high-performance liquid chromatography, and capillary electrophoresis, have been developed for the determinations of thiol compounds in biological samples.5–11 Mass spectrometry (MS) and laser-induced fluorescence (LIF), which are highly sensitive towards these analytes, are often employed in conjunction with the separation systems. Prior to detection, the complicated biological samples are subjected to sample pretreatment and separation to minimize matrix interference. These processes may, however, require long analytical times and/or derivatization. In addition, separation systems used in conjunction with MS detection are expensive.

Nanoparticles (NPs) have recently been employed in place of traditional organic matrices in MS-based determinations of analytes of interest – so-called surface-assisted laser desorption/ionization mass spectrometry (SALDI-MS). NPs that have been used in SALDI-MS include gold NPs (Au NPs),12–14 magnetic NPs,15,16 quantum dots,17 silica NPs,18 silicon nanopowder,19 silver NPs,20 and titanium dioxide NPs.21 Similar to the role of organic matrices, the NPs absorb energy from the laser irradiation and transfer it efficiently to induce the desorption and ionization of the analytes. Moreover, the NPs can act as selective probes when they are bioconjugated with recognition molecules. For example, Nile Red adsorbed 14-nm Au NPs are both selective probes and the matrix for the SALDI-MS determination of aminothiols.12

The desorption/ionization efficiency of analytes in SALDI-MS is dependent on the size of the NPs.12,14,22 For example, the signal intensities of a peptide relative to those for [Auₙ]⁺ ions (n = 3 and 5) were 1.1 and 0.7 when using 2- and 10-nm-diameter Au NPs, respectively; i.e., the smaller NPs provided better SALDI-MS sensitivity. The size of the NPs affects not only the optical properties (absorption undergoes red shifts upon increasing size), but also the surface energy. In addition, the concentration of NPs is an important factor: to achieve high sensitivity, each NP should interact with a large number of analyte molecules.23

In a previous study, we found that 14-nm-diameter Au NPs provided higher sensitivity for analytes than did 32- and 56-nm Au NPs.12 Therefore, in this study, we tested the effect of the average size of the Au NPs on the SALDI-MS determinations of GSH, Cys, and Hcys. We investigated several factors – the molar ratio of the differently sized Au NPs, the buffer concentration, the sample pH, and the salt concentration – on the sensitivity of SALDI-MS.24,25

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**EXPERIMENTAL**

**Chemicals**
Sodium tetrachloroaurate(III) dihydrate and GSH were obtained from Sigma (St. Louis, MO, USA). Citric acid, Cys, HCys, trisodium citrate, and sodium borohydride were purchased from Aldrich (Milwaukee, WI, USA). Acetonitrile (ACN) and ammonium hydroxide were obtained from Acros (Geel, Belgium). Citrate solutions were prepared using either trisodium citrate or citric acid. The values of pH of the 0.5 mM citrate solutions, which were prepared from mixtures of trisodium citrate solutions (100 mM) and 2.0 M HCl, ranged from 3.0 to 6.0. Citric acid (100 mM) and NH₄OH (25–30%) were used to prepare ammonium citrate solutions (0.5 mM; pH 3.0–8.0).

**Preparation of Au NPs**

**3.5-nm-Diameter Au NPs**
Ice-cold, freshly prepared NaBH₄ (0.1 M) solution (0.6 mL) was added over 3 min to a stirred aqueous solution (20 mL) of NaAuCl₄ (0.25 mM) and trisodium citrate (0.25 mM) in a sample vial. The solution turned pink immediately, indicating particle formation. The concentration of the 3.5-nm Au NPs in this solution was 76 nM.

**14-nm-Diameter Au NPs**
An aqueous solution (50 mL) of trisodium citrate (4 mM) was brought to a vigorous boil with stirring in a round-bottomed flask fitted with a reflux condenser. Then 0.1 M NaAuCl₄ (0.5 mM) was rapidly added and the solution was boiled for another 3 min, during which time its color changed from pale yellow to purple and to wine-red. The concentration of the 14-nm Au NPs in this solution was 15 nM.

**Characterization of Au NPs**
A double-beam UV-Vis spectrophotometer (Cintra 10e; GBC, Victoria, Australia) was used to measure the absorbance of the Au NPs in citrate solutions. For the 3.5- and 14-nm Au NPs, the maximum wavelengths of the surface plasmon resonances were 508 and 518 nm, respectively (data not shown); these values are consistent with the expected sizes of the Au NPs. The sizes and their distributions (±0.6 nm and ±2.1 nm for the 3.5- and 14-nm Au NPs, respectively) were further confirmed through transmission electron microscopy (TEM) using a H7100 microscope (Hitachi, Tokyo, Japan) operated at 75 kV.

**Preparation of samples**
The model aminothiols (concentrations ranging from 10 nM to 0.5 μM) were added separately to solutions of the two differently sized Au NPs of various concentrations and then equilibrated for 30 min. After the solutions (1.0 mL) had been centrifuged at 25000 rpm for 20 min, the supernatants (980 μL) were removed and the pellets resuspended in 0.5 mM ammonium citrate (pH 4.0, 20 μL). Aliquots (1.0 μL) of the resuspended Au NP/thiol mixtures were pipetted into a stainless-steel 96-well matrix-assisted laser desorption/ionization (MALDI) target (Bruker Daltonics, Bremen, Germany) and dried in air at room temperature prior to SALDI-MS measurements.

**Preparation of thiol extracts from lysed MCF-7 cells**
The parental MCF-7 human mammary adenocarcinoma cell line was obtained from the American Type Culture Collection (Rockville, Maryland, USA). After the cells had grown to 90% confluence in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal calf serum at 37°C, they were harvested through trypsinization. A suspension of ca. 10⁶ cells (1.0 mL) was subjected to three cycles of centrifugation (400 g, 10 min) and washing with phosphate-buffered saline (18.6 mM phosphate, 4.2 mM KCl, and 154.0 mM NaCl) and then they were resuspended in deionized water (1 mL). The cells were then ruptured through sonication for 20 min at 0°C. To remove the homogenate of the cell debris, the lysate was centrifuged at 18000 rpm for 20 min at 4°C. The supernatant was collected and deproteinized through an Amicon YM-10 membrane (Millipore Corp., Bedford, MA, USA). The two solutions of differently sized Au NPs (final concentrations: 38 and 150 pM for the 3.5- and 14-nm Au NPs, respectively) were separately added to the deproteinized MCF-7 lysates and then the mixtures were incubated at room temperature for 1 h. The Au NP/thiol mixtures were subjected to centrifugation at 25000 rpm for 20 min and then the pellets were redispersed in 0.5 mM ammonium citrate (pH 4.0, 20 μL) prior to SALDI-MS measurement.

**Preparation of thiol extracts from plasma samples**
Whole blood samples from an apparently healthy male adult were collected into K₂ EDTA Vacutainer blood collection tubes (Becton Dickinson, Franklin Lakes, NJ, USA). To obtain plasma samples, the collected whole blood samples were immediately centrifuged at 3000 rpm for 10 min at room temperature. The plasma samples were stored at −20°C. The Au NP/thiol mixtures of the plasma samples were obtained by following the same procedure as that described above for the cell lysates.

**MALDI-TOF and SALDI-TOF MS measurements**
Mass experiments were performed in the positive ion mode using a Microflex MALDI-TOF mass spectrometer (Bruker Daltonics). The samples were irradiated using a 337-nm-wavelength nitrogen laser at 10 Hz. Ions produced by laser desorption were stabilized energetically during a delayed extraction period of 200 ns and then accelerated through the TOF instrument operated in the reflection mode prior to entering the mass analyzer. The available accelerating voltages ranged from +20 to −20 kV. To obtain good resolution and signal-to-noise (S/N) ratios, the laser fluence was adjusted to slightly higher than the threshold and each mass spectrum was generated by averaging 300 laser pulses.
RESULTS AND DISCUSSION

Au NPs as assisting matrices for SALDI-MS analysis

In a previous study, we found (i) that a value of pH of 4.0 was optimal for the determination of aminothiols through SALDI-MS when using 14-nm Au NPs, (ii) that 14-nm Au NPs provided better sensitivity for GSH through SALDI-MS than did 32- or 56-nm Au NPs, and (iii) that when using 14-nm Au NPs, the SALDI-MS approaches performed without and with conducting concentration separately provide limits of detection (LODs; S/N ratio = 3) of 1.0 μM and 25 nM for GSH, respectively. In this study, we tested the behavior of smaller sizes of Au NPs in an attempt to improve the sensitivity of SALDI-MS toward aminothiols, based on the fact that the abundance of analyte ion signals and the abundances of higher-order Au clusters increases upon decreasing and increasing the size of Au NPs, respectively.

Although analyte-induced aggregation of 3.5-nm Au NPs, it is very difficult to obtain the aggregates efficiently after centrifugation. In addition, the aggregates tend to adsorb on the wall of the vial, leading to poor quantitative results. To solve this problem, we employed mixtures of the 3.5- and 14-nm Au NPs, where the larger NPs selectively capture the aminothiols and the smaller ones to improve the SALDI-MS sensitivity. The mixtures contained variable concentrations of the 3.5-nm Au NPs (0–152 pM) and a constant concentration (150 pM) of the 14-nm Au NPs. In the presence of aminothiols, aggregation of the small and large Au NPs occurred, as evidenced by the absence of an absorbance at a wavelength of 508 nm in the supernatant. In addition, the TEM image shown in Supplementary Fig. S1 (see Supporting Information) displays the existence of the 3.5- and 14-nm Au NPs in the mixtures. Based on the TEM image, we did believe the existence of cross-aggregation of the two differently sized Au NPs. Because the laser beam has an irradiation area about 50 μm, it radiated the aggregates of the different sizes of Au NPs with the analytes. Figure 1(a) reveals that the signal for [GSH + Na]+ (m/z 330.07) was absent in the spectrum of GSH (50 nM) when using only the 14-nm Au NPs. Upon increasing the concentration of the 3.5-nm Au NPs up to 76 pM, the signal for GSH increased accordingly. For example, when the concentration of 3.5-nm Au NPs increased from 0 to 76 pM, the signal for GSH increased ca. 6.5-fold (Fig. 1(b)), mainly as a result of increased ionization efficiency. The peaks at m/z 393.93, 409.96, 437.99, 452.00, 590.90, 618.93, and 646.96 are assigned as the signals of the [Au2]+, [Au2+ + CH4]+, [Au2+ + C2H6]+, [Au2+ + C3H8]+, [Au3]+, [Au3+ + C2H4]+, and [Au3+ + C4H8]+ ions.

Figure 1. Mass spectra of GSH (50 nM) obtained using a matrix of 14-nm Au NPs (150 pM) in (a) the absence and (b) the presence of 3.5-nm Au NPs (76 pM). The two Au NPs were added to GSH solutions prepared in 0.5 mM ammonium citrate (pH 4.0). The peak at m/z 330.07 is assigned to the [GSH + Na]+ ion. Inset: mass spectrum showing the m/z value up to 1100 Da. The peaks at m/z 393.93, 409.96, 437.99, 452.00, 590.90, 618.93, and 646.96 are assigned as the signals of the [Au2]+, [Au2+ + CH4]+, [Au2+ + C2H6]+, [Au2+ + C3H8]+, [Au3]+, [Au3+ + C2H4]+, and [Au3+ + C4H8]+ ions.
assigned as the signal of the $[\text{Au}_2]^{3+}$, $[\text{Au}_2^{\,+} + \text{CH}_4]^{4+}$, $[\text{Au}_2^{\,+} + \text{C}_3\text{H}_8]^{5+}$, $[\text{Au}_3^{\,+}]^{6+}$, $[\text{Au}_3^{\,+} + \text{C}_3\text{H}_8]^{7+}$, and $[\text{Au}_3^{\,+} + \text{C}_4\text{H}_8]^{8+}$ ions. Further increases in the concentration of the 3.5-nm Au NPs caused the signal to decrease, mainly because the number of adsorbed molecules on each NP decreased; in addition, background signals arising from $[\text{Au}_n]^{3+}$ ions became problematic.

Next, we measured the GSH signal by SALDI-MS using solutions containing various concentrations of the 14-nm Au NPs (75–300 pM) and the 3.5-nm Au NPs (0–76 pM). Figure 2 reveals that the intensity of the $[\text{GSH}^{\,+} + \text{Na}]^{\,+}$ signal was highly dependent on the contents of the two differently sized Au NPs. The $[\text{GSH}^{\,+} + \text{Na}]^{\,+}/[\text{Au}^{\,+}]$ signal ratios were low when the concentration of the 14-nm Au NPs was high (300 pM), mainly because of the high background noise and the low number of GSH molecules per NP. The solutions containing the 3.5- and 14-nm Au NPs at concentrations of 76 and 75 pM and of 38 and 150 pM provided comparable sensitivities.

To further improve the sensitivity, we concentrated GSH (10 nM) in a 1.0-mL solution by taking advantage of strong Au–S bonding. Supplementary Fig. S2 (see Supporting Information) reveals that the signal of the $[\text{GSH}^{\,+} + \text{Na}]^{\,+}$ ion was detectable after concentrating the sample, but it was not detectable from a solution containing 10 nM GSH that had not been concentrated. Based on the MS spectrum, we estimated the LOD (S/N ratio ~ 3) of this approach for GSH to be 2 nM. From a comparison of the signals for the $[\text{GSH}^{\,+} + \text{Na}]^{\,+}$ ions in Fig. 1(b) and Supplementary Fig. S2, we estimated that the concentration factor of this approach was 50. In contrast, when we used a 150 pM solution of the 14-nm Au NPs to capture GSH (10 nM) from a 1.0 mL solution and then conducted SALDI-MS, we did not detect any signals for GSH.

Effects of pH and salt
The pH of the sample solution is an important factor affecting the dissociation of GSH (pI = 2.86), which in turn affects the interaction between GSH and Au NPs. Because the pH also affects the stability of Au NPs to different extents depending on their sizes, we investigated the effect of the pH, even through in our previous study we had already demonstrated that pH 4.0 was optimal for the 14-nm Au NPs. Supplementary Fig. S3 (see Supporting Information) presents the intensities of the $[\text{GSH}^{\,+} + \text{Na}]^{\,+}$ signals obtained from trisodium citrate buffer solutions of various values of pH (3.0–8.0); the maximum signal appears at pH 4.0. The interaction between GSH and the Au NPs is weaker at pH 4.0 than at higher values of pH, mainly because of weaker Au–S bonding. As a result, GSH readily undergoes desorption and ionization processes at pH 4.0. In addition, the ionization of GSH occurs to a greater extent at pH 4.0 because of its lower negative charge density. Values of pH of less than pH 4.0 decreased the stability of the Au NPs, leading to poor sensitivity and irreproducibility.

Figure 2. Intensity ratios of the $[\text{GSH}^{\,+} + \text{Na}]^{\,+}$ and $[\text{Au}^{\,+}]$ ions plotted against the concentration of 14-nm Au NPs (75–300 pM) and 3.5-nm Au NPs (0–76 pM) used as probes to trap GSH (10 nM) in the concentration step. Other conditions were the same as those described in Fig. 1.

Figure 3. Calibration curves of the concentrations of three representative aminothiols determined through SALDI-MS analyses. The intensities of the (a) $[\text{GSH}^{\,+} + \text{Na}]^{\,+}$, (b) $[\text{Cys}^{\,+} + \text{H}]^{\,+}$, and (c) $[\text{Hcys}^{\,+} + \text{H}]^{\,+}$ ion intensities are plotted against the aminothiol concentrations. The concentrations of the 3.5- and 14-nm Au NPs in the sample solution (1.0 mL) were 38 and 150 pM, respectively. A sample of 1.0 μL of the concentrated GSH/Au NP mixture was used for SALDI-MS measurement. Other conditions were the same as those described in Fig. 1.
Salt also affects the stability of Au NPs to various extents depending on their sizes. Indeed, salt suppression of analyte signals in SALDI-MS has been suggested. In addition, formation of salt crystals around the nanoparticles/analytes mixtures makes it difficult for desorption/ionization of the analytes. To minimize the impact of salt on the signal for GSH, we used a citrate/NH$_4^+$ buffer (pH 4.0), rather than one prepared from sodium citrate, to prepare the GSH solution. Supplementary Fig. S4(a) (see Supporting Information) reveals the presence of five major signals in the resulting spectrum, at $m/z$ 330.07, 346.03, 352.05, 368.02, and 374.04, corresponding to the [GSH + Na]$^+$, [GSH + K]$^+$, [GSH – H + Na + K]$^+$, and [GSH – 2H + 3Na]$^+$ adduct ions, respectively. Potassium and sodium ions might originate from the deionized water, impurities in the chemicals used in this experiment, and contaminants in the MALDI-MS system. Notably, the peak height for the [GSH + Na]$^+$ ion is greater (by ca. 1.8-fold) than that obtained in the spectrum recorded from the solution of GSH in the trisodium citrate buffer (Supplementary Fig. S4(b), see Supporting Information).

Quantitative analyses of three model thiols

Figure 3 reveals that the peak heights of the analyte signals were linear with respect to the concentrations of the three model aminothiols. The correlation coefficients ($R^2$) for the determinations of GSH, Cys, and HCys were 0.998, 0.996, and 0.997, respectively, over the concentration ranges 10–500, 200–800, and 200–800 nM, respectively; their LODs (S/N ratio = 3) were 2, 20, and 44 nM, respectively. To the best of our knowledge, this LOD for GSH is the lowest that has ever been reported when using either SALDI-MS or MALDI-MS approaches. Relative to our previously reported strategy, this present approach provides a sensitivity improvement of 10-fold for the analysis of GSH. Because of the poorer ionization efficiencies of Cys and HCys, relative to that of GSH, and greater noise in the low-mass region ($m/z < 200$), their LODs were higher than that of GSH. The intensities of the signals of these analytes varied by less than 20% for the analyses of more than 50 sample spots; in comparison, the variation reached as high as 60% when using a conventional matrix (DHB).

Determination of aminothiols in lysed MCF-7 cells and plasma

To minimize protein interference, we subjected the cell lysates to deproteinization via filtration through cutoff membranes. Figure 4(a) reveals the presence of a peak at $m/z$ 330.07 for the [GSH + Na]$^+$ ion and the peak at $m/z$ 323.00 is from [2Citric acid – CH$_2$O$_3$ – H]$^+$. By using a standard addition method (plotting the peak height at $m/z$ 330.07 against the spiked GSH concentration), we estimated that the concentration of GSH in the MCF-7 cells was 24.3 ± 2.9 fmol per cell ($n = 3$). This value agrees with the normal GSH levels reported previously (23.2–34.4 fmol per cell). When we used DHB as the MALDI matrix for the same cell samples, we did not detect any signals for GSH (Fig. 4(b)).

We further validated the practicality of this SALDI-MS approach through the determination of free Cys in plasma. Figure 5(a) presents the mass spectrum of Cys in the deproteinized plasma sample after treating with 80% ACN. We employed the peak at $m/z$ 144.18 for the quantitative determination of the [Cys + Na]$^+$ ion, obtaining a value of 10.56 ± 0.86 µM ($n = 3$) that is consistent with normal levels (9.5–11.5 µM). Again, the use of DHB as the matrix did not allow us to identify the presence of Cys (Fig. 5(b)).
CONCLUSIONS

Mixtures of 3.5- and 14-nm Au NPs allow the SALDI-MS determination of the three aminothiols GSH, Cys, and Hcys. The larger Au NPs allow ready centrifugation; the smaller Au NPs provide greater desorption/ionization efficiency. This approach provides an LOD of 2 nM for GSH. This SALDI-MS method provides high sensitivity, simplicity, and selectivity for the determinations of aminothiols. The idea of using mixed Au NPs for the analyses of biomolecules may be applicable to other combinations of nanomaterials, e.g., Au and Ag NPs, Fe3O4 and Au NPs, or Au NPs and quantum dots – that display unique optical properties and affinities toward various analytes.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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REFERENCES