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# Gold Nanoparticles as Selective and Concentrating Probes for Samples in MALDI MS Analysis

Chien-Hsun Teng, Kun-Chan Ho, Ya-Shiuan Lin, and Yu-Chie Chen*

*Department of Applied Chemistry, National Chiao Tung University, Hsinchu 300, Taiwan*

**MALDI mass spectrometry is used widely in various fields because it has the characteristics of speed, ease of use, high sensitivity, and wide detectable mass range, but suppression effects between analyte molecules and interference from the sample matrix frequently arise during MALDI analysis. The suppression effects can be avoided if target species are isolated from complicated matrix solutions in advance. Herein, we proposed a novel method for achieving such a goal. We describe a strategy that uses gold nanoparticles to capture charged species from a sample solution. Generally, ionic agents, such as anionic or cationic stabilizers, encapsulate gold nanoparticles to prevent their aggregation in solution. These charged stabilizers at the surface of the gold particles are capable of attracting oppositely charged species from a sample solution through electrostatic interactions. We have employed this concept to develop nanoparticle-based probes that selectively trap and concentrate target species in sample solutions. Additionally, to readily isolate them from solution after attracting their target species, we used gold nanoparticles that are adhered to the surface of magnetic particles through S−Au bonding. A magnet can then be employed to isolate the Au@magnetic particles from the solution. The species trapped by the isolated particles were then characterized by MALDI MS after a simple washing. We demonstrate that Au@magnetic particles having negatively charged surfaces are suitable probes for selectively trapping positively charged proteins from aqueous solutions. In addition, we have employed Au@magnetic particle-based probes successfully to concentrate low amounts of peptide residues from the tryptic digest products of cytochrome *c* ($10^{-7}$ M).**

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry has become a very powerful tool for biochemical analysis[1,2] in the years since its initial development.[3] Mixtures can be analyzed simultaneously using the MALDI MS technique without the requirement of any tedious pretreatment. Suppression effects between analyte molecules and matrix interference of samples may arise, however, and limit the power of this analytical tool. Several clever strategies have been proposed to improve the results of analysis.[4−18] On-probe cleanup methods have been used frequently to remove unwanted salts or surfactants during MALDI MS analyses. Xu et al. provided a detailed review of the development of nonspecific, on-probe cleanup methods for MALDI MS samples.[16] Hydrophobic polymers such as polyethylene (PE),[17] polypropylene,[14] polyurethane,[10] paraffin,[9] and Teflon[18] have been used for effectively desalting sample substrates. For example, Blackledge and Alexander employed a PE-modified MALDI probe as a sample support and obtained a mass spectrum of bovine serum albumin, from a sample that contained 0.73% SDS, after vortexing the sample-coated probe in 50% aqueous methanol for 30 s.[17] Most of the studies to date have focused on methods for on-probe treatment.[5−18] An alternative method that avoids suppression effects in MALDI MS analysis is to isolate trace amounts of target species from complicated samples prior to their analysis.

Gold nanoparticles are used widely in various fields because of their extremely small size, visible colors, and ease of chemical modification. The Mirkin group, which has pioneered the use of inorganic nanoparticles in biochemical analysis, has reported numerous studies in which gold nanoparticles have been employed to develop methods for DNA detection and analysis.[19] The most common method for preparing gold nanoparticles is the use of trisodium citrate as a reducing agent for tetrachloroaurate;[20] the sizes of the resulting particles can be controlled by carefully adjusting the stoichiometric ratio of the reagents. It is believed

* To whom correspondence should be addressed. E-mail: yuchie@mail.nctu.edu.tw. Phone: 886-3-5131527. Fax: 886-3-5744689.

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that each particle has an $Au^0$ core and an $Au^I$ shell as a result of incomplete reduction at the nanoparticles' surfaces.[20] Citrate and chloride ions coordinate to the $Au^I$ shell, and thus, each gold nanoparticle has overall negative charge. Nanoparticles are capable of binding to oppositely charged species in aqueous solutions through electrostatic interactions based on the ionic characteristics of their surfaces.[21]

Amphiprotic species, such as peptides and proteins, have unique isoelectric points (p*I*). When the pH of a protein sample solution is below the value of the p*I* of the protein, the protein molecules have a net positive charge. Negatively charged gold nanoparticles tend to attract positively charged protein molecules to their surfaces through electrostatic interactions. On the other hand, if the pH of a protein solution is above the value of the p*I* of this protein, the protein molecules are negatively charged and repel any negatively encapsulated gold nanoparticles. On the basis of this characteristic, we have employed gold nanoparticles to bind specific proteins selectively by adjusting the pH of their sample solutions.

To speed up the separation of gold nanoparticle-conjugated proteins from a sample solution, the gold nanoparticles were bound to the surface of magnetic particles. Magnetic particles have been used widely for the isolation of target species from aqueous solutions.[22,23] Thus, once the Au@magnetic particles have adsorbed target species onto their surfaces, a magnet can be used to isolate the Au@magnetic particles quickly from the sample solutions. We have used proteins, peptides, cationic surfactants, and tryptic digest products as samples to examine the capacity of gold nanoparticles to act as the selective probes for charged species.

## EXPERIMENTAL

**Reagents.** Iron(III) chloride hexahydrate, hexadecyltrimethylammonium bromide, and trisodium citrate were obtained from Riedel-de Haën (Seelze, Germany), while iron(II) chloride tetrahydrate was purchased from Aldrich. Ammonium hydroxide was purchased from J. T. Baker. Methanol was obtained from Tedia. Tetraethoxysilane, melittin, tetradecyltrimethylammonium bromide, dodecyltrimethylammonium bromide, and decyltrimethylammonium bromide were obtained from Fluka (Buchs, Switzerland). Hydrochloric acid, trifluoroacetic acid (TFA), and acetonitrile were purchased from Merck (Darmstadt, Germany). Sodium phosphate dibasic heptahydrate, cytochrome *c*, trypsin, and myoglobin were obtained from Sigma (St. Louis, MO), while sodium phosphate was purchased from Mallinckrodt. 3-(Mercaptopropyl)trimethoxysilane (MPTMS) was obtained from Acros, and tetrachloroaurate was obtained from Showa.

**Preparation of Magnetic and Gold Nanoparticles.** Magnetic $Fe_3O_4$ particles were prepared by mixing $FeCl_3 \cdot 6H_2O$ (5.4 g), $FeCl_2$ (1.98 g), and deionized water (20 mL) at room temperature. The mixture was placed in a water bath heated at 80 °C, and then $NH_4OH$ solution (8 M, 1.5 mL) was added into this mixture. The mixture was then stirred continually for 30 min in the water bath at 80 °C. When the reaction was complete, a magnet was used to isolate the magnetic particles from the solution. The isolated magnetic particles were washed with methanol to remove any unreacted impurities. The magnetic particles were stored in a methanol solution prior to use.

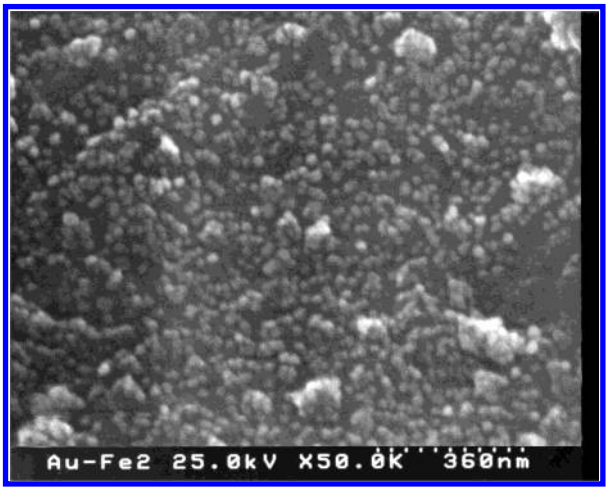


Figure 1. SEM image of the surface of an Au@magnetic particle. The gold nanoparticles cover the whole surface of the magnetic particles and retain their original sizes (~16 nm).

Gold nanoparticles were prepared using the Frens method.[18] An aqueous tetrachloroaurate solution (0.1 mg/mL, 50 mL) was heated to boiling, and then trisodium citrate (1%, 1 mL) was added to the solution while stirring. The color change of the solution from blue to brilliant red indicated the formation of monodisperse spherical particles (~16 nm).[20]

**Preparation of Au@Magnetic Particles.** We employed sol−gel techniques to covalently adhere gold nanoparticles to the surface of magnetic particles. Tetraethoxysilane (TEOS, 4.5 mL) was hydrolyzed by reacting it with HCl (0.01 N, 0.4 mL) and water (1 mL) for ~3 h at room temperature (26 ± 2 °C) until a homogeneous phase was achieved. The magnetic particles were added into the hydrolyzed TEOS solution with stirring until the mixture gelled. The gelation product was ground into approximately micrometer-sized particles (30−50 $\mu$m). A portion of these particles (2 mg) was then immersed for 24 h in a MPTMS (85%)/methanol (1:4, v/v) mixture (0.5 mL). After incubation, the upper MPTMS solution was discarded by using a pipet. The modified particles were washed with methanol (0.5 mL) and water (2 × 0.5 mL) to remove unreacted impurities. The modified particles were then immersed for 24 h in the gold nanoparticle solution (0.5 mL); we expected that the gold nanoparticles would become attached to the surfaces of the magnetic particles through S−Au bonding. The Au@magnetic particles were isolated from the solution using a magnet. The Au@magnetic particles were dried in an oven at 40 °C before use. Figure 1 presents an SEM image of the surface of the magnetic particle. The size of the gold particles attached to the surface of the magnetic particles is roughly the same as the size of the original gold nanoparticles.

**Sample Preparation for Extraction Experiments.** For extraction, a portion of a sample solution (0.5 mL) was added to the solution of gold nanoparticles (0.5 mL). The mixture was placed in a refrigerator at −20 °C for 30 min to cause flocculation of the gold particles. The gold nanoparticles were readily separated from the solution after centrifugation. The flocculated mixture was mixed with MALDI matrix for MALDI analysis.

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Various amounts of Au@magnetic particles were added to a sample solution (0.1 or 0.5 mL). The mixture was gently vortexed for 1 h. A magnet was used to isolate the magnetic particles from the solution after extraction: the magnetic particles in the sample solution were attracted to the wall on one side of the sample vial by an external magnet, and then the remaining solution was easily removed by pipet. The isolated magnetic particles were washed with water (0.1 mL) or buffer solutions to remove unwanted impurities; again, a magnet was used to separate the magnetic particles from the solution. Sinapinic acid (SA, 15 mg/mL, 1 $\mu$L) or 2,5-dihydroxybenzoic acid (2,5-DHB, 15 mg/mL, 1 $\mu$L) in acetonitrile/(1% TFA in water) (2:1, v/v) was then used as the washing solution to wash the target species from the surface of the Au@magnetic particles. The Au@magnetic particles mixed with the matrix solution stood at room temperature for 20 min before the matrix solution was removed. Two-layer sample preparation was employed for MALDI MS analysis;[24,25] i.e., a thin layer of matrix was first deposited on the target and then the matrix solution (0.5 $\mu$L) washed from the surface of the Au@magnetic particles was applied on top of the thin film of matrix prior to MALDI MS analysis.

Protein and peptide solutions were prepared in buffer solutions at different values of pH. Diammonium citrate (100 mM), ammonium phosphate (100 mM), and sodium phosphate (100 mM) solutions were used as the buffers, and citric acid, ammonium hydroxide, and sodium hydroxide were used to adjust their values of pH. Cytochrome *c* ($10^{-6}$ M) tryptic digestion [40:1 (w/w)] was performed in an $NH_4HCO_3$ buffer solution for 24 h at 38 °C. Prior to extraction experiments, the digest product was diluted 10 times using a citrate buffer (pH 4).

**Trapping Capacity of Au@magnetic Particles.** The trapping capacity of Au@magnetic particles for cytochrome *c* was estimated by using UV−visible absorption spectroscopy. We generated a calibration curve of the absorption at 408 nm versus the concentrations of cytochrome *c*. The absorption capacity of cytochrome *c* ($10^{-5}$ M, 2 mL) was determined before adding the Au@magnetic particles (20 mg) for trapping experiments. After extraction (1 h), the Au@magnetic particles were isolated from the solution by employing a magnet. The absorption capacity at 408 nm of the remaining solution was then determined by absorption spectroscopy. Based on the relative absorptions, the trapping capacity of the Au@magnetic particles for cytochrome *c* was estimated to be ~54 pmol/mg.

**Instrumentation.** All the mass spectra were obtained using a Biflex III (Bruker) time-of-flight mass spectrometer. The mass spectrometer was equipped with a 337-nm nitrogen laser, a 1.25-m flight tube, and a sample target having the capacity to load 384 samples simultaneously. The accelerating voltage was set to 19 kV. SEM images were obtained using a Hitachi S-4000 (Tokyo, Japan). UV−visible absorption spectra were obtained using an Agilent 8453 UV−visible spectrometer (Agilent).

## RESULTS AND DISCUSSION

Negatively charged gold nanoparticles were employed as selective probes to trap oppositely charged proteins from aqueous solution. We sought a convenient method for isolating the nanoparticles from the solutions once the nanoparticles had trapped the target species, and so we bound gold nanoparticles covalently to the surface of a magnetic material to generate Au@magnetic particles. We employed an aqueous solution of the analytes cytochrome *c* and myoglobin to demonstrate the feasibility of using Au@magnetic particles as selective probes. From a solution at pH 6, both cytochrome *c* and myoglobin were trapped by the Au@magnetic particles; the trapping species were confirmed by MALDI MS analysis (Figure 2a). In the corresponding experiment conducted at pH 8, only cytochrome *c* is observed in the MALDI mass spectrum (Figure 2b), and signals for neither analyte are observed in the MALDI mass spectrum when the extraction is carried out at a pH 12 (Figure 2c). These results demonstrate that Au@magnetic particles are suitable probes for selectively tapping target species from aqueous solutions.

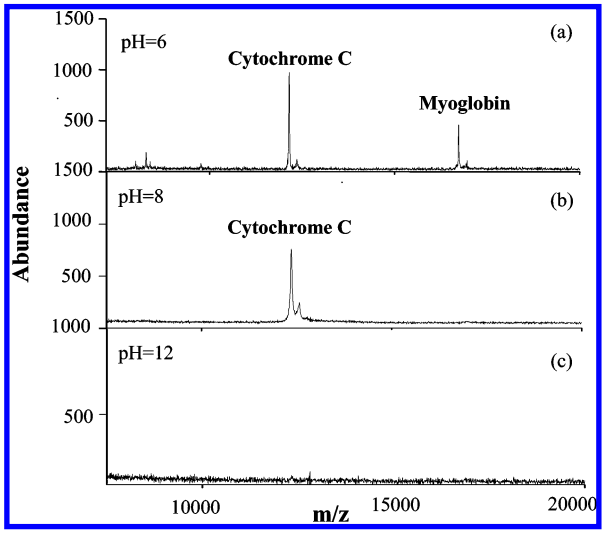


Figure 2. MALDI mass spectra obtained from a mixture (0.1 mL) of cytochrome *c* ($10^{-6}$ M) and myoglobin ($10^{-6}$ M) when using Au@magnetic particles (1 mg) as probes to trap the target species for 1 h from buffer solutions of differing pH: (a) pH 6, (b) pH 8, and (c) pH 12. SA was used as the MALDI matrix.

To examine the binding interactions between Au@magnetic particles and their target species, a mixture of equal concentration of the cationic surfactants hexadecyltrimethylammonium bromide ($C16^+$), tetradecyltrimethylammonium bromide ($C14^+$), dodecyltrimethylammonium bromide ($C12^+$), and decyltrimethylammonium bromide ($C10^+$) were used as target species. Cationic species usually bind with anions through ion-pair interactions. The ion-pair interactions of cationic surfactants with anions increase as the lengths of the alkyl chains of the cationic surfactants increase.[4] That is, the hydrophobicity also affects the formation of ion pairs at solid−liquid interfaces. Accordingly, we expected a competition effect among the four cationic surfactants in their binding to the surface of the negatively charged gold nanoparticles. Figure 3a displays the MALDI mass spectrum of equal concentrations of these four cationic surfactants (each 0.2 mM); we observe roughly equal intensities for the $C10^+$, $C12^+$, $C14^+$, and $C16^+$ species at *m*/*z* 200, 228, 256, and 284, respectively. When the Au@magnetic particles (2 mg) were used as selective probes to trap the target species from the sample solution (0.5 mL), MALDI MS analysis indicates that the ammonium cation-based surfactants having the longer alkyl chain lengths have the highest ion intensities in the mass spectrum. A competition effect in the formation of ion pairs

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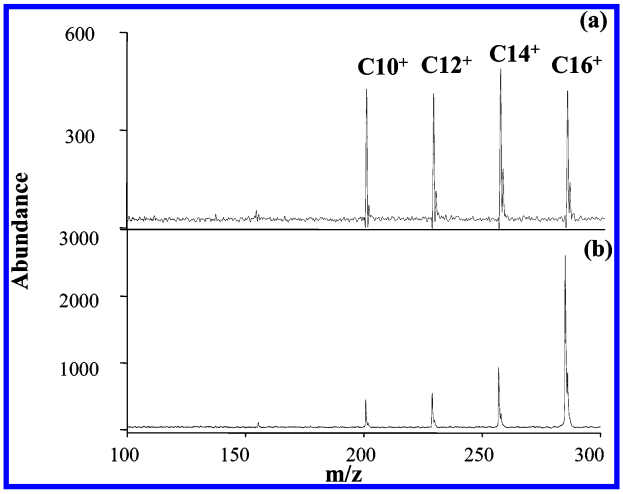


Figure 3. MALDI mass spectra of a mixture of four ammonium-based cationic surfactants, $C10^+$, $C12^+$, $C14^+$, and $C16^+$, obtained (a) by direct analysis and (b) after using Au@magnetic particles (1 mg) to trap the surfactants from a sample solution (0.5 mL). 2,5-DHB was used as the MALDI matrix.

between the cationic surfactants and the citrate ions on the surfaces of nanoparticles was observed. These results indicate that electrostatic and hydrophobic interactions are involved in the affinity process between the citrate anions on the surface of the gold nanoparticles and the cationic target species.

In addition to their role as probes that bind cationic species selectively, Au@magnetic particles can be used also as probes that concentrate traces of target species from very dilute aqueous solutions. Figure 4a presents the MALDI mass spectrum of melittin ($10^{-8}$ M, 0.5 $\mu$L) obtained without using the trapping process; we observe no analyte signal in this MALDI mass spectrum at such a low concentration. After using Au@magnetic particles as probes to concentrate the trace amount of melittin ($10^{-8}$ M) in a sample solution (0.1 mL), the protonated melittin ion peak ($MH^+$) is revealed in the MALDI mass spectrum (Figure 4b).

An additional problem faced in MALDI MS analysis of biomolecules is that the presence of urea, sodium dodecyl sulfate (SDS), and Triton X-100 can dramatically suppress the signals of analyte ions.[16] We believed that the ability to use Au@magnetic particles to selectively bind target species could be applied to exclude most of the interference during the analysis of sample solutions containing high concentrations of such salts or surfactants. We have found this situation to be the case. Figure 4c displays the MALDI mass spectrum of melittin ($10^{-7}$ M) in a sample solution containing 8 M urea; no analyte ion peak is observed in this mass spectrum, while a series of urea cluster ions, separated by 60 mass unit differences (marked with asterisks), appear in the low-mass region. When using the Au@magnetic particles to trap melittin ($10^{-7}$ M) from a sample solution (0.1 mL) containing 8 M urea, the interference is eliminated; Figure 4d presents the MALDI mass spectrum of melittin obtained after using the Au@magnetic particles (1 mg) as a probe. The $MH^+$ ion of melittin is observed in the MALDI mass spectrum at $m/z$ 2847.

The presence of 0.1% SDS in protein solutions usually has the dramatic effect of degrading the intensity of signals of the analyte in MALDI mass spectra.[16] Figure 5a presents the MALDI mass spectrum of cytochrome *c* ($10^{-6}$ M, 0.5 $\mu$L) containing 0.1% SDS; a very weak $MH^+$ ion is observed for cytochrome *c*. Figure 5b displays the MALDI mass spectrum obtained after using the Au@magnetic particles as probes to trap cytochrome *c* ($10^{-6}$ M) from a sample solution (0.1 mL) containing 0.1% SDS. An intense signal for the $MH^+$ ion of cytochrome *c* is present in this mass spectrum. The peak adjacent to the signal for the $MH^+$ ion of cytochrome *c* is a matrix (sinapinic acid) adduction of cytochrome *c*. These results indicate that our approach of using the Au@magnetic particles to trap target proteins is selective and excludes the interference of SDS.

In addition to SDS, Triton X 100, a nonionic surfactant, is frequently found in protein sample solutions. Figure 5c displays the MALDI mass spectrum of cytochrome *c* ($10^{-6}$ M, 0.5 $\mu$L) containing 20% Triton X 100. No peak for the analyte ion is observed in the MALDI mass spectrum obtained from this sample at such a high concentration of Triton X 100. Figure 5d presents the MALDI mass spectrum obtained after using the Au@magnetic particles as probes to trap cytochrome *c* ($10^{-6}$ M) from this sample solution. The peak adjacent to the signal of the $MH^+$ ion of cytochrome *c* is a matrix (sinapinic acid) adduction ion of cytochrome *c*.

All of these results indicate that target species can be selected effectively from sample solutions by using Au@magnetic particles and that the interference of salts, surfactants, and neutral species can be excluded. The time spent to trap the target species by using the Au@magnetic probes may be longer than that of an on-probe cleanup approach, but in addition to excluding interference, the target species also can be concentrated onto the surfaces of the Au@magnetic particles from a large sample solution volume during the trapping process; the simultaneous cleaning and concentrating phenomena provide the major advantages of this approach.

Although our results demonstrate that the Au@magnetic particles are effective probes that provide a capacity to select and concentrate target species from relatively simple solutions, we were interested in examining whether this approach would be successful in analyzing a more-complicated matrix solution. We believed that our approach could be employed effectively to trap, concentrate, and analyze enzymatic digest products of proteins because, generally, they contain low concentrations of peptide residues and because the net charges of these peptide residues would vary with the pH of the sample solutions. To demonstrate the trapping capacity for low concentrations of target species using the Au@magnetic particle-based probes, we used a very low concentration of the cytochrome *c* tryptic digest product as the sample. Figure 6a presents the MALDI mass spectrum obtained without any pretreatment of the cytochrome *c* ($10^{-7}$ M) tryptic digest products; we observe low-intensity ions in this mass spectrum. Furthermore, a series ions (marked with asterisks) derived from SA are observed in the mass spectrum, which may affect the observation of analyte ions revealing in this mass region. After using the Au@magnetic particles as probes to selectively trap target peptides from a sample (20 $\mu$L, pH 4) of digest product, more-intense peaks appear for the trapped species in the MALDI mass spectrum (Figure 6b). The ions at $m/z$ 1168.62, 1296.70, 1478.82, 1633.81, 1840.91, 1875.97, 2081.01, and 2209.12 correspond to the enzymatic digest products of cytochrome *c*, which

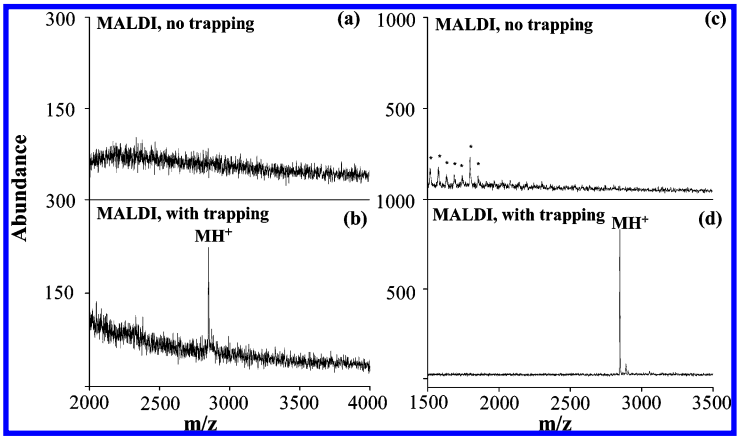


Figure 4. MALDI mass spectra of melittin obtained (a) by direct analysis ($10^{-8}$ M, 0.5 $\mu$L) and (b) after using Au@magnetic particles (1 mg) as probes to trap the trace amount of melittin ($10^{-8}$ M) in a sample solution (0.1 mL). MALDI mass spectra of a melittin ($10^{-7}$ M) solution containing 8 M urea obtained (c) by direct analysis (0.5 $\mu$L) and (d) after using Au@magnetic particles (1 mg) to trap melittin from a sample solution (0.1 mL). The peaks marked with asterisks are multimers of urea. SA was used as the MALDI matrix.

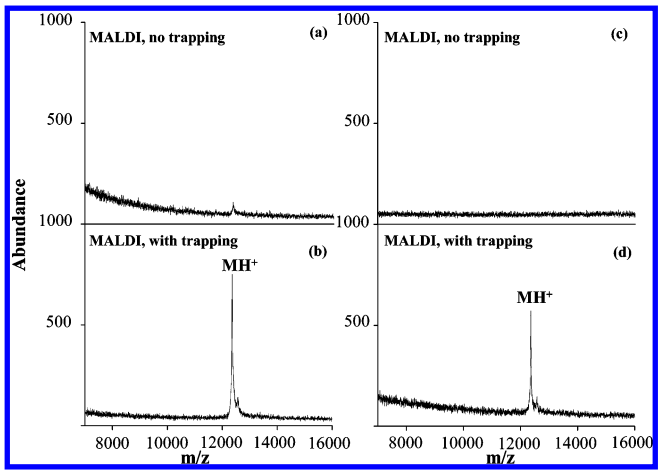


Figure 5. MALDI mass spectra of a cytochrome *c* solution ($10^{-6}$ M) containing 0.1% SDS obtained (a) by direct analysis and (b) after using Au@magnetic particles (1 mg) to trap cytochrome *c* from a sample solution (0.1 mL). MALDI mass spectra of a cytochrome *c* solution ($10^{-6}$ M) containing 20% Triton X 100 obtained (c) by direct analysis and (d) after using Au@magnetic particles (1 mg) to trap cytochrome *c* from a sample solution (0.1 mL). SA was used as the MALDI matrix.

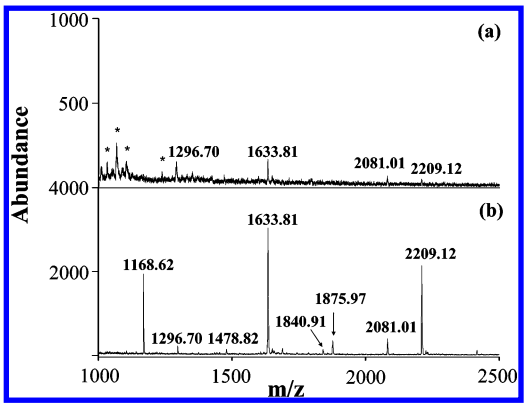


Figure 6. MALDI mass spectra of the tryptic digest products of cytochrome *c* ($10^{-7}$ M) obtained (a) by direct analysis and (b) using Au@magnetic particles (~0.4 mg) as probes to trap the digest products from a sample solution (20 $\mu$L). SA was used as the MALDI matrix. The peaks marked with asterisks were generated from SA.

Table 1. Ions Observed in the MALDI Mass Spectrum of Cytochrome *c* Tryptic Digest and a Comparison with the Output of the NCBI Database

| observed *m*/*z* | start−end sequence | miss[a] | peptide sequence | theor p*I*[b] |
|---|---|---|---|---|
| 1168.62 | 28−38 | 0 | TGPNLHGLFGR | 9.44 |
| 1296.70 | 28−39 | 1 | TGPNLHGLFGRK | 11.00 |
| 1478.82 | 89−100 | 2 | TEREDLIAYLKK | 5.85 |
| 1633.81 | 9−22 | 1 | IFVQKCAQCHTVEK | 8.06 |
| 1840.91 | 39−55 | 2 | KTGQAPGFTYTDANKNK | 9.53 |
| 1875.97 | 9−25 | 2 | IFVQKCAQCHTVEKGGK | 8.86 |
| 2081.01 | 56−72 | 1 | GITWKEETLMEYLENPK | 4.49 |
| 2209.12 | 56−73 | 2 | GITWKEETLMEYLENPKK | 4.95 |

[a] Number of missed cleavages. [b] Theoretical values of p*I* were obtained using a tool located at http://tw.expasy.org.

we confirmed by protein NCBI database searches. Furthermore, we obtained a probability-based Mowse score of 144.[24] Table 1 lists the detailed amino acid sequence and corresponding theoretical values of p*I* for each residue. All of the values of p*I* for these peptides residues are >4, which means that they all carry net positive charge in a solution at pH 4 and can be selectively attracted by the negatively charged surfaces of Au@magnetic particles.

Why are only these relatively large peptide residues observed in the mass spectra? Presumably, the digest reaction did not reach completion; this situation results in the absence of smaller peptide residues in the mass spectra. Furthermore, matrix interference in the mass region lower than 1000 also causes problems in identifying the peptide residues in this mass region. We usually observed the ions at $m/z > 1000$ to avoid interference from the matrix. Additionally, the ionization efficiencies in the MALDI process and the concentrations of these peptide residues in the digest product may be significantly higher than those of the other residues.

## CONCLUSIONS

This study has demonstrated the feasibility of using gold nanoparticles and Au@magnetic particles as selective and concentrating probes for trapping positively charged species. The

most interesting and practical application in which to employ this technique is in the analysis of enzymatic digest products of proteins. Simply by adjusting the pH of the digest's sample solutions to 4, almost all peptide residues will carry net positive charges; employing the Au@magnetic particles to concentrate the traces of peptide residues onto their surfaces, the presence of very low concentrations of digest products can be revealed in the MALDI mass spectra. In addition, the capacity of the Au@magnetic particles to exclude interference from high concentrations of salts and surfactants in the sample is another merit of this easily employed technique.

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