Analysis of Peptides and Proteins Affinity-Bound to Iron Oxide Nanoparticles by MALDI MS

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Iron oxide nanoparticles modified with oleate have been employed for the extraction of peptides and proteins from aqueous solution before matrix-assisted laser desorption/ionization (MALDI) mass spectrometric (MS) analysis. Adsorption of peptides and proteins onto the nanoparticles were mainly through electrostatic attraction and hydrophobic interaction. The analyte-adsorbed iron oxide nanoparticles could be efficiently collected from solution using a magnet. No elution step was needed. With this preconcentration strategy, the lowest detectable concentration of angiotensin I, insulin, and myoglobin in 500 μL of aqueous solution were 0.1 nM, 0.1 nM, and 10.0 nM, respectively. In addition, the nanoparticles could extract the analytes from solution with a high content of salt and surfactant, thus eliminating suppression effect during MALDI MS analysis. This method was successfully applied to concentrate the tryptic digest products of cytochrome *c*. In addition, the tryptic digestion of cytochrome *c* can be directly conducted on the iron oxide nanoparticles. (J Am Soc Mass Spectrom 2007, 18, 910–918) © 2007 American Society for Mass Spectrometry

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) has become a routine analytical tool to determine the molecular mass of biomolecules [1–3]. However, samples containing excessive amounts of salts, surfactants, or other contaminations suffer from ionization suppression and adduct formation [4, 5]. This limits the application of MALDI technique. Therefore, a simple and selective procedure for extraction and concentration of analyte from complex samples before MALDI MS is required.

Various methods have been developed to isolate the analyte from complex sample matrix. In surface-enhanced laser desorption/ionization (SELDI), the sample target played an active role in the extraction, purification, or concentration of the analyte of interest [6–9]. The target surface was derivatized for the selective retention of analyte while removing interferences through on-target washing. Several surface derivatizations have been designed to extract and concentrate the analyte through hydrophobic interaction [10, 11], ionic interaction [12, 13], or immunoaffinity [14, 15]. However, the sensitivity improvement was limited by the number of binding sites on the target. In another approach, the so-called surface-enhanced affinity capture (SEAC), micrometer-sized beads made for chromatography column were used to capture peptides and proteins from sample solution [16]. Various types of beads have been used, which include reverse-phase chromatographic beads [17, 18] and immobilized metal ion beads [19–21]. To speed up the collection of analyte-adsorbed beads from sample solution, magnetic particles, which can be simply collected using a magnet, were developed [22–24]. After collection, the microbeads were washed and placed on sample target, followed by analyzing with MALDI MS. Unfortunately, the presence of those particles on the sample target was reported to cause decrease in mass accuracy and resolution [25, 26].

Recently, nanoparticles have become interesting probes for the separation of analyte from sample solution because of their high surface area-to-volume ratio. In addition, the interference of nanoparticles during the laser desorption/ionization process may not exist because of the smallness of the particles. Previously, C_{18} functionalized silica nanoparticles have been utilized for hydrophobic interactions to extract peptides from acetonitrile, followed by liquid atmospheric pressure

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MALDI MS analysis [27]. Gold-modified magnetic nanoparticles [28] and diamond [29] were also used to selectively concentrate positively-charged proteins through electrostatic interaction. The adsorbed proteins were then eluted by adding MALDI matrix solution, such as sinapinic acid (SA) and α-cyano-4-hydroxycinnamic acid (CHCA). An aliquot of the mixed solution was deposited on sample target for acquisition of mass spectra. This method allows the MALDI MS detection of low concentration of proteins in complex matrix solution. Other types of nanoparticles have also been used to capture the analytes of interest, such as phosphopeptides [30, 31], antigen [32–34], oligonucleotides [35, 36], aminothiol [37], protein [38, 39], and bacteria [40, 41]. Among the nanoparticles used, carbon nanotube [38], Nile red-adsorbed gold [37], and TiO_2-coated magnetic nanoparticles [30] were not only employed as affinity probes but also as matrices.

The amount of analytes adsorbed onto the nanoparticles increases with decreasing the diameter of nanoparticles [42]. However, collection of the nanoparticles with diameter smaller than 10 nm by centrifugation was not easy. Additionally, the adsorbed analytes may desorb from the surface of nanoparticles during centrifugation. Magnetic nanoparticles can be easily collected by using an external magnet. Our goal in this study was to examine whether the iron oxide nanoparticles (IONPs) can be used to capture the peptides and proteins in dilute and complex sample solution. The surfaces of the iron oxide nanoparticles were modified with oleate, which contains a long carbon chain and an anionic carboxylate group. The oleate modified IONPs can attract positively-charged proteins or peptides through electrostatic attraction and hydrophobic interaction. Then a magnet can be used to isolate the IONPs quickly from the sample solution. The ability of the IONPs to act as affinity probes for charged species was evaluated. The applicability of the method to the peptide residue from the tryptic digest products of cytochrome *c* was also demonstrated. In addition, the tryptic digestion of cytochrome *c* can be directly conducted on the IONPs.

Experimental

Chemicals

Angiotensin I, insulin (from bovine pancreas), cytochrome *c* (from horse heart), myoglobin (from equine skeletal muscle), sinapinic acid (SA), ammonium bicarbonate, sodium phosphate dibasic, and sodium oleate were purchased from Sigma (St. Louis, MO). α-Cyano-4-hydroxycinnamic acid (CHCA) was obtained from Aldrich (Milwaukee, WI). Ferrous chloride tetrahydrate was purchased from Fluka (Buchs, Switzerland). Iron(III) chloride anhydrous was purchased from Riedel-de Haën (Seelze, German). Hydrochloric Acid was purchased from J. T. Baker (Phillipsburg, NJ). Sodium hydroxide was purchased from Hayashi Pure Chemical Industries Ltd. (Osaka, Japan). Trypsin was from Promega (Madison, WI). All chemicals were used as received without further purification. All other chemicals were of reagent grade. Water purified with a Barnstead NANOpure system (Dubuque, IA) was used for all solutions.

Preparation of IONPs and Surface Modification

Iron oxide nanoparticles in aqueous solution were prepared by a well-known coprecipitation method [43, 44]. Briefly, a reactant solution was prepared by mixing 0.2 g $FeCl_2$ $4H_2O$ and 0.34 g $FeCl_3$ into 1.7 mL of deaerated 0.2 M HCl solution. An aliquot of 160 μL of resulting reactant solution was dropwise added into 200 mL aqueous NaOH solution (0.01 M) under vigorous stirring. The solution mixture was stirred for 30 min until the black precipitates of iron oxide nanoparticles formed. Thus prepared iron oxide nanoparticles were then separated from the solution by using a magnet and the supernatant was removed from the precipitate by decantation. The iron oxide nanoparticles were redispersed by adding 50 mL deoxygenated deionized water and the solution was further centrifuged (8000 rpm and 10 min) to obtain flocculates. This purification cycle was repeated three times to remove excess salts. The redispersion of the final flocculates was done by adding 0.01 M HCl aqueous solution (typically 15 mL) with stir to convert the anionic charges on the nanoparticles into positive. The resulting cationic colloidal particles were stabilized because of the positive surface charges, and the solution appeared black.

The surface modification of the IONPs was then conducted to attach anionic carboxylate groups onto iron oxide nanoparticles following the previously reported procedure [45, 46]. This procedure is quite straightforward: dilute the previously obtained cationic iron oxide nanoparticles by dissolving 2 mL into 8 mL HCl solution (0.01 M). Then, 2 mL sodium oleate solution (0.033 M) was added dropwise into the concentration adjusted iron oxide nanoparticles aqueous solution which was kept at 80 °C with mild stir. The surface modification reaction was allowed to complete at least 10 min. A centrifugation followed by redispersion was then performed to remove the excess amount of oleates. Thus prepared IONPs solution was centrifuged by 12,000 rpm for 10 min. The flocculates at the bottom of ultracentrifugation vial were separated from the excess amount of sodium oleates by decanting the upper portion. The flocculates were redispersed into deionized water and then the final solution was adjusted to give its pH value close to 5.

Characterization of IONPs

The shape and size distribution of IONPs were characterized by transmission electron microscope (TEM) using TEM (JEOL, Tokyo, Japan) at an accelerating 200 kV. TEM samples were prepared by dipping 200 mesh

Formvar coated copper grid into the IONP aqueous solution. The iron content in the IONP solution was measured by an atomic absorption spectrometry (AAS). For such measurements, 10 μL of IONP solution mixed with 990 μL of 12 M HCl to convert IONPs into Fe^{2+} solution followed by AAS quantitative analysis (AA3110; Perkin Elmer, Wellesley, MA). The dissolution and Fe^{3+}/Fe^{2+} transformation required 30 min. The AAS measurements indicated that the IONPs suspension contained 31.6 mM Fe(II)/Fe(III).

Adsorption Capacity of IONPs

The adsorption capacity of IONPs for peptides and proteins in 5 mM phosphate buffers at pH 2 to 10 was investigated. The amount of analytes adsorbed was determined from the change in analyte concentrations before and after addition of the IONPs into the solutions. Sixty μL of IONPs solution is added to a sample vial. The magnetic particles in the solution were attracted to the bottom of the sample vial by an external magnet, and the supernatant solution was removed by pipet. An aliquot of 1 mL of 10^{-5} M analyte solution was added. The solution mixture was thoroughly mixed with a shaker for 1 h. After extraction, the magnetic nanoparticles were collected from the solution by using a magnet. Quantitation of the analyte concentrations was performed by a UV-visible spectroscopy (Lambda20; Perkin Elmer, Wellesley, MA) at 409 nm for myoglobin and at 220 nm for insulin and angiotensin I.

Sample Preparation and Extraction Procedure

CHCA and SA solutions, both freshly prepared in 1:1:0.001 acetonitrile/water/TFA solution, were used as the MALDI matrices for peptides and proteins, respectively. Protein and peptide solutions were prepared in deionized water and stored at −20 °C. MALDI samples were prepared by mixing 1 μL of analyte solution and 1 μL of matrix solution on the target and letting them dry at room temperature.

Thirty μL of IONPs solution was added to a 1.5 mL vial. The magnetic particles in the solution were attracted to the bottom of the sample vial by an external magnet, and the supernatant solution was removed by pipet. Then 500 μL of peptide or protein solution was added to the sample vial. The mixture was thoroughly mixed with a shaker for 1 h. Again, a magnet was used to isolate the magnetic nanoparticles from the solution after extraction. The isolated magnetic nanoparticles were washed with 100 μL of DI water to remove impurities. Then the collected magnetic nanoparticles were deposited on a stainless steel target and allowed to dry. Matrix solution (1 μL) was applied on top of the magnetic particles before MALDI MS analysis. Cytochrome *c* (1.2×10^{-6} M) tryptic digestion [30:1 (wt/wt)] was performed in a 25 mM NH_4HCO_3 buffer solution (pH 7.9) for 24 h at 37 °C. Before extraction, the digest products were diluted to desired concentration with NH_4HCO_3 buffer solution.

In ZipTip pretreatment, a SCX pipet tip (Millipore, MA) containing strong cation exchange resin was first activated by passing through 0.1% TFA aqueous solution. Then 500 μL of peptide or protein solution was passed through the ZipTip. After rinsing two times with 10 μL of deionized water, the analytes were eluted with a 0.001:1:1 (vol/vol) TFA-acetonitrile-water mixture (10 μL). MALDI samples were prepared by mixing 1 μL of elution and 1 μL of matrix solution on the target and letting them dry at room temperature.

MALDI-TOF MS

All the mass spectra were obtained using an Autoflex II (Bruker Daltonics, Bremen, German) time-of-flight mass spectrometer equipped with a 1.22 m flight tube. The samples were irradiated with a 337 nm nitrogen laser at 10 Hz. The accelerating voltage was set to 19 kV. The laser energy was 40 to 50 μJ/pulse. All spectra were recorded in the positive linear mode except spectra from the tryptic digestion experiments which were recorded in the positive reflectron mode (flight length 2.6 m).

Results and Discussion

Adsorption of Peptide and Protein

IONPs were chosen as the extractors in this study because of their magnetic property and high surface-to-volume ratios, which facilitate the easy isolation of analyte-adsorbed nanoparticles from the sample solution. To prevent nanoparticles aggregation and allow efficient analyte binding, the surface of IONPs was modified with oleate, which contains a carboxylate group and a long carbon chain (C_{18}). Oleate molecules may trap positively-charged peptides and proteins through ionic interaction and hydrophobic interaction. To explore the extraction capability of IONPs, both size distribution and analytes adsorption were examined before conducting MS analysis. From the TEM image of IONPs (data not shown), the IONPs were well dispersed and possessed a size distribution of 10.3 ± 1.3 nm. The ability of IONPs to extract angiotensin I, insulin, and myoglobin from aqueous solution at different pH was investigated with UV-VIS absorption spectrometry.ëAsëshownëinëFigureë1,ëtheëamountsëofëthe analytes adsorbed were sensitive to the solution pH. The maximum adsorption occurred at pH 4 for both insulin (curve b) and angiotensin I (curve a), while the maximum adsorption for myoglobin (curve c) was at pH 6. At the optimal pH, the fractions of analytes that adsorbed to the IONPs were 82, 53, and 70% for insulin, angiotensin I, and myoglobin, respectively. Based on these results, the adsorption capacity of the IONPs for angiotensin I, insulin, and myoglobin were estimated to be 48 mg/g, 323 mg/g, and 818 mg/g, respectively. The

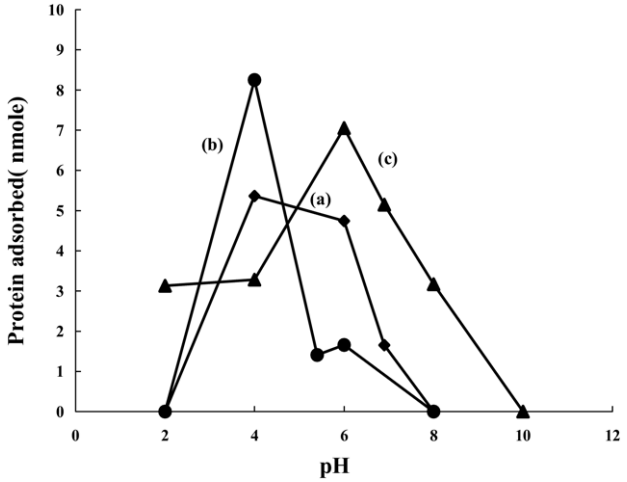

Figure 1. Amounts of (**a**) angiotensin I, (**b**) insulin, and (**c**) myoglobin adsorbed on nanoparticles as a function of solution pH. One mL of 10^{-5} M analyte solution mixed with 60 μL of IONPs solution. The data were made by averaging three measurements from three separate samples. The relative standard deviations of the measurements were less than 10.0%.

isoelectric point (pI) values of angiotensin I, insulin, and myoglobin are 6.9, 5.4, and 6.9, respectively. We found that the maximum adsorption all occurred when the pH of the protein solution was below the value of pI. At pH <pI, the protein molecules are positively charged. The pKa value of the oleic acid is around 5.0. The oleic acid dissociates into carboxylate, and the surface of IONPs carries negative charges at pH >4. Therefore, the negatively charged IONPs tend to capture positively charged protein molecules to their surface through electrostatic attraction. When the pH of the protein solution equals the value of pI, 51% of myoglobin molecules and 15% of insulin and angiotensin I molecules still attach to the surface of IONPs, even without electrostatic attraction between the protein molecules and IONPs. The adsorption at pH = pI indicates that hydrophobic interaction exists between the long carbon chain of oleate and protein molecules. In addition, the hydrogen bonding may also involve in the adsorption process. At pH >pI, the fraction of protein molecules adsorbed to the IONPs rapidly decrease due to the repulsive force between the negatively charged protein and the negative surface of IONPs. The UV-VIS absorption results suggest that the IONPs can extract peptide and protein molecules from solution through electrostatic attraction, hydrophobic interaction, and hydrogen bonding.

Analysis of Peptides and Proteins by MALDI MS

To demonstrate mass spectrometric analysis, angiotensin I, insulin, and myoglobin were extracted by IONPs from solution and analyzed using MALDI MS. In our method, the collected analyte-adsorbed IONPs were directly put onto the sample target and 1 μL of matrix solution was added. There is no elution step during sample preparation to alleviate sample transfer losses

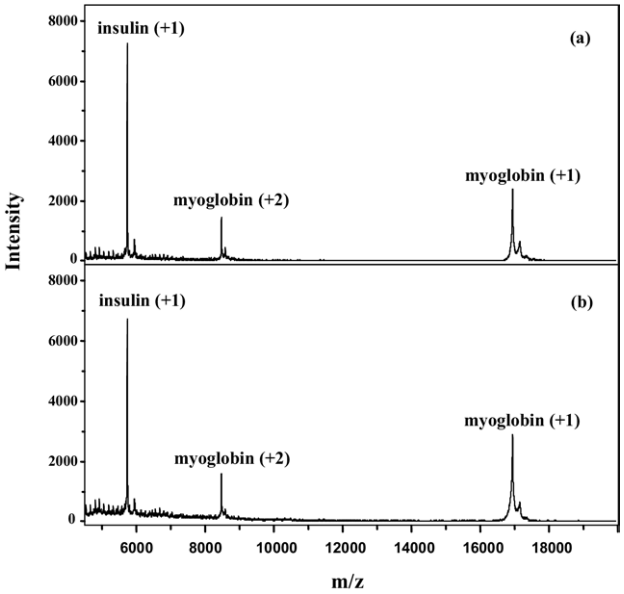

Figure 2. Mass spectra of a protein mixture of insulin ($5*10^{-8}$ M) and myoglobin ($1*10^{-6}$ M) obtained when using IONPs to trap the protein for 1 h. The spectra were obtained (**a**) without and (**b**) with removal of the IONPs before MALDI MS analysis. SA was used as the MALDI matrix.

and reduce preparation time. Due to the small size of IONPs, we assumed that the presence of IONPs would not interfere with desorption and ionization process. To demonstrate this assumption, 5 μL of SA solution mixed with the protein-adsorbed IONPs and 1 μL of the slurry was used for acquisition of mass spectrum without removal of IONPs. Then the IONPs were collected

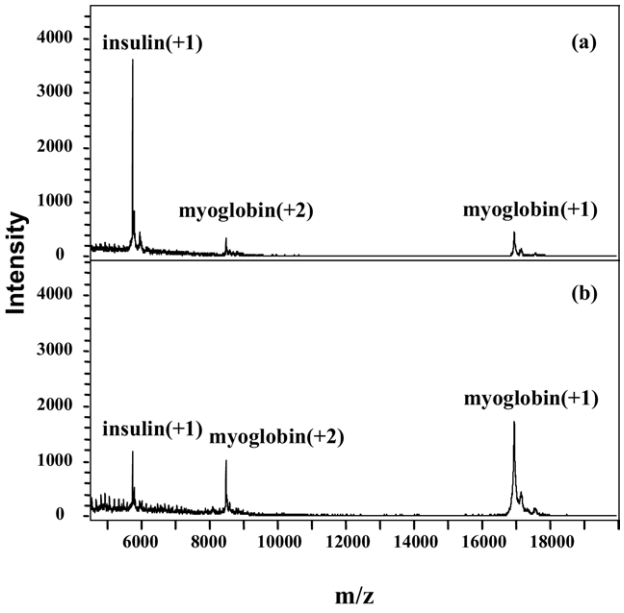

Figure 3. Mass spectra of a protein mixture of insulin ($1*10^{-8}$ M) and myoglobin ($1*10^{-6}$ M) when using IONPs to trap the protein from buffer of different pH: (**a**) pH 4, (**b**) pH 6, and (**c**) pH 10. SA was used as the MALDI matrix.

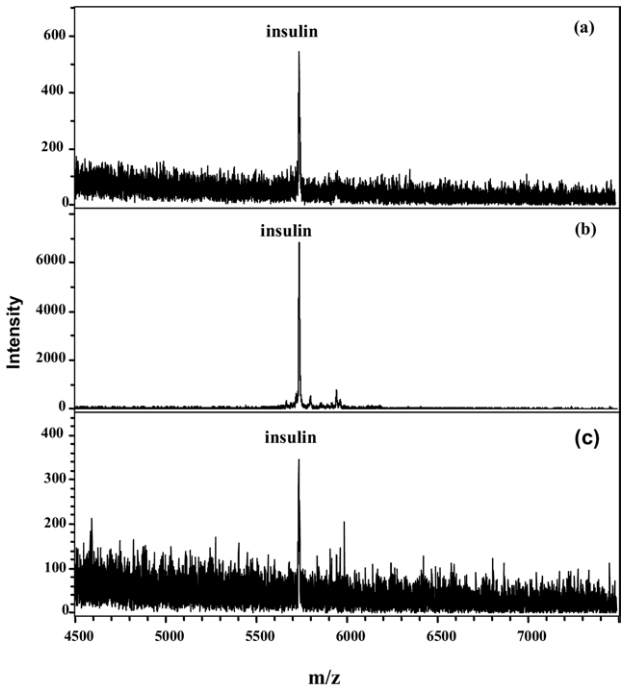

Figure 4. Mass spectra of insulin obtained (**a**) without any pretreatment, and (**b**) with preconcentration of 10 nM, and (**c**) 0.1 nM insulin solution by IONPs; other conditions as in Figure 2.

by a magnet from the remaining 4 μL of the slurry and 1 μL of the supernatant was analyzed by MALDI MS. Figure 2 shows the mass spectra of the protein with and without IONPs. There was no distinct difference in these two mass spectra. It suggests that the presence of small IONPs does not deteriorate the signal intensities, resolution, and mass accuracy of the mass spectra as shown in Figure 2a. In MALDI, the reproducibility of intra- and inter-sample was usually poor. We also examined the reproducibility of the sample preparation. The signal intensities of angiotensin I, insulin, and myoglobin varied within 2.5 to 11.8% over 50 sample spots (five samples).

Figure 1 shows that the adsorption of peptides and proteins was significantly affected by the pH of the solutions. We employed a mixture of insulin and myoglobin to demonstrate the selective capture of protein. From a solution of pH 4, both insulin and myoglobin were captured by IONPs, and the adsorbed proteins were confirmed by MALDI MS (Figure 3a). At pH 6, the signal intensity for insulin decreased due to the net negative charges on insulin molecules. However, a fraction of insulin was still adsorbed to IONPs through hydrophobic interaction. From pH 4 to 6, the signal intensity of myoglobin in the mass spectrum significantly increased (Figure 3b), which indicates higher adsorption of myoglobin at pH 6. These results were consistent with the results obtained in Figure 1.

The IONPs plays a role as a solid-phase extraction (SPE) support. The increased surface area-to-volume ratio of the particle in comparison to microparticles can allow for efficient extraction and concentration of analytes from very dilute solution. Figure 4a shows the mass spectrum of insulin (10 nM) without preconcentration process. The signal/noise (S/N) ratio was determined to be 4.3. No signals were obtained when the insulin concentration was lower than 10 nM. With the use of IONPs to preconcentrate the insulin from 500 μL of solution, the signal was greatly enhanced with S/N ratio of 63.3 as shown in Figure 4b. Compared with regular MALDI, mixing 1 μL of analyte solution with 1 μL of matrix solution, the use of IONPs provides a 15-fold higher peak intensity. With preconcentration by IONPs, the lowest concentration that can be observed was 0.1 nM (Figure 4c). The SCX ZipTip, which would attract positively charged peptide and protein through electrostatic attraction, was also used to preconcentrate insulin from 500 μL solution (10 nM). The S/N ratio obtained was 20.3. Compared with the ZipTip result, the signal intensity obtained by the use of IONPs was more than three times larger. The same experiments were performed for myoglobin and angiotensin I, and the results are summarized in Table 1. The results indicate that the signal enhancements for the three analytes are 2- to 3-fold higher when compared to those with ZipTip. In addition, ZipTip requires more sample manipulation and increases the possibility of contamination or sample loss. With preconcentration by IONPs, the lowest detectable concentrations of myoglobin and angiotensin I were 10.0 nM and 0.1 nM.

Mass Analysis of Peptide and Protein in Complex Sample Solution

Samples from biological sources often contain a complex mixture of salts and surfactants. However, the presence of these contaminants will suppress the analyte signals in MALDI MS. We investigated the ability of using IONPs to capture analytes and eliminate the interference from high concentration of salts or surfactants. Figure 5a shows the mass spectrum of insulin and myoglobin mixture in a sample solution containing 0.1% SDS. Only a very weak signal was observed for

Table 1. The signal-to-noise ratios of analytes without and with preconcentration by IONPs and ZipTip

Analytes	Without pretreatment	Preconcentration by IONPs	Preconcentration by ZipTip
Angiotensin I[a]	3.8	32.4	13.6
Insulin[a]	4.3	63.3	20.3
Myoglobin[a]	9.6	68.9	26.9

[a]The concentrations of angiotensin I, insulin, and myoglobin were 5 nM, 10 nM, and 500 nM, respectively.

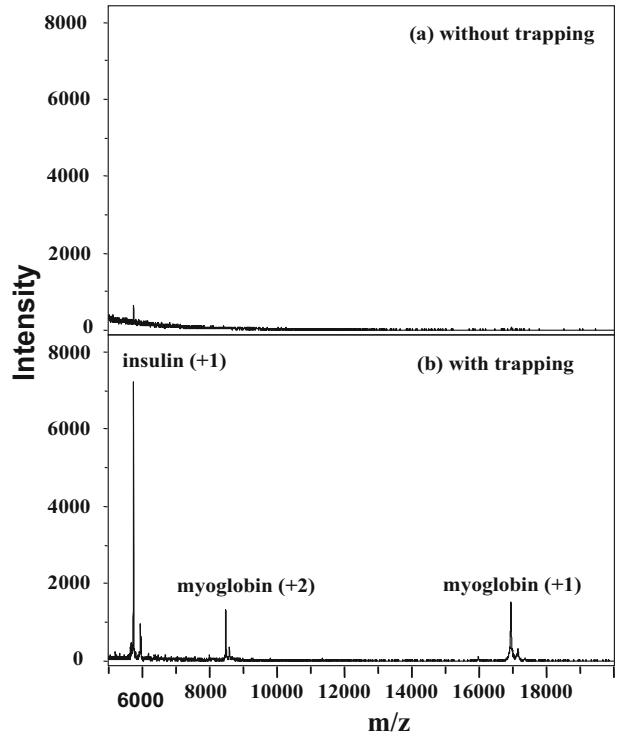

Figure 5. Mass spectra of insulin and myoglobin solution containing 0.1% SDS obtained (**a**) without any pretreatment, and (**b**) with extraction of mixture solution by IONPs. Mass spectrum of insulin and myoglobin solution containing 8 M urea obtained (**c**) without any pretreatment, and (**d**) with extraction of mixture solution by IONPs; other conditions as in Figure 2.

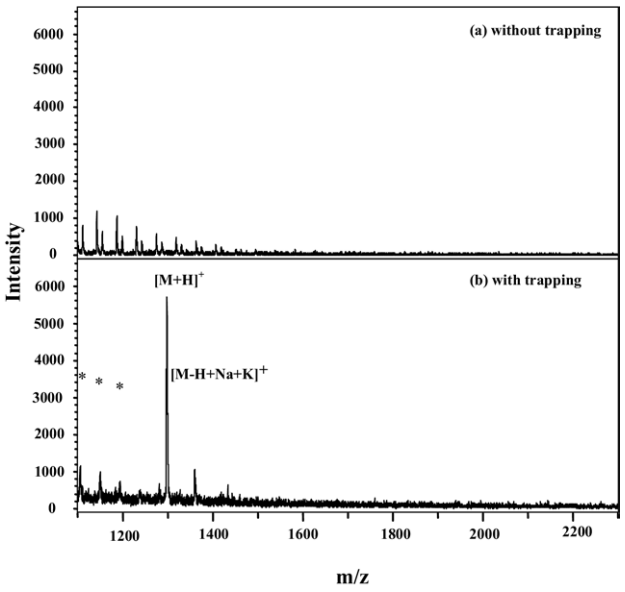

Figure 6. Mass spectrum of angiotensin I solution containing 20% TritonX-100 obtained (**a**) without any pretreatment, and (**b**) with extraction of mixture solution by IONPs. CHCA was used as the MALDI matrix.

insulin, and no myoglobin ion was detected. When using IONPs to extract insulin and myoglobin from a solution mixture containing 0.1% SDS, the intense signals for both insulin and myoglobin were present in the mass spectrum (Figure 5b). Urea is also frequently found in protein samples solutions. Figure 5c shows the mass spectrum of insulin and myoglobin mixture in a sample solution containing 8 M urea. No insulin or myoglobin ion was detected. When using IONPs to extract insulin and myoglobin from a solution mixture containing 8 M urea, the intense signals for both insulin and myoglobin were present in the mass spectrum (Figure 5d). The use of IONPs enhanced the signal intensities in the presence of the contaminations; however, the signal intensities were still lower compared with the analyte solutions of same concentration without salts or surfactants.

The presence of TritonX-100, a nonionic surfactant, in MALDI samples usually degrades analyte signal. Figure 6a shows the mass spectrum of angiotensin I containing 20% TritonX-100. No angiotensin I ion was detected except a series of TritonX-100 cluster ions appeared in the mass spectrum. Figure 6b shows the mass spectrum of angiotensin I after IONPs extraction. The angiotensin I ion was detected at *m/z* 1297.67, and the signal at *m/z* 1359.40 corresponded to $[M - H + Na + K]^+$. Those peaks marked with asterisks were generated from TritonX-100. Although slight amount of TritonX-100 was adsorbed onto the IONPs through hydrophobic interaction, the angiotensin I could be efficiently isolated from sample solution by using IONPs. These results demonstrate that the analytes can be extracted from contaminated sample solution by using IONPs and the interference

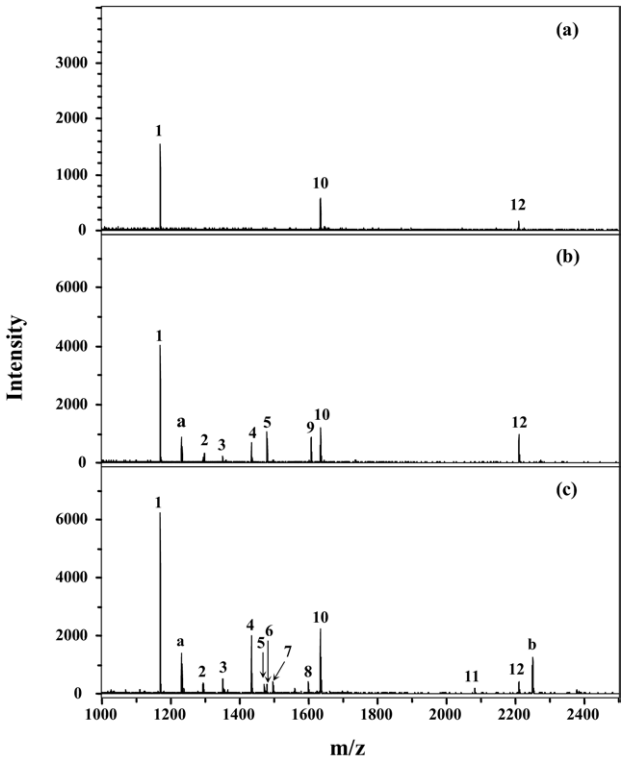

Figure 7. Mass spectrum of the tryptic digest products of cytochrome *c* obtained (**a**) without preconcentration, (**b**) with preconcentration of the digest products (50 μL) by IONPs, and (**c**) with preconcentration of cytochrome *c* (500 μL), followed by on-particle enzymatic digestion. CHCA was used as the MALDI matrix. Peak identities are listed in Table 2.

of salts and surfactants can be minimized. This developed procedure provides cleaning and concentration at the same time.

Mass Analysis of Enzymatic Digest Products

The applicability of the method for analyzing tryptic digest products of cytochrome *c* was investigated. Figure 7a shows the mass spectrum of cytochrome *c* ($2.5*10^{-7}$ M) tryptic digest products without trapping by IONPs. There were three low-intensity signals in the mass spectrum. Figure 7b shows the mass spectrum of cytochrome *c* tryptic digest products ($5*10^{-8}$ M, 50 μL) after using IONPs to capture the peptide fragments of digest products. More intense peaks appeared in the mass spectrum. The ions at *m/z* 1168.64, 1296.74, 1350.72, 1433.82, 1478.86, 1606.97, 1633.69, and 2209.19 correspond to the enzymatic digest products of cytochrome *c*, and were confirmed by protein NCBI database searches. The probability-based Mowse score was 126. Signals were enhanced without the need to preseparate the enzyme and IONPs from the peptide fragments for MALDI MS analysis. In another important application, IONPs were used to preconcentrate low concentration of cytochrome *c*, and the enzymatic digestion of cytochrome *c* was conducted directly on the particles. An aliquot of 500 μL cytochrome *c* solution ($5*10^{-8}$ M) mixed with 10 μL of IONPs for 1 h. A magnet was used to collect the IONPs, and the supernatant was removed by a pipet. Trypsin in NH_4HCO_3 buffer solution (2.5 μL, 20 ng/μL) was added to mix with the cytochrome *c*-adsorbed IONPs. The mixture reacted for 24 h at 37 °C. A magnet was used again to collect the peptide-adsorbed IONPs, followed by MALDI analysis. The mass spectrum of on-particle tryptic digest of cytochrome *c* is shown in Figure 7c. The ions at *m/z* 1168.67, 1296.72, 1350.79, 1433.85, 1470.74, 1478.88, 1495.77, 1598.84, 1633.69, 2081.04, and 2209.14 correspond to the enzymatic digest products of cytochrome *c*, and were matched by protein NCBI database searches with a probability-based Mowse score of 170. Table 2 lists the detailed amino acid sequence. Peak a (*m/z* = 1230.58) and peak b (*m/z* = 2248.97) were not matched by NCBI database searches. Peak a may be caused by the impurity of sample. Peak b was assigned to be the incomplete digestion fragment, because the cleavage site on cytochrome *c* may be blocked by the adsorption of cytochrome *c* on the IONPs. This method integrates isolation, concentration, purification, and digestion on nanoparticles. Compared with the concen-

Table 2. Ions observed in the MALDI mass spectrum of cytochrome *c* tryptic digest and a comparison with the output of the NCBI database

Peak no.	Observed *m/z*	Start-end sequence	Miss[a]	Peptide sequence
1	1168.67	28-38	0	TGPNLHGLFGR
2	1296.72	28-39	1	TGPNLHGLFGRK
3	1350.79	89-99	1	TEREDLIAYLK
4	1433.85	26-38	1	HKTGPNLHGLFGR
5	1470.74	40-53	0	TGQAPGFTYTDANK
6	1478.88	89-100	2	TEREDLIAYLKK
7	1495.77	61-72	0	EETLMEYLENPK
8	1598.84	39-53	1	KTGQAPGFTYTDANK
9	1606.97	88-100	3	KTEREDLIAYLKK
10	1633.69	9-22	1	IFVQKCAQCHTVEK
11	2081.04	56-72	1	GITWKEETLMEYLENPK
12	2209.14	56-73	2	GITWKEETLMEYLENPKK

[a]Number of missed cleavages.

tration typically used in tryptic digestion for peptide mapping, the concentration of cytochrome *c* used in this experiment is lower by two orders of magnitude.

Conclusions

We prepared iron oxide nanoparticles modified with oleate and used them as SPE support to concentrate peptide and protein from dilute solution. With preconcentration, the lowest detectable concentration of angiotensin I, insulin, and myoglobin were 0.1 nM, 0.1 nM, and 10 nM, respectively. Sensitivity may be further enhanced by using a larger volume of sample solution and a longer extraction time. In addition, the use of IONPs can eliminate the suppression of analyte signals due to salts and surfactants in MALDI samples. We have successfully applied the developed method to the analysis of enzymatic digest products of proteins. In the future, the modification of the iron oxide nanoparticle surfaces can be developed to achieve selective capture of target biomolecules.

Acknowledgments

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