# Enrichment of Peptides using Novel $C_8$-functionalized Magnetic Nanoparticles for Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometric Analysis

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**Abstract:** $C_8$-functionalized magnetic nanoparticles were synthesized by coating magnetic $Fe_3O_4$ nanoparticles with silica–amine groups using 3-aminopropyltriethoxysilane and by subsequently modifying the amine groups with chloro(dimethyl)octylsilane to produce octyl groups on the surface of the MNPs. The $C_8$-functionalized MNPs were used to enrich peptides from tryptic protein digests of myoglobin and α-casein. The enriched peptides were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF–MS). MALDI-MS was also used to investigate desalting of the $C_8$-functionalized MNPs. Sample solutions were prepared in 1.0 M NaCl, and the successful removal of salt was observed. Enrichment with $C_8$-functionalized MNPs was very effective for separating and concentrating tryptic peptides.

**Key words:** 3-aminopropyltriethoxysilane, chloro(dimethyl)octylsilane, MALDI, magnetic nanoparticles

## Introduction

Recently, magnetic materials have been used for separating and enriching proteins or peptides.[1-5] Magnetite ($Fe_3O_4$) nanoparticles are popular due to their superparamagnetic properties, which endow the nanoparticles with strong magnetic responsibility, reducing sample preparation time.[4,5] The surface of magnetic nanoparticles (MNPs) is commonly modified with specific functional groups for various applications.[6] MNPs coated with alkyl groups can be used to enrich peptides through hydrophobic interactions between alkyl groups and peptides. Laboratory-prepared $C_8$-MNPs were used to enrich peptides from serum using $FeCl_3 \cdot 6H_2O$ and 1,6-hexadiamine to coat the $Fe_3O_4$ surface with amine functional groups to prepare amine-functionalized MNPs, which were then modified with chloro(dimethyl)octylsilane.[5] MNPs synthesized with $FeCl_2$ and $FeCl_3$ and modified with oleate ($C_{17}H_{33}COO^-$) were also used to extract peptides and proteins from aqueous solutions.[7] Alternatively, MNPs were enclosed in a silica shell after reacting with tetraethylorthosilicate and subsequently modified with chloro(dimethyl)octylsilane[4] to generate $C_8$-MNPs. In another study, MNPs were reacted directly with trimethoxypropylsilane or octadecyltrimethoxysilane to provide $C_3$- and $C_{18}$-functionalized MNPs.[8] In the present study, $C_8$-MNPs were synthesized using a method in which the surface of the MNPs was modified by 3-aminopropyltriethoxysilane (APES)[9,10] to generate an amino silane coating and then by chloro(dimethyl)octylsilane to attach octyl groups to the amine groups. The synthesized $C_8$-MNPs were very effective for peptide enrichment and desalting.

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## Materials and Methods

### Materials

Myoglobin derived from horse heart, α-casein from bovine milk, bradykinin, angiotensin I, adenocorticotropic hormone, 3-aminopropyltriethoxysilane, 2,5-dihydroxybenzoic acid (DHB), ammonium bicarbonate, sodium chloride (NaCl), chloro(dimethyl)octylsilane, iron(II) chloride hexahydrate, iron(III) chloride tetrahydrate, pyridine, phosphoric acid, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sequencing-grade modified trypsin was obtained from Promega (Madison, WI, USA; catalog number v5113). Ethanol was acquired from Merck (Darmstadt, Germany), acetonitrile (ACN) was obtained from Burdick & Jackson (Muskegon, MI, USA), and ammonia solution 28.0–30.0% was purchased from Samchun Chemicals (Seoul, South Korea).

### Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-MS) analysis

MALDI-MS experiments were performed on an AXIMA-CFR time-of-flight mass spectrometer (Shimadzu, Tokyo, Japan) equipped with a 337-nm nitrogen laser in reflectron positive-ion mode. Mass spectra were measured from *m/z* 500 to 6,000 after external calibration using standard peptides (bradykinin, angiotensin I, and adenocorticotropic hormone).

All samples were deposited on the MALDI plate via a two-step method, in which 1 μL of the matrix solution was loaded onto the MALDI plate, allowed to dry, and covered with 1 μL of a mixed sample/matrix solution (1:1, v/v). For identification, a database from Swiss Prot (http://expasy.org/tools/findpept.html) was used. The matrix solution contained 10 mg of DHB in 1 mL of water/ACN (50:50, v/v) with 1% phosphoric acid.

### Protein digestion and sample preparation

For tryptic digestion of standard protein samples (myoglobin or α-casein), 100 μg of each protein was dissolved in 100 μL of 50 mM aqueous ammonium bicarbonate with 10 μL of 0.5 μg/μL trypsin solution. The solutions were incubated overnight at 37°C. The digested solutions were then diluted with 0.1% TFA to produce 5.8 pmol/μL tryptic myoglobin peptides or 4.0 pmol/μL tryptic α-casein peptides. To confirm desalting, 1.0 M aqueous NaCl was used as the diluent instead of 0.1% TFA.

### Synthesis of $C_8$-functionalized MNPs

$C_8$-functionalized MNPs were synthesized in three steps: synthesis of MNPs, coating of MNPs with amino silane, and attachment of octyl groups (Fig. 1). First, $Fe_3O_4$ MNPs were prepared by co-precipitating $Fe^{2+}$ and $Fe^{3+}$ ions from a basic solution containing $FeCl_2 \cdot 4H_2O$ (2.0 g) and $FeCl_3 \cdot 6H_2O$ (5.4 g) dissolved in 100 mL of water.[7] An $NH_4OH$ solution (75 mL; 28.0-30.0%) was added at 25°C with stirring, immediately producing black particles with magnetic properties. The precipitate was heated at 80°C for 30 min, washed with water and ethanol, dried, and crushed into fine particles (~10 nm diameter). Second, the surface of the synthesized MNPs was coated with amino silane[9] by dissolving 0.43 g of the $Fe_3O_4$ MNPs in a tube containing 9.7 mL of ethanol, adding 0.3 mL of APES, and gently mixing overnight at 25°C. Using a magnet, precipitates were collected on the bottom of the tube while the supernatant was removed. The particles were rinsed with ethanol, dried at 80°C, and collected. Finally, octyl groups were attached to the surface of the amine functional groups of MNPs[5] by dispersing 10 mg of the APES-bound MNPs in 1.0 mL of anhydrous pyridine, and 0.1 mL of chloro (dimethyl)octylsilane was added under vibration and mixed for 12 h at 25°C.

### Enrichment and desalting processes

To investigate the effectiveness of the $C_8$-MNPs for the enrichment of peptides, 30 μL of a 10 mg/mL $C_8$-MNP suspension was added to 10 μL of the prepared peptide solution (~5 pmol/μL) and mixed by vibrating at room temperature for 60 min to allow the peptides to adsorb to the carbon chain of the $C_8$-MNPs. Using a small permanent magnet, the $C_8$-MNPs and captured peptides were pulled to the bottom of the tube, and the supernatant was transferred to another tube (loading solution). The isolated nanoparticles were washed twice with water (10 μL each), and the water used for washing was analyzed for the presence of peptides (washing solution). To detach the peptides from the $C_8$-MNP, 10 μL of the DHB matrix solution was added to the tube containing the washed nanoparticles. Using a magnet, the nanoparticles were isolated at the bottom, and the supernatant containing peptides was collected (enriched solution). The solution collected from each step (10 μL) was analyzed using MALDI-MS.

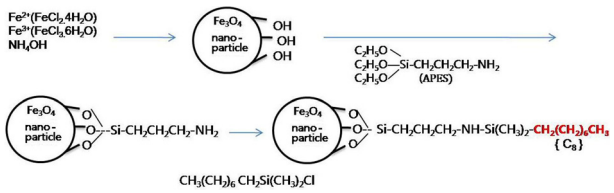


**Figure 1.** Schematic diagram of the synthesis of $C_8$-MNPs.

## Results and Discussion

### Advantages of using $C_8$-modified particles

Generally, the synthesis of $C_8$-MNPs consists of three steps: synthesis of MNPs, modification of MNPs with active functional groups, and attachment of octyl groups. While the synthesis of MNPs is performed exclusively with $Fe^{2+}$ and $Fe^{3+}$ ions in a basic solution, various methods are used for the second and the third steps. Recently, APES was introduced to modify the MNPs surface.[9,10] Modification of MNPs with APES is advantageous because it provides amine functional groups, which are used to covalently attach other molecules. The attachment of octyl groups was reported using chloro(dimethyl) octylsilane on 1,6-hexadiamine-modified amine groups of MNPs[5] or on tetraethylorthosilicate-modified silanol groups of MNPs.[4] In the present study, $C_8$-MNPs were synthesized using APES to attach amine functional groups, and chloro (dimethyl)octylsilane was used to modify the amine functional groups, producing octyl groups. A schematic diagram of the synthetic procedure is shown in Figure 1.

### Enrichment of peptides in 0.1% TFA and 1.0 M NaCl

Enrichment using the $C_8$-MNPs was investigated using tryptically digested peptide solutions from myoglobin and α-casein in 0.1% TFA and 1.0 M NaCl. Figures 2 and 3 show the mass spectra of tryptically digested myoglobin and α-casein, respectively, for samples in 0.1% TFA (left) and 1.0 M NaCl (right) prior to enrichment (A), after enrichment - the eluted peptide solution from the magnetic beads (B), from the loading solution - the supernatant removed from the mixture of $C_8$-MNPs and peptides (C), and from the washing solution (D). No peptide peak was observed from the loading solution and the washing solution; thus, the $C_8$-MNPs efficiently adsorbed the peptides during the loading and washing steps. Similar quality mass spectra were obtained for the samples in 0.1% TFA before (A-1) and after enrichment (B-1), as shown in Figures 2 and 3. For the samples in 1.0 M NaCl, significant improvement was observed from the mass spectra

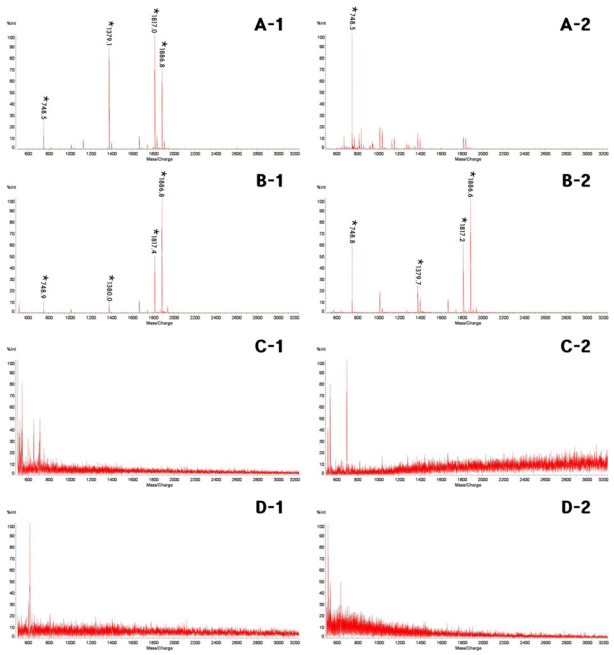


**Figure 2.** MALDI mass spectra of myoglobin tryptic digests in 0.1% TFA (A-1, B-1, C-1, and D-1) and 1.0 M NaCl (A-2, B-2, C-2, and D-2): (A) tryptic peptides before enrichment; (B) tryptic peptides after enrichment; (C) loading solution; and (D) washing solution. Each identified peptide peak is marked with an asterisk and *m/z* value.

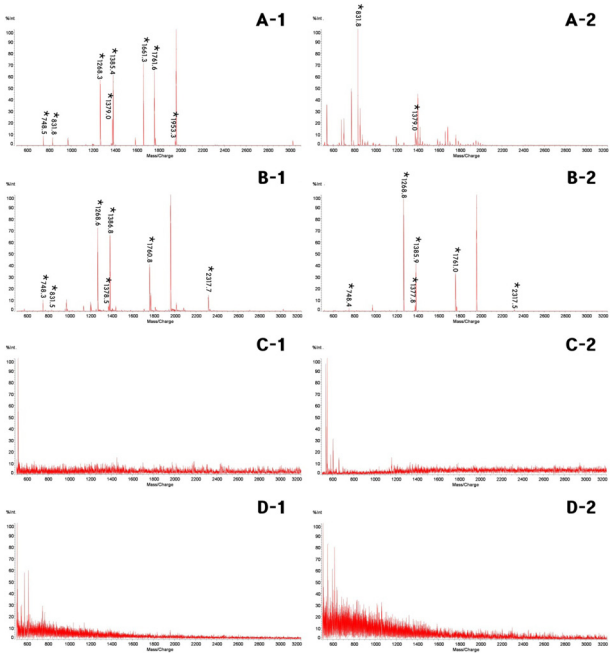


**Figure 3.** MALDI mass spectra of α-casein tryptic digests in 0.1% TFA (A-1, B-1, C-1, and D-1) and 1.0 M NaCl (A-2, B-2, C-2, and D-2): (A) tryptic peptides before enrichment; (B) tryptic peptides after enrichment; (C) loading solution; and (D) washing solution. Each identified peptide peak is marked with an asterisk and *m/z* value.

**Table 1.** Identified peptides from typtic digestion of myoglobin and α-casein.

| Theoretical monoisotopic $[M+H]^+$ | Peptide sequence | position | modification | Missed cleavages |
|---|---|---|---|---|
| myoglobin | | | | |
| 748.435 | (K)/ALELFR/(N) | 135-140 | | 0 |
| 1378.842 | (K)/HGTVVLTALG-GILK/(K) | 65-78 | | 0 |
| 1815.902 | GLSDGEWQQVLN-VWGK/(V) | 2-17 | | 0 |
| 1853.962 | (K)/GHHEAELK-PLAQSHATK/(H) | 81-97 | | 1 |
| 1885.022 | (K)/YLEFISDAIIHV-LHSK/(H) | 104-119 | | 0 |
| α-casein | | | | |
| 748.370 | (K)/TTMPLW | 209-214 | | 0 |
| 831.384 | (K)/EDVPSER/(Y) | 99-105 | | 0 |
| 1267.704 | (R)/YLGYLEQLLR/(L) | 106-115 | | 0 |
| 1379.665 | (R)/EQLSTSEENSKK/(T) | 141-152 (s2)[a] | | 1 |
| 1384.730 | (R)/FFVAPFPEVFGK/(E) | 38-49 | | 0 |
| 1386.646 | (K)/TVDMESTEVFTK/(K) | 153-164 (s2)[a] | | 0 |
| 1660.794 | (K)/VPQLEIVPN-SAEER/(L) | 121-134 | PHOS[b] | 0 |
| 1759.945 | (K)/HQGLPQEVL-NENLLR/(F) | 23-37 | | 0 |
| 1951.952 | (K)/YKVPQLEIVPN-SAEER/(L) | 119-134 | PHOS[b] | 1 |
| 2316.137 | (K)/EPMIGVNQELAY-FYPELFR/(Q) | 148-166 | | 0 |

[a]The peptides are originated from alpha-S2 casein. The other casein peptides are from alpha-S1 casein.
[b]Phosphorylation was observed where the phosphorylated amino acids are underlined.

after enrichment (B-2) compared to the mass spectra before enrichment (A-2). For example, while one peptide and two peptides were identified from the tryptically digested myoglobin and α-casein in 1.0 M NaCl before enrichment, respectively, four and six peptides were successfully identified after enrichment using the $C_8$-MNPs for the tryptically digested myoglobin and α-casein, respectively. These results indicate that the $C_8$-MNPs were very effective for desalting.

### Interaction between $C_8$-MNPs and phosphopeptides

Phosphopeptides of tryptically digested α-casein were observed at *m/z* 1661.3 and 1953.3 from the sample in 0.1% TFA as shown in Fig. 3(A-1). However, phosphopeptides were not observed after enrichment as shown in Fig. 3(B-1). The phosphopeptides are believed to have been adsorbed

on the surface of the MNPs due to additional interactions between the phosphate groups and $Fe_3O_4$ nanoparticles.[11,12] Enrichment of tryptically digested α-casein in 1.0 M NaCl enabled successful identification of six peptides that did not include phosphopeptides.

## Conclusions

A novel method for synthesizing $C_8$-functionalized MNPs was proposed and used to enrich and desalt tryptic peptides of myoglobin and α-casein. Peptides from tryptically digested proteins were successfully adsorbed on the surface of MNPs during the loading step and removed during elution. Based on the enrichment results for samples in 1.0 M NaCl, the salt was effectively removed during enrichment using $C_8$-MNPs, allowing peptide peaks to be observed.

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## References

1. Kim, Y. P.; Cho, K.; Lee, D.; Piao, Y.; Ahn, Y. H.; Yoo, J. S.; Hyun, T.; Kim, H. S. *Rapid Commun. Mass Spectrom.* **2007**, 21, 3435.
2. Zhou, W.; Yao, N.; Yao, G. P.; Deng, C. H.; Zhang, X. M.; Yang, P. Y. *Chem. Commun.* **2008**, 5577.
3. Chen, H. M.; Qi, D. W.; Deng, C. H.; Yang, P. Y.; Zhang, X. M. *Proteomics* **2009**, 9, 380.
4. Chen, H. M.; Xu, X. Q.; Yao, N.; Deng, C. H.; Yang, P. Y.; Zhang, X. M. *Proteomics* **2008**, 8, 2778.
5. Yao, N.; Chen, H. M.; Lin, H. Q.; Deng, C. H.; Zhang, X. M. *J. Chromatogr. A* **2008**, 1185, 93.
6. Thorek, D. L. J.; Chen, A.; Czupryna, J.; Tsourkas, A. *Ann. Biomed. Eng.* **2006**, 34, 23.
7. Chang, S. Y.; Zheng, N. Y.; Chen, C. S.; Chen, C. D.; Chen, Y. Y.; Wang, C. R. *J. Am. Soc. Mass Spectrom.* **2007**, 18, 910.
8. Hsiao, H. H.; Hsieh, H. Y.; Chou, C. C.; Lin, S. Y.; Wang, A. H. J.; Khoo, K. H. *J. Proteome Res.* **2007**, 6, 1313.
9. Shaw, S. Y.; Chen, Y. J.; Ou, J. J.; Ho, L. *Enzyme Microb. Technol.* **2006**, 39, 1089.
10. Yamaura, M.; Camilo, R. L.; Sampaio, L. C.; Macedo, M. A.; Nakamura, M.; Toma, H. E. *J. Magn. Magn. Mater.* **2004**, 279, 210.
11. Chen, C. T.; Chen, Y. C. *Anal. Chem.* **2005**, 77, 5912.
12. Lee, A.; Yang, H. J.; Lim, E. S.; Kim, J.; Kim, Y. *Rapid Commun Mass Spectrom.* **2008**, 22, 2561.