

Synthesis of $\text{Fe}_3\text{O}_4@\text{SiO}_2@\text{PMMA}$ Core–Shell–Shell Magnetic Microspheres for Highly Efficient Enrichment of Peptides and Proteins for MALDI-ToF MS Analysis**

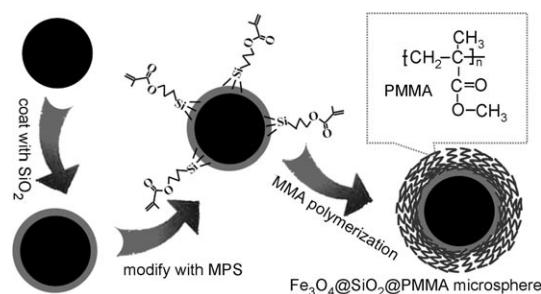
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Over the past decade, magnetic nanomaterials have received increasing attention because of their unique physical and chemical properties and their many potential applications in various fields such as drug delivery and cell separation.^[1] Recently, the application of magnetic nanomaterials to proteomics research has received much attention.^[2] Peptide mapping by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) along with database searching is a major and well-known tool in modern protein analysis.^[3] Although MALDI-ToF MS is highly sensitive to trace amounts of peptides or proteins, it seems to be insufficient for the detection of low-abundance peptides or proteins extracted from biological tissues, as these peptides/proteins are not only present at extremely low concentrations (< 1 nM), but their MS signals suffer strong interference from those of highly abundant proteins as well as contaminants introduced into the sample during pretreatment procedures. Magnetic nanomaterials were recently developed to address this issue. They are often used as affinity probes for the enrichment of low-abundance peptides or proteins, because their strong magnetic properties facilitate the isolation of nanomaterial–target peptide/protein complexes from the sample solution.^[4] For example, C_8 -modified magnetic nanoparticles have been applied to conveniently separate and enrich peptides for proteomics.^[4g,h] Magnetic silica nanospheres with a highly ordered periodic mesostructure have been synthesized for size-selective bioseparation of proteins.^[4k] Although much progress has been made in the application of magnetic nanomaterials to the enrichment of peptides and proteins, the design of novel functionalized magnetic nanomaterials with well-defined nanostructures and surface properties for application in proteomics remains an area of intense research interest.

Poly(methyl methacrylate) (PMMA) is a common synthetic organic polymer traditionally used in various industrial applications. Recent research has revealed that PMMA can

be used as a powerful absorbent for the enrichment of peptides and proteins.^[5] Therefore, the design and synthesis of magnetic nanomaterials with PMMA at the surface is of particular interest in projects requiring fast and convenient enrichment of peptides or proteins. Herein we report the facile synthesis of core–shell–shell composite $\text{Fe}_3\text{O}_4@\text{SiO}_2@\text{PMMA}$ microspheres by combining a sol-gel approach with a seeded aqueous-phase radical polymerization method, along with application of the microspheres to the enrichment of peptides and proteins for mass spectrometric analysis.

In the synthesis protocol (Scheme 1), according to a previously reported method,^[6] magnetite core–shell $\text{Fe}_3\text{O}_4@\text{SiO}_2$ microspheres were synthesized by a sol-gel



Scheme 1. Synthetic procedure for core–shell–shell $\text{Fe}_3\text{O}_4@\text{SiO}_2@\text{PMMA}$ microspheres.

process to coat a thin layer of dense amorphous silica on Fe_3O_4 particles.^[4h,i] The $\text{Fe}_3\text{O}_4@\text{SiO}_2$ microspheres were then modified with 3-methacryloxypropyltrimethoxysilane (MPS),^[7] a polymerizable silane coupling agent, and $\text{Fe}_3\text{O}_4@\text{SiO}_2$ -MPS microspheres thus obtained. An aqueous-phase radical polymerization of methyl methacrylate (MMA) was then carried out in the presence of $\text{Fe}_3\text{O}_4@\text{SiO}_2$ -MPS microspheres, resulting in $\text{Fe}_3\text{O}_4@\text{SiO}_2@\text{PMMA}$ microspheres with a well-defined core–shell structure (see the Supporting Information for synthesis details). Because the $\text{Fe}_3\text{O}_4@\text{SiO}_2@\text{PMMA}$ microspheres possess a strong magnetic core and an organic hydrophobic PMMA exterior, they hold great promise for rapid magnetic separation and high absorption of trace peptides or proteins in solution-phase systems. In fact, we found that $\text{Fe}_3\text{O}_4@\text{SiO}_2@\text{PMMA}$ microspheres exhibit some remarkable features that are useful for the extraction of peptides and proteins and thus their analysis by MALDI-ToF MS.

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Observation by transmission electron microscopy (TEM) shows that the magnetite particles obtained are nearly spherical in shape and have an average diameter of ca. 170 nm (Figure 1a). The selected area electronic diffraction

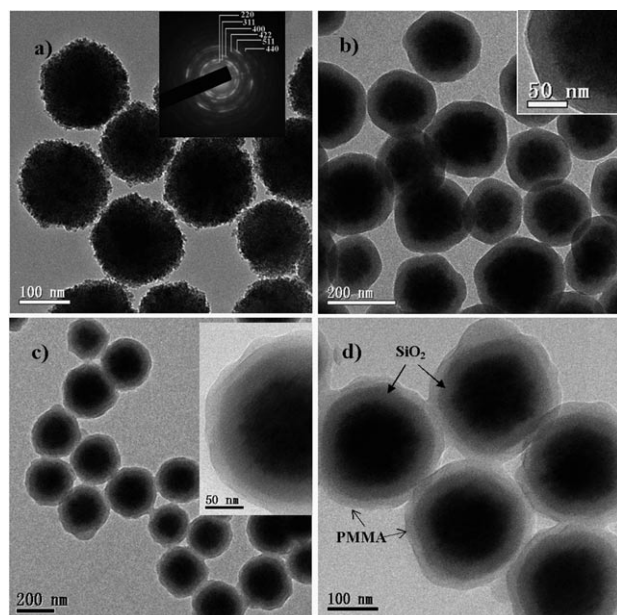


Figure 1. TEM images of: a) Fe_3O_4 particles, b) $\text{Fe}_3\text{O}_4@SiO_2$ microspheres, and c) d) $\text{Fe}_3\text{O}_4@SiO_2@PMMA$ microspheres.

(SAED) pattern (Figure 1a, inset) recorded on a single particle shows spotty diffraction rings that can be indexed precisely to magnetite with polycrystalline features. TEM images of higher magnification reveal that each magnetite particle is composed of many smaller magnetite nanoparticles of about 10 nm diameter (Figure S1, Supporting Information). A representative TEM image of the obtained $\text{Fe}_3\text{O}_4@SiO_2$ core-shell microspheres (Figure 1b) shows that the dark magnetite particles are individually coated with a uniform gray silica shell with a thickness of ca. 35 nm. Through a robust sol-gel approach, the thickness of the silica shell can be tuned from tens to hundreds of nanometers. TEM images of the obtained $\text{Fe}_3\text{O}_4@SiO_2@PMMA$ microspheres show that they are well dispersed and have a mean diameter of 270 nm (Figure 1c). High-magnification TEM images (Figure 1c [inset] and 1d) reveal a sandwich structure of the composite microspheres with a dark Fe_3O_4 core, a gray SiO_2 middle layer, and a light-gray PMMA shell about 20 nm in thickness. The clarity in observation of this unique structure is due to the distinct mass contrast between these three components.

Characterization by scanning electron microscopy (SEM) (Figure S2, Supporting Information) also indicates that the $\text{Fe}_3\text{O}_4@SiO_2$ microspheres possess a nearly spherical shape with a smooth surface, a diameter of 240 nm, and a size deviation of 12%. After further coating with PMMA, the obtained $\text{Fe}_3\text{O}_4@SiO_2@PMMA$ microspheres are slightly larger and more uniform in diameter and morphology than

$\text{Fe}_3\text{O}_4@SiO_2$ microspheres, in good agreement with the TEM results (Figure 1c,d).

The surface modification of $\text{Fe}_3\text{O}_4@SiO_2$ microspheres and further coating with PMMA were investigated by Fourier-transform infrared (FTIR) spectroscopy. In Figure 2a, the

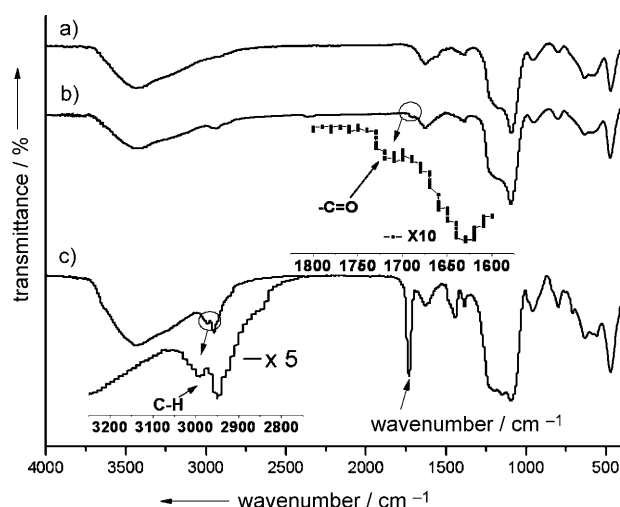


Figure 2. FTIR spectra of: a) $\text{Fe}_3\text{O}_4@SiO_2$ microspheres, b) MPS- $\text{Fe}_3\text{O}_4@SiO_2$ microspheres, and c) $\text{Fe}_3\text{O}_4@SiO_2@PMMA$ microspheres. The insets show enlargements of the selected IR absorption bands (circled regions).

absorption peaks for the $\text{Fe}_3\text{O}_4@SiO_2$ microspheres at 1090 cm^{-1} is assigned to the Si–O–Si vibration, whereas the peaks around 1630 and 3400 cm^{-1} are attributed to the absorbed water and hydroxy groups. After modification with MPS, new peaks are detected for the $\text{Fe}_3\text{O}_4@SiO_2$ -MPS microspheres at 1720 and 2940 cm^{-1} , which can be assigned to the ester C=O and C–H bonds from the silane MPS. As a result of the presence of large quantities of PMMA at the surface of the microspheres after coating with PMMA, the FTIR spectrum of $\text{Fe}_3\text{O}_4@SiO_2@PMMA$ microspheres shows strong peaks at 1730 and 2950 cm^{-1} , which are assigned to the ester C=O and C–H bonds of the repeating MMA units.

Notably, the introduction of a dense silica coating on the magnetite particles is necessary for this study, because it not only provides a silica-like surface for magnetite that makes surface modification with organic silane molecules easier and more effective, but also protects the magnetite cores in the $\text{Fe}_3\text{O}_4@SiO_2@PMMA$ microspheres from being etched in practical applications. Magnetic characterization was carried out by magnetometry at 300 K using a superconducting quantum interference device (SQUID). The magnetization values were measured to 49.5 and 36.7 emu g^{-1} for $\text{Fe}_3\text{O}_4@SiO_2$ and $\text{Fe}_3\text{O}_4@SiO_2@PMMA$ microspheres, respectively. Figure 3 shows the hysteresis loops of the two samples, and it is apparent that both show superparamagnetic properties due to the presence of nanometer-sized magnetite particles in the core. Similar to the $\text{Fe}_3\text{O}_4@SiO_2$ microspheres, the $\text{Fe}_3\text{O}_4@SiO_2@PMMA$ microspheres can be well dispersed in aqueous solution without visible sedimentation over 8 h, which is demonstrated by photographs of their solution in

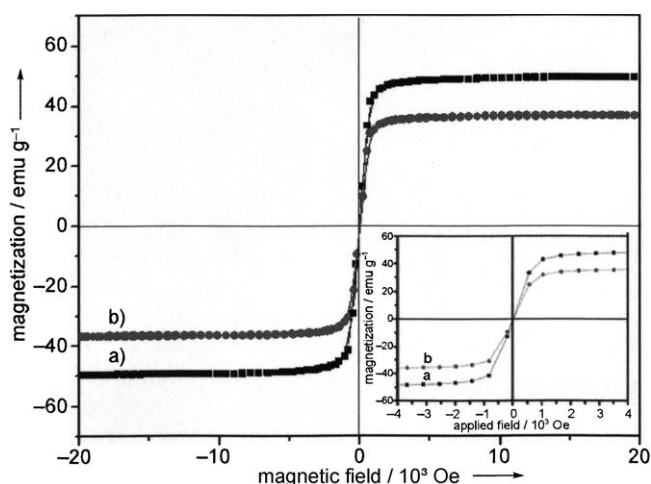


Figure 3. The hysteresis loops of: a) $\text{Fe}_3\text{O}_4@SiO_2$ microspheres and b) $\text{Fe}_3\text{O}_4@SiO_2@PMMA$ microspheres.

bulk (Figure S2 insets, Supporting Information). Such stable dispersal is due to the electrostatic repulsion between these negatively charged microspheres and is beneficial to their application in high-capacity adsorption. On the other hand, these microspheres can be readily separated from their dispersion (ca. 1 wt %) in 0.5 min with a magnetic field (1000 Oe) owing to their high magnetite content and thus good magnetic response. These unique properties ensure a rapid switch in the separation–redispersion process that can afford a fast and efficient enrichment or separation with the aid of an applied magnetic field.

To study the enrichment effect of $\text{Fe}_3\text{O}_4@SiO_2@PMMA$ microspheres on peptide analysis, a standard peptide, angiotensin II (DRVYIHPF, $M_r = 1046.2$ Da, isoelectric point (pI) = 6.74) and a standard protein, cytochrome *c* (0.5 mg L^{-1} , $M_r = 12384$ Da, pI = 9.59) were used as models. After incubation for 10 min with continuous agitation and separa-

tion for 0.5 min with the help of a magnet, followed by a three-cycle rinse with water, the microspheres with the captured peptides were mixed with a MALDI matrix (α -cyano-4-hydroxycinnamic acid, α -CHCA) solution and applied to the MALDI target directly. For comparison, the original peptide solutions without enrichment as well as after treatment with a commercial ZipTip C_{18} pipette tip were also analyzed by MALDI-ToF MS under the same experimental conditions, and the results are displayed in Figure 4 (see the Supporting Information for detailed procedures).

The main merit of this enrichment process is that the $\text{Fe}_3\text{O}_4@SiO_2@PMMA$ microspheres can effectively concentrate peptides or proteins; the adsorbed species can then be directly analyzed by MALDI-ToF MS without interference to the signals of the target molecules. At a concentration of 4 nM, angiotensin II without enrichment was barely detectable by MS, with a signal-to-noise (S/N) ratio of only 13.21. The S/N increased to 82.34 after treatment with a ZipTip C_{18} pipette tip. However, after enrichment with the PMMA-coated $\text{Fe}_3\text{O}_4@SiO_2$ microspheres, the peak intensity of angiotensin II became much higher, with an increase in S/N up to 798.67. After enrichment with $\text{Fe}_3\text{O}_4@SiO_2@PMMA$ micro-

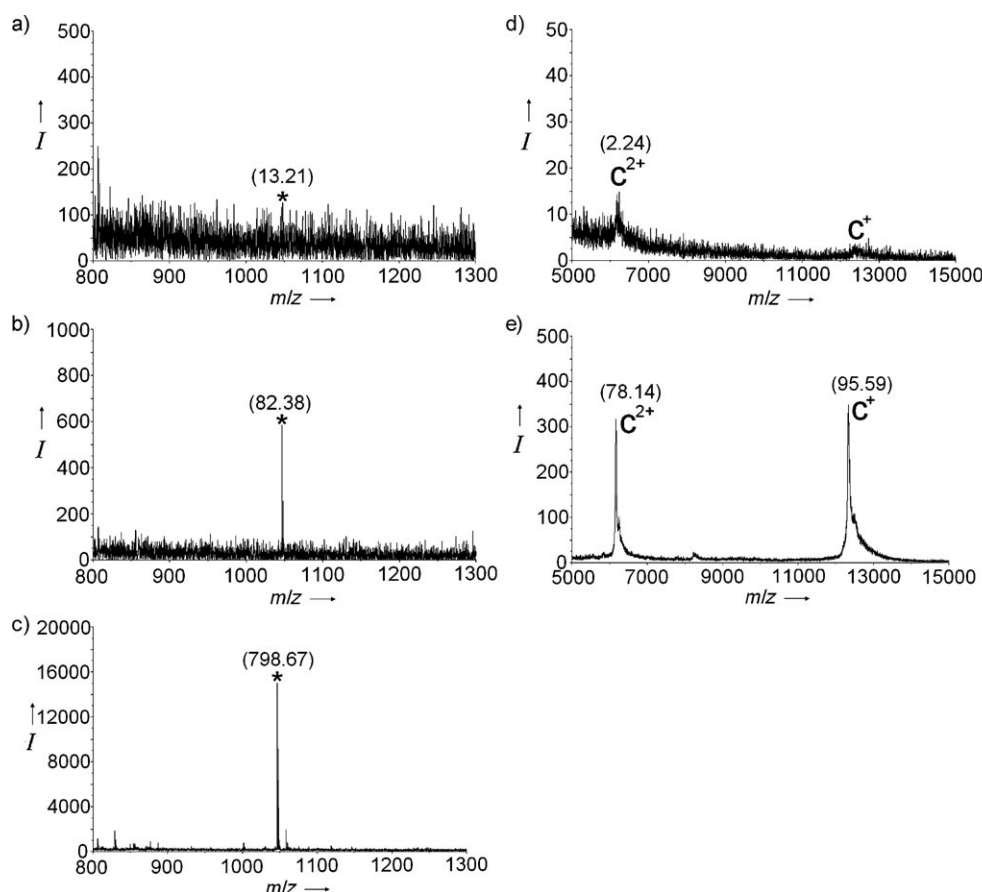


Figure 4. MALDI-ToF MS data for: a) angiotensin II (4 nM) without treatment, b) angiotensin II (4 nM) after treatment with ZipTip C_{18} , c) angiotensin II (4 nM) enriched with PMMA-coated $\text{Fe}_3\text{O}_4@SiO_2$ microspheres, d) cytochrome *c* (0.5 mg L^{-1}) without enrichment, and e) cytochrome *c* (0.5 mg L^{-1}) after enrichment with PMMA-coated $\text{Fe}_3\text{O}_4@SiO_2$ microspheres. The peaks marked with asterisks represent angiotensin II; c^+ and c^{2+} respectively correspond to the singly and doubly charged peaks of cytochrome *c*. The S/N ratios are indicated in parentheses. A solution volume of 0.4 mL was used for each sample. Analysis of peptides and proteins was performed in the reflector and linear ToF detection modes, respectively. I = absolute intensity.

spheres, the S/N of cytochrome *c* at a concentration of 0.5 mg L^{-1} increased to 95.59, which is much higher than that without enrichment. The remarkable increase in S/N after enrichment with $\text{Fe}_3\text{O}_4@\text{SiO}_2@\text{PMMA}$ microspheres followed by direct MALDI-ToF MS analysis can be attributed to the optimized structure of the microspheres. The PMMA polymer surface has linear hydrophobic chains, which endows the PMMA-modified microspheres with good hydrophobic properties. Because more than half of the naturally occurring amino acids have a hydrophobic aliphatic or aromatic group, and are widely represented in nearly all proteins and peptides, the PMMA-modified magnetic microspheres could be used as hydrophobic probes for peptides and proteins. Similarly, other biomolecules with hydrophobic groups could also be enriched by these microspheres. Like other typical polymer microspheres,^[8] the PMMA beads are porous. For target molecules that are trapped deep inside the particles, their detection by MALDI-ToF MS would be very difficult, and thus the intensity and S/N for samples enriched by pure PMMA would be low.^[5] However, in the case of the core-shell particles, the $\text{Fe}_3\text{O}_4@\text{SiO}_2$ particle core serves to block the target molecules, effectively constraining them to the thin outer layers of the microsphere. Hence, samples treated with the core-shell particles exhibit high-intensity signals in MS analysis.

The second merit of the enrichment process reported herein is fast isolation without reiterative centrifugation. The cores of $\text{Fe}_3\text{O}_4@\text{SiO}_2@\text{PMMA}$ microspheres endows them with powerful magnetic properties. After target molecule adsorption, the target-microsphere complexes can be easily separated from the mixture, by simple application of a magnetic field, within 0.5 min prior to redispersion in water for rinsing or application to the MALDI plate. This makes isolation of the trapped peptides very convenient and rapid, saving significant amounts of time dedicated to experimental protocols.

The third merit of this $\text{Fe}_3\text{O}_4@\text{SiO}_2@\text{PMMA}$ microsphere-mediated peptide enrichment process is that the co-concentration of salts can be avoided. Peptides and proteins from real proteome samples typically contain salts, which can disrupt the co-crystallization of analyte and matrix molecules; this severely compromises the MALDI process and results in poor MS data.^[9] Therefore, a desalting step is required after conventional protein concentration processes in order to obtain a satisfactory analysis. Herein, the desalting effect of $\text{Fe}_3\text{O}_4@\text{SiO}_2@\text{PMMA}$ microspheres was investigated as well. Figure S3 in the Supporting Information shows that only three peptides could be detected in a BSA digest solution at a concentration of 2 nM, and their signals disappeared when the solution contained 100 mM urea. After the BSA digest (2 nM, 0.4 mL, containing 100 mM urea) was desalted by a commercial ZipTipC₁₈ pipette tip, five peptides were observed with low S/N values. However, after enrichment with $\text{Fe}_3\text{O}_4@\text{SiO}_2@\text{PMMA}$ microspheres, 15 peptides with a sequence coverage of 24% for 2 nM BSA digests and 14 peptides with a sequence coverage of 22% for 2 nM BSA digests with 100 mM urea could be assigned (Table S1, Supporting Information), indicating effective enrichment was possible for both samples regardless of contamination

by urea. The good enrichment and desalting properties of $\text{Fe}_3\text{O}_4@\text{SiO}_2@\text{PMMA}$ microspheres could be attributed to the nonpolar PMMA polymer, which exhibits strong hydrophobic interactions with peptides and weak interactions with hydrophilic molecules such as salts. Thus these PMMA-coated $\text{Fe}_3\text{O}_4@\text{SiO}_2$ microspheres can effectively enrich peptides at trace quantities without concomitant concentration of salts.

The proposed enrichment process has been proven effective in a real proteomic analysis. Proteins extracted from human eye lens were selected, and after 2D electrophoretic separation (Figure S4, Supporting Information), one protein spot with relatively weak intensity was digested in-gel and analyzed. No significant protein (protein scores > 64 are significant) could be identified when the solution was analyzed directly or after treatment with a ZipTipC₁₈ pipette tip. However, after enrichment with PMMA-coated $\text{Fe}_3\text{O}_4@\text{SiO}_2$ microspheres, a protein with a score of 142 was identified (Figure S5 and Table S2, Supporting Information), thereby showing the excellent potential of this method for proteomic applications.

Considering the interactions between organic polymer and target biomolecules such as hydrophobic-hydrophobic and hydrophilic-hydrophilic interactions, we believe that other functional polymers as the shell of magnetic core-shell microspheres can interact with specific biomolecules. For example, poly(acrylic acid) (PAA), which contains numerous hydrophilic carboxylate groups, should have the ability to capture hydrophilic biomolecules such as glycoproteins or glycopeptides. Further studies on the synthesis and related applications of core-shell magnetic microspheres with other polymer shells are ongoing in our research group.

In summary, core-shell-shell $\text{Fe}_3\text{O}_4@\text{SiO}_2@\text{PMMA}$ composite microspheres were readily synthesized by combining a sol-gel approach and a seeded aqueous-phase radical polymerization method. The synthesized $\text{Fe}_3\text{O}_4@\text{SiO}_2@\text{PMMA}$ microspheres have magnetite cores, organic hydrophobic PMMA exteriors, and good dispersal qualities. These microspheres can enrich low-concentration peptides and proteins effectively, rapidly, and conveniently. Furthermore, this work can be extended to the design of core-shell magnetic microspheres with other polymers as coatings for bioseparation applications.

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- [1] a) A. Lu, E. L. Salabas, F. Schüth, *Angew. Chem.* **2007**, *119*, 1242; *Angew. Chem. Int. Ed.* **2007**, *46*, 1222; b) Y. Deng, C. Deng, D. Yang, C. Wang, S. Fu, X. Zhang, *Chem. Commun.* **2005**, 5548; c) P. Nandigala, T. Chen, C. Yang, W. Hsu, C. Heath, *Biotechnol. Prog.* **1997**, *13*, 844; d) J. Liu, Z. Sun, Y. Deng, Y. Zou, C. Li, X. Guo, L. Xiong, Y. Gao, F. Li, D. Zhao, *Angew. Chem.* **2009**, *121*, 5989; *Angew. Chem. Int. Ed.* **2009**, *48*, 5875; e) Y. Deng, C. Wang, X. Shen, W. Yang, L. Jin, H. Gao, S. Fu, *Chem. Eur. J.* **2005**, *11*, 6006; f) Y. Deng, W. Yang, C. Wang, S. Fu, *Adv. Mater.* **2003**, *15*, 1729;

- g) Y. Deng, D. Qi, C. Deng, X. Zhang, D. Zhao, *J. Am. Chem. Soc.* **2008**, *130*, 28; h) J. Ge, Y. Hu, M. Biasini, W. Beyermann, Y. Yin, *Angew. Chem.* **2007**, *119*, 4420; *Angew. Chem. Int. Ed.* **2007**, *46*, 4342; i) L. Li, Y. Feng, Y. Li, W. Zhao, J. Shi, *Angew. Chem.* **2009**, *121*, 6002; *Angew. Chem. Int. Ed.* **2009**, *48*, 5888; j) L. Zhang, S. Qiao, Y. Jin, Z. Chen, H. Gu, G. Lu, *Adv. Mater.* **2008**, *20*, 805.
- [2] a) J. Villanueva, J. Philip, D. Entenberg, C. Chaparro, M. Tanwar, E. Holland, P. Tempst, *Anal. Chem.* **2004**, *76*, 1560; b) C. Johnson, N. Zhukovsky, A. Cass, J. Nagy, *Proteomics* **2008**, *8*, 715; c) S. Ficarro, G. Adelmant, M. Tomar, Y. Zhang, V. Cheng, J. Marto, *Anal. Chem.* **2009**, *81*, 4566; d) K. Wang, S. Chuang, P. Lin, L. Huang, S. Chen, S. Ouarda, W. Pan, P. Lee, C. Lin, Y. Chen, *Anal. Chem.* **2008**, *80*, 6159; e) J. Bao, W. Chen, T. Liu, Y. Zhu, P. Jin, L. Wang, J. Liu, Y. Wei, Y. Li, *ACS Nano* **2007**, *1*, 293; f) Y. Deng, C. Deng, D. Qi, C. Liu, J. Liu, X. Zhang, D. Zhao, *Adv. Mater.* **2009**, *21*, 1377.
- [3] a) S. Fields, *Science* **2001**, *291*, 1221; b) A. Abbott, *Nature* **2001**, *409*, 747; c) M. Mann, P. Hojrup, P. Roepstorff, *Biol. Mass Spectrom.* **1993**, *22*, 338; d) J. Yates, S. Speicher, P. Griffin, T. Hunkapiller, *Anal. Biochem.* **1993**, *214*, 397.
- [4] a) C. Lo, W. Chen, C. Chen, Y. Chen, *J. Proteome Res.* **2007**, *6*, 887; b) Y. Li, T. Leng, H. Lin, C. Deng, X. Xu, N. Yao, P. Yang, X. Zhang, *J. Proteome Res.* **2007**, *6*, 4498; c) X. Xu, C. Deng, P. Yang, X. Zhang, *J. Proteome Res.* **2007**, *6*, 3849; d) Y. Li, X. Xu, B. Yan, C. Deng, W. Yu, P. Yang, X. Zhang, *J. Proteome Res.* **2007**, *6*, 2367; e) Y. Li, D. Qi, H. Lin, C. Deng, X. Xu, P. Yang, X. Zhang, *Chem. Commun.* **2008**, 564; f) G. Yao, D. Qi, C. Deng, X. Zhang, *J. Chromatogr. A* **2008**, *1215*, 82; g) H. Chen, C. Deng, X. Zhang, *Adv. Mater.* **2009**, *21*, 2200; h) H. Chen, X. Xu, N. Yao, C. Deng, P. Yang, X. Zhang, *Proteomics* **2008**, *8*, 2778; i) X. Xu, C. Deng, M. Gao, W. Yu, P. Yang, X. Zhang, *Adv. Mater.* **2006**, *18*, 3289; j) Y. Li, B. Yan, C. Deng, X. Zhang, *Proteomics* **2007**, *7*, 2330; k) L. Zhang, S. Qiao, Y. Jin, H. Yang, S. Budihartono, F. Stahr, Z. Yan, X. Wang, Z. Hao, G. Lu, *Adv. Funct. Mater.* **2008**, *18*, 3203.
- [5] a) W. Jia, X. Chen, H. Lu, P. Yang, *Angew. Chem.* **2006**, *118*, 3423; *Angew. Chem. Int. Ed.* **2006**, *45*, 3345; b) W. Shen, H. Xiong, Y. Xu, S. Cai, H. Lu, P. Yang, *Anal. Chem.* **2008**, *80*, 6758; c) H. Xiong, X. Guan, L. Jin, W. Shen, H. Lu, Y. Xia, *Angew. Chem.* **2008**, *120*, 4272; *Angew. Chem. Int. Ed.* **2008**, *47*, 4204.
- [6] H. Deng, X. Li, Q. Peng, X. Wang, J. Chen, Y. Li, *Angew. Chem.* **2005**, *117*, 2842; *Angew. Chem. Int. Ed.* **2005**, *44*, 2782.
- [7] J. Ge, Y. Hu, T. Zhang, Y. Yin, *J. Am. Chem. Soc.* **2007**, *129*, 8974.
- [8] a) X. Gao, S. Nie, *Anal. Chem.* **2004**, *76*, 2406; b) N. Gaponik, I. Radtchenko, G. Sukhorukov, H. Weller, A. Rogach, *Adv. Mater.* **2002**, *14*, 879; c) M. Kuang, D. Wang, H. Bao, M. Gao, H. Möhwald, M. Jiang, *Adv. Mater.* **2005**, *17*, 267.
- [9] a) N. Zhang, A. Doucette, L. Li, *Anal. Chem.* **2001**, *73*, 2968; b) A. Brockman, B. Dodd, R. Orlando, *Anal. Chem.* **1997**, *69*, 4716; c) M. Weng, Y. Chen, *Rapid Commun. Mass Spectrom.* **2004**, *18*, 1421.