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ZnO−Poly(methyl methacrylate) Nanobeads for Enriching and Desalting Low-Abundant Proteins Followed by Directly MALDI-TOF MS Analysis

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A fast solid-phase microextraction method using core−shell ZnO−poly (methyl methacrylate) nanobeads (ZnO−PMMA) as adsorbent was established. This fast method with high enriching efficiency and salt tolerance capability depends on the structure of the core−shell nanobeads. First, the large surface area of the PMMA shell makes the dispersive nanobeads capture samples quickly, by virtue of multi-interactions between ZnO−PMMA and samples except for the interaction with salts. Second, the small nanosize of the ZnO-core (2.1 nm) and the flexible hydrophobic PMMA shell, which can prevent the cores from aggregation, make the nanobeads form a homogeneous layer on the matrix-assisted laser desorption/ionization (MALDI) plate and do not hinder the cocrystallization of the matrix and samples. Third, the ZnO core also prevents PMMA from fragmentation and ionization in mass spectrometer. In this article, ~80% bovine serum albumin digests were enriched by ZnO−PMMA from 100 amol/μL solution within 10-min incubation, and the solid phase can be directly analyzed by MALDI mass spectrometry. Mass intensity can be increased 5−10-fold (ZnO−PMMA enrichment vs lyophilization). High-quality mass spectra can be obtained, even with the presence of saturated NaCl (6.2 M), saturated NH_4HCO_3 (2.6 M), or 1 M urea. This method has been successfully applied to human colorectal cancer proteome research, and eight new proteins have been found.

Extensive proteome analysis has been developed and has identified numerous proteins in various cancer tissues in the past few years. By finding out the potential candidates of biomarkers in cancer tissues, clinical proteomics with the aim of solving a specific clinical problem represents a promising tool directed to the improvement of tumor diagnosis.[1,2] In order to get high sensitivity, good accuracy, high throughput, and rapid data interpretation as well as quantitation for clinical proteome research, mass spectrometry (MS) is chosen as the main analysis method.[3] However, identification of low-abundant proteins is still a challenge for two reasons: a large percentage of sample would be lost during concentration before MS analysis; MS signal suppression would be caused by inorganic salts, chaotropes, and detergents, which come from sample pretreatment. Lyophilization is normally used to concentrate the diluted samples; however, the inevitable sample loss and coconcentration with the contaminants hinder the identification of low-abundant proteins. Therefore, sample enriching and desalting before MS analysis is vital for identification of low-abundant proteins. In order to get better enriching efficiency, simplify the enriching and desalting process, on-plate desalting and peptide enrichment protocol is one of the strategies worldwide. A number of polymer membranes, such as PVDF,[4] polyacrylamide,[5] silicone polymer,[6] poly(methyl methacrylate) (PMMA)-C_{60}, and PSt-C_{60}[7] have been used. However, the sample volume of on-plate protocol is limited, which is not restricted by in-microtube desalting and enriching protocol. Solid-phase microextraction in-microtube is the other normally used enriching and desalting method. Many materials are chosen as adsorbents, such as agarose beads,[8] C_4 or C_{18} particulate,[9] polymeric microbeads,[10] immobilized magnetic beads,[11,12] carbon nanotubes,[13,14] and others. In our previous work, nanozeolite particles[15] and $CaCO_3$−PMMA, which has a core of $CaCO_3$ and a

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shell of PMMA bonded onto $CaCO_3$ core,[16] were used as adsorbents to enrich and desalt trace peptides/proteins.

Herein, we report a new method based on another improved core−shell ZnO−PMMA nanobead. PMMA shell was polymerizated by virtue of the inherent free radicals pre-existing on ZnO nanoparticle surfaces. This kind of shells can make the cores stable because the polymer shell is so dense that ions or molecules cannot penetrate to destroy the cores.[17] The core−shell ZnO−PMMA nanobeads produced by an improved synthesis are more stable and dispersive in solution.[18] The PMMA shell has a strong enriching efficiency for protein/peptide. The ZnO core with the diameter of 2.1 nm can be directly spotted on a matrix-assisted laser desorption/ionization (MALDI) plate and form a homogeneous layer so as to avoid an eluting step and reduce sample loss. This enrichment method could make the limit down to 100 amol/μL bovine serum albumin (BSA) digests. However, the limit was ~1 fmol/μL in our previous methods reported.[15,16] Meanwhile, the method has a strong decontaminating capability due to the weak interaction between PMMA and inorganic salts/chaotropes/detergents; thus, proteins/peptides can be easily enriched from the highly contaminated sample solution. In the previous desalting methods reported, tolerance of salt concentration in a low-abundant sample system was ~1 M.[7,16] With this novel method, even with the presence of saturated NaCl (6.2 M), saturated NH_4HCO_3 (2.6 M), or 1 M urea, high-quality mass spectra of digested BSA (1 fmol/μL) can be obtained, but no peaks could be detected when the same samples were enriched by lyophilization. By virtue of low limits of detection, rapid enrichment, good reproducibility, high recovery, and powerful desalting ability of this method, we have applied it to proteome research on human colorectal cancer (CRC). CRC is the third most prevalent cancer in the world; however, few biomarkers have been detected and used because of their low abundance and high salt interference.[19−23] We have identified eight unreported proteins from the CRC proteome by this new method.

EXPERIMENTAL SECTION

Nanobeads Preparation. ZnO−PMMA core−shell nanobeads were prepared through polymerization of methyl methacrylate initiated by the inherent free radicals on the ZnO surface, and pure PMMA nanobeads were synthesized under the same conditions, according to our previous research.[18]

Sample Preparation. Colorectal cancer tissues were collected from freshly isolated surgical resections in the operating room of the Affiliated Tumor Hospital in Fudan University. A lysis buffer [8 M urea (Sigma), 4% CHAPS (Sigma), and Protease Inhibitor Cocktail (Sigma)] was added in order to extract the total proteins. The sample was purified by a Clean-up Kit (GE Healthcare) before separation. Protein concentration was determined using the Bradford method.

For two-dimensional electrophoresis (2-DE) sample preparation, 200 μg of proteins from CRC were first separated by 18-cm nonlinear pH 3−10 IPG strips (GE Healthcare). Then the strip was reduced, alkylated, and embedded on the top of a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel. After the electrophoresis separation, the 2-DE gel was visualized by a silver staining method. The stained gel was scanned using the ImageScanner and analyzed with ImageMaster 2-D software (GE Healthcare).

Proteins in solution and in gel were all digested by trypsin (sequencing level, freshly diluted in 25 mM NH_4HCO_3; Roche) at 37 °C overnight. Before in-gel digestion, the excised spots should be treated according to the previous work.[7] In short, the destained and dried gel was incubated with trypsin at 4 °C for 15 min and covered with 30 μL of 25 mM NH_4HCO_3. After in-gel digestion, peptides were extracted sequentially.

All of the steps above were followed by the standard procedures.[7]

Enrichment of Peptides and Proteins. A 0.5-μL aliquot of ZnO−PMMA suspension (2.5 μg/μL for peptides and 10 μg/μL for proteins) was added into the solution of peptides or proteins. This solution was then incubated for 10 min with vortexing at 37 °C and then centrifugated at 16 000 rpm for 20 min. After the supernate was decanted, the deposit was resuspended in 0.8 μL of matrix (5 mg/mL α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid; Sigma) and spotted on the MALDI plate.

MALDI-TOF Mass Spectrometry Analysis and Data Analysis. All mass spectra were acquired by a 4700 Proteomics Analyzer MALDI-TOF/TOF-MS (Applied Biosystems, Framingham, MA). The instrument was operated at an accelerating voltage of 20 kV. A 200-Hz pulsed ND:YAG laser (355 nm) was used for MALDI. The MS instrument was calibrated by trypsin-digested peptides of horse heart myoglobin (MYO, Sigma) with known molecular masses. Peptides were analyzed in reflector TOF detection mode. Intact proteins were analyzed in linear TOF detection modes. All spectra were taken from the signal average of 2000 laser shots. The laser intensity was kept constant. The five strongest peaks in each mass spectrum were automatically selected for MS/MS analysis. GPS Explorer software (version 3.6, Applied Biosystems) with Mascot (version 2.1, Matrix Science, London, UK) as a search engine was used to identify proteins against the IPI_Human_3.07 database for CRC. All proteins were identified using the peptide fingerprint mass spectra combined with tandem mass spectra: The searching parameters were set up as follows: the enzyme was trypsin, the number of missed cleavages was allowed up to 1, the variable modification was oxidation of methionine, the peptide mass tolerance was 150 ppm, and the tandem mass tolerance was 0.5 Da. For the IPI_Human_3.07 database, protein scores greater than 59 were considered significant (probability $p < 0.05$).

RESULTS AND DISCUSSION

Performances of the ZnO−PMMA Enriching and Desalting Method. Diluted sample should be concentrated before being analyzed by mass spectrometry in order to obtain spectra of good

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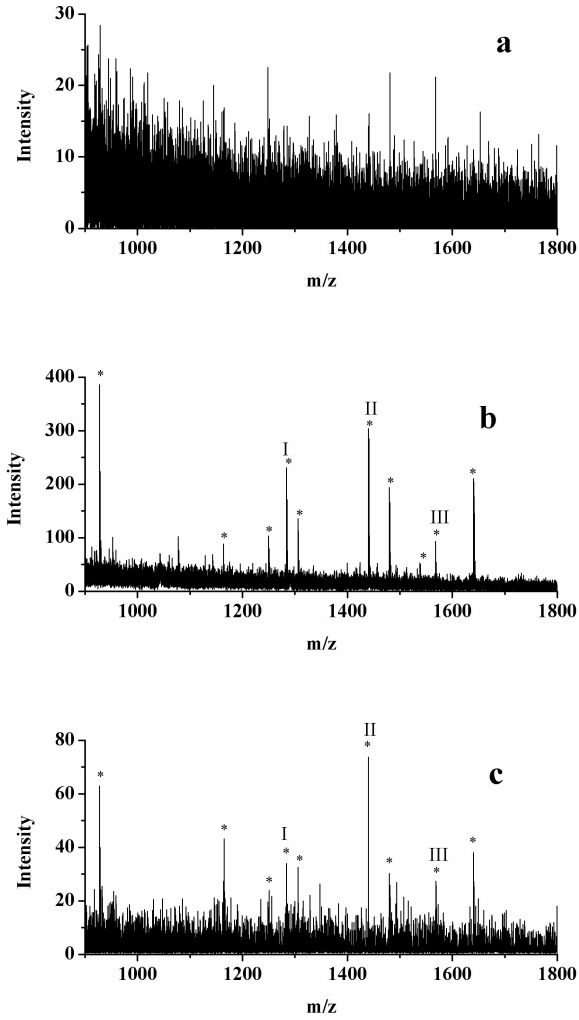

Figure 1. MALDI-TOF mass spectra of 100 amol/μL BSA digests (a) directly analyzed without any enrichment, (b) enriched by ZnO-PMMA nanobeads, and (c) lyophilized. MALDI-TOF MS experiments were performed on a 4700 Proteomics Analyzer (Applied Biosystems), with the Nd:YAG laser at 355 nm, a repetition rate of 200 Hz, and an acceleration voltage of 20 kV. Analysis of peptides was performed in the reflector TOF detection mode. All spectra were taken from a signal average of 2000 laser shots. Asterisks mark peaks assigned to peptides from BSA. Peptide I: amino acid sequence HPEYAVSVLLR; *m/z* 1283.4; PI 6.75. Peptide II: amino acid sequence RHPEYAVSVLLR; *m/z* 1439.6; PI 8.75. Peptide III: amino acid sequence DAFLGSFLYEYSR; *m/z* 1567.7; PI 4.37.

quality; therefore, concentration with high efficiency is the crucial step in sample pretreatment. Figure 1 shows diluted trypsin-digested BSA (100 amol/μL) is enriched by the ZnO−PMMA nanobeads or lyophilized from 200 μL. The 0.8-μL matrix was used to redissolve the deposit either from ZnO−PMMA enriching or from lyophilization and then spotted on the MALDI plate for analysis. The 1-μL BSA digests with a concentration of 100 amol/μL are scarcely detected without enriching, as shown in Figure 1a. After being enriched by the ZnO−PMMA nanobeads, the peptide signals become much stronger in the mass spectrum (Figure 1b). Therefore it illuminated that the new method had a high enriching efficiency. Furthermore, it also indicated that this new enriching method had a general enriching efficiency based on these three peptides, which were peptides I, II, and III with the neutral, alkaline, and acid isoelectric points (PI), respectively (marked in Figure 1b and c). Even compared with the lyophilization method, the new enriching method had an obvious better enriching efficiency (Figure 1c). The absolute intensity can be increased 5−10-fold (ZnO−PMMA enrichment vs lyophilization). Furthermore, for the purpose of calculating the enriching factor of the new method, 1-μL samples of BSA digests with concentrations ranging from 12 to 28 fmol/μL were directly loaded on the MALDI plate. The typical peptide I of the BSA digest, with the nearly neutral isoelectric point (PI) of 6.75 and middle m/z of 1283.4 Da, was used as the test peptide. From the curve of absolute intensity to concentration made by undiluted peptide I and the average intensity of the enriched peptide I, more than 80% of peptide I could be enriched from the 100 amol/μL solution (Supporting Information, Figure S-1). This good enriching efficiency of the nanobeads could be due to the multi-interactions between the ZnO−PMMA nanobeads and peptides, which mainly include hydrogen bonding between the carbonyl of PMMA and the amide nitrogen of peptide/protein and dipole−dipole interactions owing to the PMMA shell. Good reproducibility could be due to the flexible hydrophobic PMMA shell, which can prevent the nanobeads from aggregation on the MALDI plate. Our previously reported nanozeolites[15] were also directly spotted on the MALDI plate after enriching in order to prevent sample loss; however, they would aggregate on the MALDI plate and the inhomogeneity of solid particles can cause relatively unstable MS signals.

The adsorption capacity of ZnO−PMMA nanobeads for peptides was also studied. The 200-μL aliquot of 0.1−1 fmol/μL digested BSA was enriched by 0.25 μg of ZnO−PMMA nanobeads, followed by MS analysis. The MS intensity of peptide I (as mentioned above) increased from 206 to 501 when the concentration of BSA digests increased from 100 to 600 amol/μL. However, after the concentration increased over 600 amol/μL, the intensities of peptide I were kept around 500. Therefore, 0.25 μg of nanobeads reached adsorption saturation when being added into 120 fmol of digested BSA. Considering the 80% enriching efficiency of the nanobeads calculated above, we can estimate that their adsorption capacity for peptides is 26.3 mg/g.

The core−shell ZnO−PMMA enriching method has a fast and simple process. In order to determine the incubation time, 1.25 μg of nanobeads was used to incubate 1 fmol/μL digested BSA in 200 μL of solution, followed by a 20-min high-speed centrifugation; then all the prepared samples were detected by MS. From 30-s to 10-min incubation, the MS signals were increased; after 10 min, the MS signals were not further increased. This fast incubation depends on the structure of the core−shell nanobeads. In solution, the shells are spread due to the flexibility of PMMA chains, with the result that the large surface area of the shells makes the dispersive nanobeads capture the sample quickly and shortens the incubation time. In contrast, it is time-consuming for a 90-min incubation step to use our previously reported nanozeolites[15] because of the relatively low efficiency for protein/peptide enriching. Subsequently, another adsorbent, $CaCO_3$−PMMA, with a fast incubation step was reported.[16] Since the $CaCO_3$ core with an average diameter of 70 nm can be destroyed in acidic acetonitrile solution, after centrifugation, the supernate composed of peptides/proteins together with PMMA are mixed with MALDI matrix and can form a homogeneous sample layer

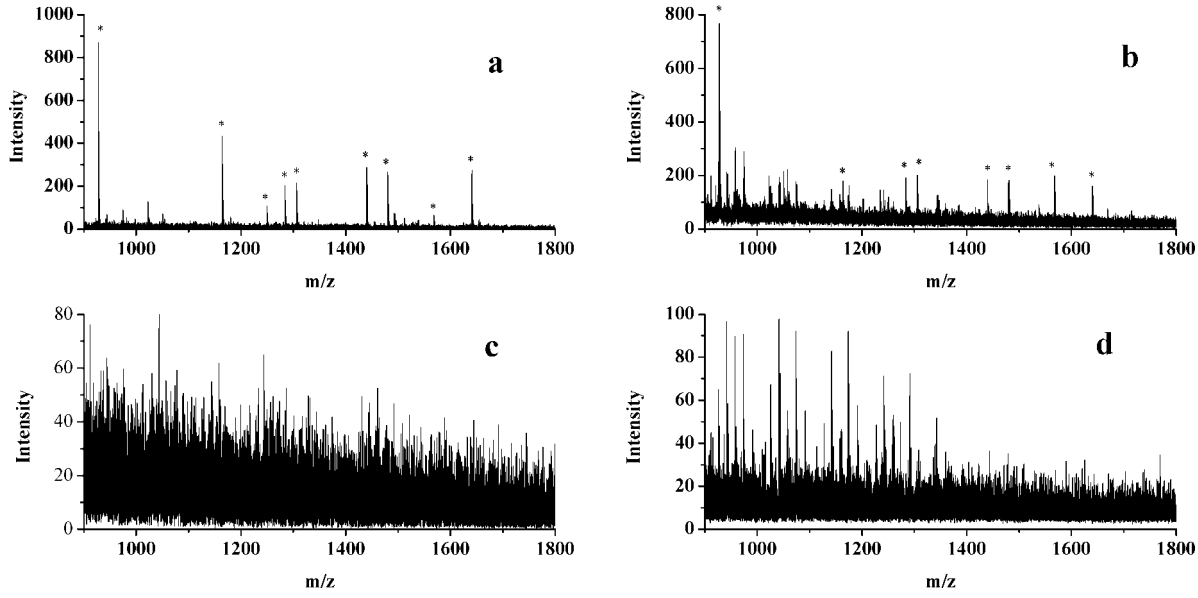

Figure 2. MALDI-TOF mass spectra of (a) 20 fmol of BSA digest and 1.25 μg of ZnO−PMMA, (b) 20 fmol of BSA digest and 1.25 μg of PMMA, (c) 1.25 μg of ZnO−PMMA, and (d) 1.25 μg of PMMA. Asterisks mark peaks assigned to peptides from BSA. MS parameters were the same as Figure 1.

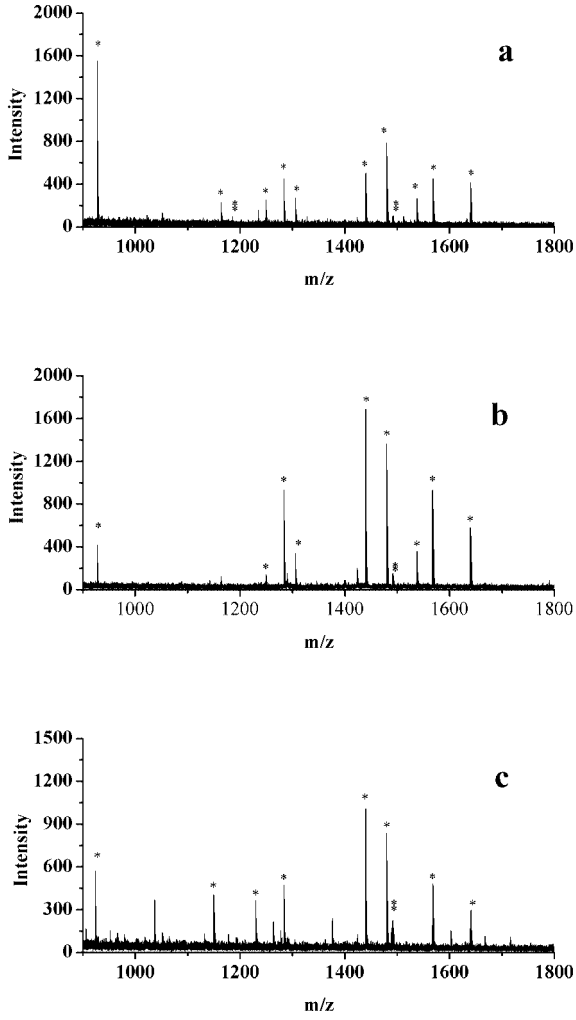

Figure 3. MALDI-TOF mass spectra of BSA digest (1 fmol/μL) enriched by ZnO−PMMA in the presence of (a) saturated NaCl (6.2 M), (b) saturated NH_4HCO_3 (2.6 M), and (c) 1 M urea. Single asterisks mark peaks assigned to peptides from BSA with H^+ and double asterisks with Na^+. MS parameters were the same as in Figure 1.

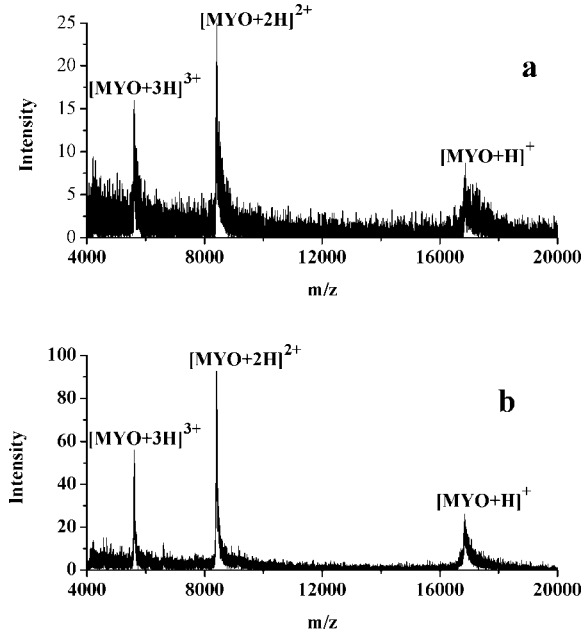

Figure 4. MALDI-TOF mass spectra of (a) 1 μL of 400 fmol/μL intact MYO directly loaded on MALDI plate and (b) 50 fmol/μL intact MYO enriched by ZnO−PMMA nanobeads from 200 μL of solution. Analysis of proteins was performed in the linear TOF detection mode. The other parameters were the same as in Figure 1.

on the MALDI plate. However, this particle-free MALDI plate method has additional dissolution and centrifugation steps. Herein, there is no need to destroy the core−shell structure before MS analysis, because ZnO (2.1 nm) cores are much smaller than that of PMMA−$CaCO_3$, the nanobeads can form a homogeneous layer on the MALDI plate and do not hinder the cocrystallization of the matrix and the peptides, as proven in Figure 2. MALDI-TOF mass spectrum of digested BSA (20 fmol) mixed with ZnO−PMMA (1.25 μg) is displayed in Figure 2a. From this spectrum (combined with Figure S-1, Supporting Information) we can conclude that MS signals of the digests are not suppressed in the presence of the nanobeads. In addition, it is not advisable to use pure PMMA

Table 1. Selected List of Identified Proteins from Human Colorectal Cancer[a]

spot no.	protein description	gene name	IPI_Human accession no.	MW (Da)/PI	protein score	no. of matched peptides	sequence coverage (%)
		Regulation of Transcription					
1	hepatocarcinoma high expression protein		IPI00477874	28148/6.08	62	6	26
		Energy and Metabolism					
2	cytochrome P450 4F12	CYP4F12	IPI00373933	60744/8.49	63	10	16
		Cellular Organization/Cytoskeleton					
3	VCP protein	VCP	IPI00478540	89340/5.17	72	4	5
4	filamin-A-interacting protein 1	FILIP1	IPI00297210	138024/8.46	84	28	24
5	LIM domain containing preferred translocation partner in lipoma	LPP	IPI00023704	65704/7.18	72	9	12
6	transmembrane protein 2	TMEM2	IPI00170706	154276/8.41	64	10	9
		Cellular Communication and Signal Transduction					
7	syndecan-3	SDC3	IPI00414286	56307/4.66	77	12	20
		Protein Synthesis and folding					
8	150 kDa oxygen-regulated protein precursor	RP1	IPI00000877	111266/5.16	192	30	22
9	endoplasmic reticulum protein ERp29 precursor	ERP29	IPI00024911	28975/6.77	166	7	27
		Transport/Cargo Protein					
10	early endosome antigen 1	EEA1	IPI00329536	162367/5.53	79	33	18

[a] The proteins were extracted from human colorectal cancer. Search parameters, IPI_Human_3.07 database; enzyme used, trypsin; maximum of missed cleavages, 1; peptide mass tolerance, 150 ppm; tandem mass tolerance, 0.5 Da; variable modification, oxidation of methionine. Every extraction solution of protein in gel digests was enriched by the ZnO−PMMA nanobeads. GPS Explorer software from Applied Biosystems with Mascot as a search engine was used to identify proteins. All proteins were identified by using the peptide fingerprint mass spectra combined with tandem mass spectra. Protein scores greater than 59 are significant (probability $p < 0.05$).

nanobeads as the adsorbent. Pure PMMA, without the strong covalent bond with ZnO, is unstable in the MALDI source and can make the spectra complicated (shown in Figure 2). In the presence of pure PMMA, some fragments from the polymer together with BSA digests are detected, and signals of BSA digests are suppressed (Figure 2b). The same amount of PMMA or ZnO−PMMA without BSA digests is analyzed by MS (Figure 2c and d, respectively). Results indicate that the ZnO core can prevent the PMMA shell from fragmentation and ionization in MALDI source.

Furthermore, the ZnO−PMMA nanobeads enriching method has a high salt-tolerance capability. Figure 3 displays the MALDI-TOF mass spectra of BSA digests (1 fmol/μL), which are enriched from a series of 200-μL solutions with high concentrations of different type salts. Samples in the presence of salts would hardly obtain quality mass spectra. However, using this new enriching method, mass spectra of high quality can be obtained even in the presence of 6.2 M NaCl (saturated), 2.6 M NH_4HCO_3 (saturated), and 1 M urea (shown in Figure 3a−c). By virtue of the weak interaction between PMMA shells and contaminants, such as inorganic salts and chaotropes, these contaminants can be easily separated from the samples and decanted with the supernate after centrifugation. Our previous work[16] indicated that another widely utilized technique, Zip-Tip C_{18}, had a relatively low efficiency for enriching and desalting low-abundant samples compared with $CaCO_3$−PMMA, because of its low loading capacity of less than 10 μL of solution and monointeraction between samples and C_{18}. Moreover, samples with large volume and salt of a high concentration, especially with saturated salt, are difficult to be treated with Zip-Tip C_{18} and the repeated wash and elution steps in the Zip-Tip C_{18} protocol would also make the sample loss inevitable. Therefore, this novel method based on ZnO−PMMA nanobeads might be widely utilized for enriching and desalting low-abundant proteins/peptides in the future.

Enriching intact proteins is also a good performance of the core−shell ZnO−PMMA nanobeads. The MS signal of 1 μL of standard intact MYO with a concentration of 400 fmol/μL is very weak (total intensity, close to 19) when being directly loaded on the MALDI plate (Figure 4a). However, the total intensity can increased up to 149 when 50 fmol/μL intact MYO is enriched by ZnO−PMMA nanobeads from 200 μL of solution (shown in Figure 4b). In this case, an enriching factor of 63 could be estimated based on the intensity enhancement.

Proteome Analysis of Colorectal Cancer Separated from 2-D Gel. By virtue of the superiority of this ZnO−PMMA method for identification, we applied it to the protein spots separated from the 2-DE gel (Supporting Information, Figure S-2), in order to find out more candidates for biomarkers from the colorectal cancer patients. A total of 101 protein spots were identified as 74 nonredundant proteins. From these spots, eight proteins, which had never been reported in CRC, have been identified successfully. They are 150-kDa oxygen-regulated protein precursor, filamin-A interacting protein 1, LIM domain containing preferred translocation partner in lipoma, early endosome antigen 1, syndecan-3, hepatocarcinoma high expression protein, cytochrome P450 4F12, and transmembrane protein 2. Another two proteins, VCP protein and endoplasmic reticulum protein ERp29 precursor in CRC, which have not been identified by mass spectrometer before, have been validated. All the proteins mentioned above are pointed out on the 2-DE gel in Supporting Informtion, Figure S-2. Table 1 shows the data of the proteins from GPS Explorer with Mascot against IPI_Human_3.07 database.

Some of the newly detected proteins might have the correlation of tumor growth or metastasis. For example, filamin-A interacting protein 1 (spot 6 shown in Table 1) induces the degradation of filamin-A and controls the start of neocortical cell migration from the ventricular zone.[24] LIM domain containing preferred translo-

cation partner in lipoma (LPP, spot 9 shown in Table 1), as a novel coregulatory binding partner for PEA3, was found to upregulate the transactivation capacity of PEA3.[25] PEA3 is thought to play an important role in promoting tumor metastasis.[26] Syndecan-3 (spot 7 shown in Table 1) is a transmembrane heparin sulfate proteoglycan, which has potentials for signal transduction and can interact with tyrosine kinases c-Src and c-Fys, Actin-binding proteins and F-actin, and other important components.[27,28] It is still difficult to absolutely ascribe these new proteins to ZnO−PMMA; however, the superiority of ZnO−PMMA validated by both standard proteins and the proteome of CRC illustrates this method can be applied for identifying low-abundant proteins/peptides.

CONCLUSION

This is the first use of the novel core−shell ZnO-PMMA nanobeads with the 2.1-nm core as adsorbent and realize a simple, fast, and reproducible protein and peptide enriching method with a high-enriching rate and high salt-tolerance capability. This is also the first time to use core−shell ZnO−PMMA nanobeads for human colorectal cancer proteome analysis. We have found eight new proteins from the CRC proteome by this new method. In order to find more potential biomarkers, high-throughput proteome research in CRC with this new enriching method will be our future work.

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