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Facile synthesis of novel magnetic silica nanoparticles functionalized with layer-by-layer detonation nanodiamonds for secretome study of human hepatoma cell

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Abstract

Novel magnetic silica nanoparticles functionalized with layer-by-layer detonation nanodiamonds (dNDs) were prepared by coating single submicron-size magnetite particles with silica and subsequently modified with dNDs. The resulting layer-by-layer dNDs functionalized magnetic silica microspheres (Fe_3O_4@SiO_2@[dNDs]n) exhibit well-defined magnetite-core-silica-shell structure and possess high content of magnetite, which endow them with high dispersibility and excellent magnetic responsibility. Meanwhile, dNDs is known for its high affinity and biocompatibility towards peptides or proteins. Thus, a novel convenient, fast and efficient pretreatment approach of low-abundance peptides or proteins was successfully established with Fe_3O_4@SiO_2@[dNDs]n microspheres. The signal intensity of low-abundance peptides was improved by at least two to three orders of magnitude in mass spectrometry analysis. The novel microsphere also showed good tolerance to salt. Even with high concentration of salt, peptides or proteins could be isolated effectively from sample. Therefore, the convenient and efficient enrichment process of this novel layer-by-layer dNDs-functionalized microsphere make it promising candidate for isolation of protein in huge volume of culture supernatant for secretome analysis. In the application of Fe_3O_4@SiO_2@[dNDs]n in the secretome of hepatoma cell, 1473 proteins were identified and covered a broad range of p*I* and molecular weight, including 377 low molecular weight proteins.

Keywords: Detonation nanodiamond-functionalized magnetic silica microsphere, Pretreatment, Low-abundance peptides or proteins, Secretome, Mass spectrometry

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1. Introduction

The secretome, an "omic" term used to describe proteins secreted from cells or tissues, has recently received much attention due to the vital roles of secreted proteins in lots of important processes such as cell growth, development, cell signaling and binding. Profiling of cancer cell line secretomes has been shown to be a promising strategy for identifying potential cancer biomarkers [1-4]. However, analysis of secreted proteins is quite challenging due to the inherent low concentrations of secreted proteins and the presence of high amounts of interfering salts and other compounds. Therefore, pretreatment and separation of proteins is almost mandatory for subsequent secretomic analysis [3-4]. Ultrafiltration, precipitation and dialysis are the most often used pretreatment methods. However, these traditional techniques suffer from their own drawbacks. Dialysis methods are time-consuming and result in substantial protein loss, which is a critical concern when dealing with small sample volumes. Depending on the molecular weight cut off (MWCO) of the membrane followed by centrifugal vacuum drying, proteins can be purified, separated, or concentrated in either fraction by ultrafiltration approach. But ultrafiltration approach is also time-consuming and costly. As for protein precipitation, some important parameters should be considered, such as temperature, pH and protein concentration in solution. Meanwhile, the precipitates in the procedure are hardly dissolvable, thus this method gives poor protein yield [5]. Furthermore, the low molecular weight (LMW) fraction of secretome, which is considered to be a valuable reservoir of biomarkers, such as hormones, cytokines, and growth factors, are also prone to lose during the conventional pretreatment approaches. Therefore, it is an urgent task to develop novel sample preparation techniques for pretreatment of low-abundance proteins or peptides from culture supernatant.

For rapid enrichment of low-abundance proteins or peptides from biological samples, proteomics using nanotechnology resources establish a novel analytical platform known as nanoproteomics [6]. The employment of nanomaterials smaller than 100 nm to enrich low-abundance proteins or peptides provides a promising way to increase the limit of detection and identified efficiency of proteins with their unique size-related physical and chemical properties. Some scientists have developed several advanced materials for the concentration of low-abundance peptides in complex samples, including, functionalized nano-CdS (for peptides) [7], $CaCO_3$, ZnO_2, SnO_2 or TiO_2–poly(methyl methacrylate) nanoparticles (for peptides) [8, 9], MCM-41 porous nanoparticles (for endogenous peptides) [10] and mesoporous silica particles (for peptides in plasma) [11], polymeric beads (for peptides/proteins) [12], porous glass beads (for proteins) [13], multi-walled carbon nanotubes (for proteins) [14] and so on. In the secretome analysis, Cao et al. described a versatile and effective approach for enrichment of secreted proteins based on nanozeolites LTL (Linder type-L) [15]. Nanozeolite LTL displays the absorption of protein or peptides due to its special columned morphology and negative surface charge. However, 600 μg of nanozeolites in 10 mL of conditioned media must be centrifuged at high speed for a

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long time to collect it. The concentrated secretome proteins or peptides were further eluted under gel electrophoresis, which could cause low molecular weight proteins (lower than 10 KDa) loss. Paule et al. also showed the combination of SEAN (size exclusion/affinity nanoparticles) and proteomics enables the analysis of secreted proteins with low molecular weight and low abundance [16]. However, these approaches mentioned above need centrifugation to separate nanoparticles from sample, making the pretreatment process very laborious, time-consuming and apt to lose low-abundant peptides because of co-separation with nanoparticles during centrifugation. Therefore, searching for a simple, fast and effective enrichment process for secretome analysis is urgently needed.

To make up for the shortcoming mentioned above, some surface functionalized magnetic particles have been widely developed for the peptide or protein extraction and enrichment owning to their distinguished properties, including high magnetic sensitivity, excellent dispersibility in aqueous solution and chemical stability, which guarantee a fast, efficient magnetic separation, as well as good recycling [17, 18]. Previous reports have shown that magnetic beads functionalized with reversed-phase (RP) group (such as C_8-functionalized magnetic microspheres) are broadly used for SPE-based strategy, which are an ideal candidate for the efficient enrichment of peptides from biological samples through hydrophobic–hydrophobic interaction or hydrophilic interaction [19]. Other carbon-based composites, including Fullerene (C_{60})-functionalized magnetic microspheres (Fe_3O_4@nSiO_2@C_{60}) [20], carbon nanotube (CNT)-decorated magnetic microspheres and graphene-encapsulated magnetic microspheres (Fe_3O_4@nSiO_2@G) have been developed for the enrichment of low-concentration peptides due to the strong hydrophobic interactions between the carbon skeletons and peptides [21, 22]. However, due to the huge interaction between peptides or proteins with the layer of carbon material outside the above materials, the recovery of peptides or proteins isn't good. Thus, these advanced material microspheres have never been applied for the secretome analysis. Functionalized magnetic nanoparticles have also been developed for specific enrichment of peptides/proteins with post-translational modifications, such as phosphorylation and glycosylation. For example, several metal ion-immobilized magnetic nanoparticles, such as zirconium arsenate-modified magnetic nanoparticles (ZrAs-Fe_3O_4@SiO_2) have been prepared for the enrichment of phosphorylated peptides, which exploit the high affinity of positively charged metal cations to negatively charged phosphorylated peptides [23]. The hydrazide-functionalized magnetic nanoparticles have been fabricated for specific enrichment of glycosylated peptides [24,25]. Therefore, the functionalized magnetic material has a broad application prospect in the life science.

Detonation nanodiamond (dND), with its prominent features of very small particle sizes (3-10 nm), high surface area, chemical stability, biological compatibility, and non-toxicity is well believed to be a promising material for biological applications. Especially, its high adsorption capacity makes dNDs a good candidate for effective concentration and extraction of peptides and

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proteins in dilute and contaminated sample [26-28]. However, dNDs are not easy to isolate and tedious centrifugation steps are often required for further separation, which would limit its analytical applications in the huge volume samples, such as the culture supernatant of cell. The most advantage of magnetic microsphere is its super paramagnetic properties, which prevent the magnetic microspheres from aggregating and enables them to re-disperse rapidly after the removal of a magnetic field. Therefore, the combination of dNDs with magnetic microsphere will benefit their further development, which will be an interesting advanced composite material as a result of their strong sorption and super paramagnetic properties. It is worth noting that, in previous reports, core-shell diamond particles were usually prepared by layer-by-layer (LbL) deposition on the surface of diamond as a support for solid-phase extraction and high-performance liquid chromatography. The core-shell particles shown significantly larger surface area than the original solid particles and the highest efficiencies yet reported for a diamond-based chromatographic support [29].

Thus, we fabricate a novel dND-functionalized magnetic silica microsphere [Fe_3O_4@SiO_2@[dND]n] through layer-by-layer deposition of nanodiamond on magnetic silica microsphere. While the novel materials maintaining excellent magnetic properties and admirable adsorption, the process of enrichment is very fast, convenient and efficient. The novel material was tested for their efficiency in the enrichment of the digestion of model proteins and a mixture of model proteins. Moreover, the multiple functional groups outside of [Fe_3O_4@SiO_2@[dND]n] played electrostatic interaction, hydrophilic and hydrophobic interaction with proteins in biological sample. Thus it could be successfully applied to convenient, efficient and fast enrichment of the secreted proteins in culture supernatant from human hepatoma cells.

2. Experiment

2.1 Reagents

Detonation nanodiamond was purchased from Gansu Lingyun Nanomaterial Corp. (Lanzhou, China). Proteins and reagents were purchased from Sigma-Aldrich (Mississauga, ON, Canada), including the following: bovine serum albumin (BSA), β-casein, cytochrome *C* (Cyto *C*), (3-aminopropyl)-triethoxysilane (APTEOS), poly-L-lysine hydrochloride (PL, molecular weight >18,000), 2-(N-morpholino) ethanesulfonic acid (MES), ammonium bicarbonate (NH_4HCO_3), glycine. 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) were purchased form Alfa Aesar (Ward Hill, MA, USA). Porcine trypsin was purchased from Promega (Madison, WI, USA). Analytical grade acetonitrile (ACN) and trifluoroacetic acid (TFA) were from Merck Millipore (Darmstadt, Germany). All of other chemicals were of analytical grade, and were purchased from Shanghai Chemical Reagent Co. (Shanghai, China). All regents applied in SDS-electrophoresis were obtained from Bio-Rad (Hercules, CA, USA).Water used for all experiments was purified using a Milli-Q Plus system (Millipore, Bedford, MA, USA).

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2.2 Synthesis of Fe_3O_4@SiO_2@[dND]n microspheres

Magnetic silica microspheres with a magnetite core of about 220 nm in diameter and silica shell of about 20 nm in thickness were synthesized according to our previous reported method [30]. After thoroughly washed with ethanol, 0.050 g of magnetic silica microspheres were dispersed in mixed solution of 160 mL ethanol, 40 mL deionized water and 5.0 mL APTEOS by ultrasonic vibration. Then, the obtained dispersion was subject to mechanical stirring at 40 °C, and 0.50 mL of ammonia aqueous solution were added into the dispersion with syringe. After stirring for 6 h, the resulting APTEOS-modified magnetic silica microspheres were recovered and washed with ethanol repeatedly.

dND particles were further carboxylated and oxidized with strong oxidative acid treatments following the procedure of Huang and Chang [31]. Carboxylic acid groups on the surface of heat- and acid-treated dND particles were activated by carbodiimide and subsequently reacted with NHS to provide a reactive site for covalent attachment to PL, as previously described [32,33]. dND particles (0.50 g) and 0.20 g of NHS were prepared in a 50 mM MES buffer solution (pH=6.1). Under the assistant of ultrasonic, 0.25 g of EDC was added quickly, and the mixture was continually stirred at room temperature for 1 h. The suspension was then centrifuged and rinsed thoroughly with MES buffer solution to remove excess EDC, NHS and byproduct urea.

0.25 g of APTEOS-modified magnetic silica microspheres and 0.05 g of the estered dND particles were redispersed in 50 ml of MES buffer solution and then continually stirred for 1 h. The obtained dND-functionalized magnetic silica microspheres were separated with a magnet, washed with MES buffer solution by ultrasonic to remove any weakly adsorbed or unbound dND particles. Then, 0.10 wt% solution of PL was made from 0.050 g of PL powder in 50 mL of MES buffer solution, and 0.10 g of dND-functionalized magnetic silica microspheres was poured into this solution and stirred for 1 h to amine functionalize the outer surface of the dND particles in the first layer. The PL-dND functionalized magnetic silica microsphere was then washed extensively with ultrapure water. In the next step, the PL-dND functionalized magnetic silica microspheres was then poured into the suspension of the estered dND particles as in the first deposition of dNDs. This general procedure was repeated until the desired number of PL-dND bilayers was deposited. At last, the final step was the block reaction of the unreacted epoxy groups of dND particles with 0.10 wt% solution of PL.

2.3 Characterization of Fe_3O_4@SiO_2@[dND]n microspheres

dND particles, Fe_3O_4@SiO_2 particles, Fe_3O_4@SiO_2@dNDs particles, Fe_3O_4@SiO_2@$(dNDs)_n$ particles were characterized by using Fourier transform infrared (FT-IR), respectively. FT-IR spectra were collected using a Nexus 470 FT-IR spectrometer (Nicolet, Madison, WI, USA). All samples were dehydrated before analysis. The size and morphology of Fe_3O_4@SiO_2 particles and Fe_3O_4@SiO_2@$(dNDs)_n$ were observed using a JEOL model JEM-2011 (HR) transmission

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electron microscope (TEM) (JEOL, Tokyo, Japan) at 200 kV, respectively. The mean size and size distribution of the Fe_3O_4@SiO_2@$(dNDs)_n$ were measured by dynamic light scattering (DLS) (Malvern, Autoszer 4700) in aqueous solution with pH 4. All DLS measurements were carried out with a wavelength of 532 nm at 25 ^{0}C and an angle detection of 90^0..

2.4 Pretreatment of standard peptides or proteins

Suspension of Fe_3O_4@SiO_2@[dND]n microspheres (20.0 μL, 5 μg/μL) was added into a solution (1.0 mL) containing certain concentrations of standard peptides or proteins. The solution containing peptides or proteins and Fe_3O_4@SiO_2@[dND]n microspheres was shaken at room temperature for 5 min. After decanting supernate with the help of magnet, the residue was rinsed with water three times. And the peptides or proteins retained on the microsphere surface were eluted with eluting solutions (50% ACN aqueous solution containing 0.1% TFA, 5.0 μL). The elute containing the captured peptides or proteins was separated from Fe_3O_4@SiO_2@[dND]n microspheres with the help of magnet. The eluted procedure was repeated for better recovery of peptides or proteins.

To evaluate the enrichment efficiency of Fe_3O_4@SiO_2@[dND]n microspheres for proteins, a series of the mixed standard proteins were treated by Fe_3O_4@SiO_2@[dND]n microspheres. Then the recovery and reproducibility of these proteins was investigated, which was the key factor for the further application in real biological sample. BSA, Cyto *C* and β-casein were combined as the standard protein mixture. The elute containing the captured proteins was separated and analyzed by SDS-PAGE electrophoresis.

2.5 Pretreatment of secretory proteins

The human hepatoma cell line Huh-7 was cultured at 37 □ in 5% CO_2 in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% glutamine until approaching approximately 60-70% confluence. Cells were washed two times with PBS and two times with serum- and phenol red-free DMEM cell medium. Cells were then incubated in the serum- and phenol red-free cell medium at 37 °C. After 36 h, the culture supernatants containing secreted proteins were collected and centrifuged at 1000×g for 5 min (4 °C) and subsequently filtered using a 0.22 μm filter (Millipore, Bedford, MA, USA).

For the treatment of the culture supernatants from human hepatoma cells (10.0 mL), 0.50 mL of suspension of Fe_3O_4@SiO_2@[dND]n microspheres was applied, and then shaken at 4 °C for 20 min. After the collection of magnetic microspheres by magnet, the supernatant was transferred and treated with the same amount of Fe_3O_4@SiO_2@[dND]n nanoparticles again as mentioned above. Then two parts of enriched microspheres were mixed and washed by 0.20 mL PBS buffer (pH, 7.4) for three times. At last, the secreted proteins enriched on the microspheres were eluted by a series of ACN aqueous solution (10%, 30% and 50%) for 5 min, and the combined elute was dried by vacuum freeze-drying.

The enriched secreted proteins were dissolved in 50 mM NH_4HCO_3 buffer. Then trypsin was

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added to the protein solution (1:30, w/w) and incubated at 37 □ overnight. The tryptic peptides were fractionated on a waters UPLC using a C18 column (BEH C18 2.1×50 mm, 1.7 μm, Waters, Milford, MA, USA). Peptides were eluted at a flow rate of 600 μL/min with a linear gradient of 5−40% solvent B (ACN) over 15 min, the solvent A is 20mM ammonium formate with pH adjusted to 10. The absorbance at 214 nm was monitored, and a total of 21 fractions were collected. The fraction was separated by nano-HPLC (Eksigent Technologies, Redwood, CA, USA) on the secondary reversed phase analytical column (C18, 3 μm, 150 mm×75 μm, Eksigent, Redwood, CA, USA). Peptides were subsequently eluted using the following gradient conditions with phase B (98% ACN/0.1% formic acid) from 5 to 45% B (5-100 min) and total flow rate was maintained at 300 nL/min. Electrospray voltage of 2.5 kV versus the inlet of the Triple TOF 4600 mass spectrometer was used.

2.6 Mass spectrometry analysis

All MS analysis of standard peptides was performed on a 4700 Proteomics Analyzer (TOF/TOF) (Applied Biosystems, Framingham, MA, USA). The instrument was operated at an accelerating voltage of 20 kV. All mass spectra were obtained in the positive-ion reflection mode with a mass range from 700 to 3200 Da and each spectrum was accumulated by 1000 laser shots typically with automatic mode. Tryptic myoglobin peptides were used to calibrate the mass instrument with an internal calibration mode. Mass accuracy was within 100 ppm. Mass spectral data were examined and processed using Data Explorer 4.0 software supplied by Applied Biosystems. GPS Explorer software (Applied Biosystems) with Mascot (Matrix Science, London, UK) as a search engine was used to identify proteins. All standard proteins were identified using the combination of PMF and MS/MS against Swiss-Prot database. The search parameters were set up as follows: enzyme was trypsin, the number of missed cleavage sites was allowed up to 1, the variable modification was oxidation of methionine, the mass tolerance of precursor ions and fragments were 100 ppm and 0.2 Da, respectively.

Triple TOF 4600 mass spectrometer (Applied Biosystems, Framingham, MA, USA) was operated in information-dependent data acquisition mode to switch automatically between MS and MS/MS acquisition. MS spectra were acquired across the mass range of 350–1250 m/z using 250 ms accumulation time per spectrum. Tandem mass spectral scanned from 100–1250 m/z in high sensitivity mode with rolling collision energy. The most intense precursors in the top of 25 were selected for fragmentation per cycle with dynamic exclusion time of 25 s. Tandem mass spectra were extracted and charge state de-convoluted by MS Data Converter from Applied Biosystems. Database searches to identify the peptides were performed with Mascot (v.2.3, Matrix Science) against the Swiss-Prot human database. The search parameters included the following: (i) precursor ion mass tolerance less than 25 ppm; (ii) fragment ion mass tolerance less than 0.1 Da; (iii) up to two missed cleavage sites allowed; (iv) variable modifications, oxidation (M, +15.9949). In the database searching, Percolator was applied.

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2.7 Bioinformatics analysis of secretory proteins

The theoretical p*I* and molecular weight of the identified proteins were exported by Mascot. Protein sequences were retrieved from the Uniprot database. The bioinformatical confirmation of secretory proteins was carried out as follows. SecretomeP (http://www.cbs.dtu.dk/services/SecretomeP) was used first to predict non-classical secretory proteins (with no signal-peptide and SecretomeP score >0.5) [34]. Then, the transmembrane hidden Markov model (TMHMM) algorithm (http://www.cbs.dtu.dk/services/TMHMM) was applied to map the putative transmembrane domains in the rest of proteins with confirmed signal peptide by SecretomeP [35]. The bio-functions of the identified proteins were analyzed with the Ingenuity Pathways Analysis (IPA, Ingenuity Systems, Mountain View, CA) [36].

3. Results and discussion

3.1 Preparation and characterization of Fe_3O_4@SiO_2@$(dNDs)_n$

The overall synthetic procedure of Fe_3O_4@SiO_2@dNDs is illustrated in Scheme 1(a). Monodisperse Fe_3O_4 microspheres (~220 nm) were synthesized by using a solvothermal method, followed by coating with silica through a sol-gel process. After covalent grafting of APTES onto the silica-coated Fe_3O_4, the functionalized Fe_3O_4 nanoparticles were readily grafted to the dNDs via covalent amide bonds. Meanwhile, the surface of dNDs was modified with a layer of PL. Then, one more layer of dND was assembled on the PL-functional dND-coated Fe_3O_4@SiO_2 through covalent bonds. This process was repeated until the core-shell particles contained five bilayers of PL-nanodiamond. Finally, the unreacted epoxy groups of dNDs on the microsphere surface were blocked with 0.10 wt% solution of PL. The product was denoted Fe_3O_4@SiO_2@$(dNDs)_n$.

The newly synthesized materials were characterized by different techniques, including FT-IR and transmission electron microscopy (TEM). FT-IR was used to evaluate the preparation procedure. Fig. 1 shows the FT-IR spectra of dNDs, Fe_3O_4@SiO_2, Fe_3O_4@SiO_2@dNDs and Fe_3O_4@SiO_2@$(dNDs)_n$, respectively. In the spectrum of dNDs (Fig. 1a), bands in the region 3424 cm^{-1} can be assigned to the surface hydroxyl O–H stretching vibrations of adsorbed water and/or surface carboxylic groups. 2850~2910 cm^{-1}, 1676 cm^{-1}, and 1194~1112 cm^{-1} can be assigned to C–H stretching vibration, C=O stretching vibration, and C–O–C stretching vibration, respectively. In the spectrum of Fe_3O_4@SiO_2 (Fig. 1b), 1056 cm^{-1} and 570 cm^{-1} can be assigned to be Si–O–Si stretching vibration and Fe–O–Fe vibration. After the first deposition of dNDs on Fe_3O_4@SiO_2 particles (Fig. 1c), the intensity of the peak at 2850~2910 cm^{-1} ascribed to C–H stretch mode increased to some extent. In addition, the new peaks at 1634 cm^{-1} and 1542 cm^{-1} ascribed to carboxyl C=O stretch vibration of dNDs and/or PL and the stretching vibration of C–N also confirm the successful grafting of dNDs. In the spectra of Fe_3O_4@SiO_2 particles coated with 5 layers of dNDs (Fig. 1d), comparing with the intensity of the peaks ascribed to $–CH_2$ stretch vibration, C=O stretch vibration and C–N stretch vibration was increased slightly, which suggested that dNDs were successfully bonded on Fe_3O_4@SiO_2 particles surfaces via PL through a

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layer-by-layer synthetic approach.

Fig. 2 presents the TEM images obtained from the core-shell structured magnetic silica microspheres before (Fig. 2a) and (Fig. 2b, Fig.2c and Fig. 2d) after functionalized with dNDs. Because the difference of density between Si and Fe is large, the black Fe_3O_4 particles had a spherical shape with an average diameter of ～220 nm and were coated with an amorphous SiO_2 layer with the thickness of ～20 nm (Fig. 2a). As shown in Fig. 2b and 2c, after 5 times of coating procedure with dNDs, the magnetic silica microspheres have been successfully functionalized with uniform dNDs layers via PL. Although the Fe_3O_4@SiO_2@$(dNDs)_n$ microspheres were sonicated in ethanol before TEM measurements, the functionalized dNDs on microspheres was stable, which also suggested dNDs were covalently bonded on Fe_3O_4@SiO_2@$(dNDs)_n$ microspheres surfaces via PL successfully. Furthermore, no free Fe_3O_4 nanoparticles or dNDs were observed in Fig. 2d, indicating that the Fe_3O_4 nanoparticles, PL and dNDs have been integrated into an entity. As shown in Fig. S1, it was also confirmed by dynamic light scattering (DLS) that the hydrodynamic diameters for the Fe_3O_4@SiO_2@$(dNDs)_n$ microspheres (619±80 nm) increased by ～400 nm in comparison with those of the Fe_3O_4@SiO_2 particles (290±35 nm) dispersed in aqueous solution. Meanwhile, it also proved that the Fe_3O_4@SiO_2@$(dNDs)_n$ microspheres has good dispersibility in aqueous solution, which might be valuable for the further application in biological analysis. Together with the FTIR spectra, TEM images, and the increasing size of Fe_3O_4@SiO_2@$(dNDs)_n$ microspheres, these suggests that dNDs is indeed covalent binding on magnetic particles via PL.

The synthesized Fe_3O_4@SiO_2@$(dNDs)_n$ microspheres possess the super paramagnetic properties, which can prevent the aggregation of microspheres. The microspheres display good dispersibility in water or ethanol, which might be due to the numerous functional groups of dNDs surfaces and the PL linkers. Additionally, it exhibits magnetic field-induced aggregation, indicating fast response of magnetic microspheres in reply to magnetic field. It is found that the 80% Fe_3O_4@SiO_2@$(dNDs)_n$ microspheres could be separated from buffer solution easily with a magnet in 15s (Supporting information Fig. S2). The excellent dispersion and super paramagnetic properties make the novel layer-by-layer microspheres fascinating and promising for enrichment of peptides and proteins in biological samples.

3.2 Enrichment of standard peptides by Fe_3O_4@SiO_2@$(dNDs)_n$

With a high surface area (400 m^2/g) and abundant functional groups, dNDs provide an ideal substrate for the enrichment of low abundant peptides [26]. In this study, the novel magnetic silica nanoparticles functionalized with dNDs were prepared for the rapid and accessible pretreatment approach. The collection efficiency of the composite materials also increased due to the magnetic ferrite “core”. The performance of enrichment of peptides by Fe_3O_4@SiO_2@$(dNDs)_n$ was evaluated by the standard peptides of Lambinin B and tryptic digests of BSA. The enrichment strategy was illustrated in Scheme 1b. After treatment by the novel microspheres, peptides were

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eluted by ACN aqueous solution. Then, the elution could be either analyzed directly by MALDI-MS or dried by vacuum freeze-drying for further MS analysis. The standard peptide of Lambinin B (CDPGYLGSR, Mr 966.43) was applied for the evaluation of enriched equilibration time by Fe_3O_4@SiO_2@$(dNDs)_n$. The microspheres were incubated with standard peptides of Lambinin B (0.50 fmol/μL, 1 mL) for 2, 4, 6, 8, 10, 15, 25 and 30 min, respectively. After eluted by 10 μL of 50% ACN aqueous solution containing 0.1% TFA for 5 min, 1 μL of elute was analyzed by MALDI-TOF MS. The signal intensity percentage of peptide was measured with respect to the different incubation time. As shown in Fig. S3, the enrichment saturates within 6 min and this value can be considered as the equilibrium time for the further peptide treatment. The short equilibrium time is attributed to the significant interaction between the peptides and dNDs on the microsphere surface as well as the PL linker.

To evaluate the enrichment capacity of the novel microspheres, 1 mL of tryptic digests of BSA (from 1 to 50 fmol/μL) was treated with 20 μg of Fe_3O_4@SiO_2@$(dNDs)_n$ microsphere，respectively. The tryptic digests of BSA enriched by the microspheres were eluted by 5.0 μL of 50% ACN aqueous solution containing 0.1% TFA, and then 1 μL of elute was deposited on MALDI stainless plate for MS analysis. Each concentration was repeated six times. Four typical tryptic peptides of BSA, m/z at 927.5 (YLYEIAR), 1163.3 (LVNELTEFAK), 1479.6 (LGEYGFQNALIVR) and 1567.7 (DAFLGSFLYEYSR), were used for the enrichment isotherm. As illustrated in Fig. 3, the average signal intensity of four peptides was enhanced from 304.9 to 2000.4 with the concentration of tryptic BSA peptides increased to 20 fmol/μL and saturated with further concentration. Attributed to the dNDs functionalized on magnetic particles and a much larger surface area-to-mass ration of dNDs , the enrichment of peptides to the novel microspheres saturated at 70 mg/g. Therefore, Fe_3O_4@SiO_2@$(dNDs)_n$ microsphere has the potential to be a good material for the enrichment of peptides in diluted sample solution.

Generally, real biological samples always contain salts, or other contaminants for the maintenance of a non-toxic environment for cells, stabilization of solvated sample and other biological activity. However, these salts, or other contaminants play a strong negative effect on the quality of mass spectrometry analysis and can even completely suppress the signals of MS. Therefore, a desalting or accessional step is necessary after conventional pretreatment processes. The previous reports have revealed that the potential of using dNDs as an adsorbent for desalting and subsequent MALDI-MS analysis. In the present study, we also explored the power of Fe_3O_4@SiO_2@$(dNDs)_n$ for desalting. In the presence of saturated NaCl solution, a mass spectrum with high quality of 1 μL of tryptic BSA peptides (2 fmol/μL) can hardly be obtained (Fig. 4a). The peptide signals were greatly suppressed by the high concentration of salts. When the identical sample was pretreated by the novel microspheres, peptides can be captured on the layer-by-layer dNDs outside the silica-coated ferrite “core” by hydrophobic interaction and other molecular interaction. Then the salt was washed by aqueous solution. After the elution with 50% ACN

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aqueous solution containing 0.1% TFA, a very clear peptide fingerprinting was observed with highly enhanced signals (Fig. 4b). It can be safe to draw the conclusion that Fe_3O_4@SiO_2@$(dNDs)_n$ has good salt tolerance capability. Therefore, with the aid of silica-coated ferrite "core", the easy separation of target-molecule-bound nanoparticles using an external magnetic field and the powerful salt tolerance capability make the novel microspheres easy and powerful for pretreatment of peptides in diluted and complicated sample.

3.3 Enrichment of standard proteins by Fe_3O_4@SiO_2@$(dNDs)_n$

To examine the recovery and reproducibility of Fe_3O_4@SiO_2@$(dNDs)_n$ microspheres in low-abundance protein enrichment, a mixture of model protein (S) including BSA (3 μg/mL), β-casein (2 μg/mL) and Cyto *C* (5 μg/mL) was used. Fig. 5a displays the silver stained SDS-PAGE of standard proteins with a series of varying amounts from 0.2S to 4S. The amount of BSA, β-casein and Cyto *C* in 1S sample is 0.75 μg, 0.5 μg and 1.25 μg, respectively. Quantity analysis of each standard protein in SDS-PAGE gel was performed by Quantity One software (Version 4.3, Bio-Rad Laboratories). As shown in Fig. S4, the amount of each standard protein versus the gray value of each lane gives good linearity, such as $y=54.62\ln(x)+192.92$, $R^2=0.93$ (BSA, MW= 69 248Da, p*I*=5.83), $y=47.34\ln(x)+88.99$, $R^2=0.98$ (β-casein, MW=23 583Da, p*I*=5.13) and $y=87.03\ln(x)+184.49$, $R^2=0.96$ (Cyto *C*, MW=11 832Da, p*I*= 9.59). After incubated in 1 mL of the mixed standard protein BSA (3 μg/mL), β-casein (2 μg/mL) and Cyto *C* (5 μg/mL) for 6 min, 300 μg Fe_3O_4@SiO_2@[dNDs]n microspheres were sequential resuspended in the elution buffer (20% ACN aqueous solution containing 0.1% TFA and 50% ACN aqueous solution containing 0.1% TFA). Each elution was vacuum dried and then separated under the same SDS-page condition, respectively (Fig. 5b and Fig. 5c). To evaluate the reproducibility of the novel pretreatment approach for proteins, the enriching procedure was repeated five times, respectively. As calculated by the linearity of gray value with the amount of standard proteins, the recovery of each protein summed by two SDS-page is 93(±2.03)%，99(±2.93)%，89(±2.25)%, respectively. Based on these results, the novel Fe_3O_4@SiO_2@[dNDs]n microsphere is of good practical values in that it displays high enrichment efficiency in the enrichment of low-abundance peptides or proteins. After regeneration of protein, the TEM image of Fe_3O_4@SiO_2@$(dNDs)_n$ (Fig. S5) shows the stability of the functionalized dNDs outside microspheres as the former one (Fig. 2c). Thus, the functionalized magnetic microsphere has the capability for re-use.

In the synthesis of the novel magnetic microspheres, PL was used as blocking agent to stop layer-by-layer deposition of dNDs. Thus, the outer-layer of the Fe_3O_4@SiO_2@$(dNDs)_n$ microspheres shows positive charges under the incubation buffer (Tris-HCl, pH 7.4). Meanwhile, the BSA and β-casein carry negative charges in the same conditions due to their theoretical p*I* values of BSA and β-casein are about 5.0. Owning to the electrostatic interactions between negative charge of model proteins and positive charge of PL, the novel microspheres can be applied in the enrichment of BSA and β-casein efficiently. Because the electrostatic interactions

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can be easily broken in the low concentration of organic solvent eluent, the model proteins of BSA and β-casein were easier eluted than that of Cyto C in 20% ACN aqueous solution containing 0.1% TFA (Fig. S4b). However, the functional group of dND shows high hydrophilic and hydrophobic interaction between the novel microspheres and proteins. Even though it carries positive charges at pH 7.4, Cyto *C* (p*I*=9.59) can also be enriched by the hydrophilic/hydrophobic interactions. In Fig. S4c, the Cyto *C* can be totally eluted in the high concentration organic solvent eluent. Therefore, the mechanism of the novel microspheres for enrichment of proteins is complex interaction of electrostatic interaction, hydrophilic and hydrophobic interaction.

3.4 Application to secretome analysis

To intensively evaluate the feasibility of the magnetic microspheres for the secreted protein enrichment, this material was further applied to complex biological samples. Cancer cell secretome profiling has been shown to be a promising strategy for identifying potential cancer biomarkers. In this study, Fe_3O_4@SiO_2@[dND]n microspheres were used to capture secreted proteins in Huh-7 cells, followed by separation and detection using 2-D LC/ESI-MS/MS. As a result, a total of 1473 unique proteins were identified with duplicate experiments.

The identified proteins were further analyzed using bioinformatics programs designed to predict protein secretion pathways. Among the identified 1473 proteins, 611 proteins were predicted to be secreted in the classical secretory pathway based on the presence of a signal peptide and the absence of transmembrane domains by SecretomeP program and TMHMM. And 409 proteins were verified to be secretory proteins released through non-classical secretory pathway by the SecretomeP program. Additionally, 132 proteins were determined by TMHMM algorithm as membrane secretory proteins but could be categorized as neither classical nor non-classical secretion [15]. Collectively, 50.4% (743/1473) of the identified proteins can be verified as secretory proteins by these analyses, and this ratio is consistent with previous studies [37].

The MW, p*I* (Isoelectric point), GRAVY (the grand average of hydropathicity) of each protein was then investigated to examine the universality of our method. As a result, the 1473 identified proteins showed a wide MW distribution (Fig. 6a) and a broad p*I* distribution (Fig. 6b) even including very basic proteins (up to p*I* 12.24). Among them, the percentage of the proteins with MW below 30 kDa reaches 25.6% (377/1473). Many of the identified LMWPs (low molecular weight proteins) are known to be associated with cancer development and metastasis, such as platelet-derived growth factor subunit B (PDGFB), neurotensin/neuromedin N (NEUT), extracellular superoxide dismutase [Cu-Zn] (SODC), etc [38-41]. As shown in Fig. S6 shows the distribution of GRAVY of the secreted proteins in Huh-7 cells enriched by Fe_3O_4@SiO_2@$(dNDs)_n$ microspheres, the GRAVY of the mostly classical secretory proteins and non-classical secretory proteins bellows zero. The most secretory proteins enriched in the culture supernatant from Huh-7 cell were hydrophilic protein matched with the characteristic of secretory protein. Thus, the above

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results show the high efficiency and universality of this method. The detailed information on all proteins identified including the Swiss-Prot accession names, MW, p*I*, etc. is provided in Supporting Information Table S1.

The bio-functions of the identified proteins were further analyzed with the IPA tool (Fig. S7) Major functions of the identified secreted proteins are "Cellular Growth and Proliferation", "Cell Death and Survival", "Protein Synthesis", "Infections Disease" and "Cellular Movement". These bio-functions are reported to play important roles in the development and invasion of cancer [42-44]. And these observations support the viewpoint that the secretome of cancer cells represents an attractive pool for biomarker discovery.

Some well-known hepatocellular carcinoma biomarkers such as alpha fetoprotein (AFP), apolipoprotein E (APOE), osteopontin (OPN) and dickkopf-related protein 1 (DKK1) were identified in this study [45-47]. In addition, some proteins included in our list were scarcely identified in previous studies of HCC-derived secretome, such as EGF-like repeat and discoidin I-like domain-containing protein 3 (EDIL3), NEUT, and bone morphogenetic protein 4 (BMP4), which show high efficiency of the novel material for the secreted protein enrichment [48-50]. Moreover, these proteins are found to be involved in the development, prognosis and treatment of HCC. It was reported that high levels of autocrine EDIL3 may contribute to the receptive microenvironment for the survival of detached HCC cells and may involve in cancer cell spreading [51,52]. Dysfunctional activation of the NEUT/IL-8 pathway was detected in HCC which is associated with increased inflammatory response in microenvironment, enhanced epithelial mesenchymal transition in cancer, and worse prognosis in HCC patients [39]. BMP4 signaling was previously shown to play a critical role in the differentiation of cancer stem cells and might be a potent therapeutic agent in HCC [53]. These results further underscore the value of Fe_3O_4@SiO_2@[dND]n microspheres in secreted proteins enrichment and analysis.

4. **Conclusions**

In summary, magnetic Fe_3O_4@SiO_2@[dND]n microspheres were prepared and applied for the first time in enrichment and desalting of low-abundance of peptides and proteins. Due to the high absorption capacity of dNDs for biomolecules, the novel magnetic microspheres showed the excellent performance in concentration of peptides and proteins in highly diluted and complicated samples. The enrichment process was fast and efficient. Furthermore, the successful application of Fe_3O_4@SiO_2@[dND]n in the direct harvesting of secreted proteins from large sample volumes demonstrated it is a promising tool for proteomics and secretome research.

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Scheme 1. Schematic illustrating the facile synthesis of novel magnetic silica nanoparticles functionalized with dNDs by poly-L-lysine via a process of diimide-acivated amidation (a), and its application to the pretreatment of peptides and proteins in sample (b).

Fig. 1. FT-IR spectra of (a) dND particles, (b) Fe_3O_4@SiO_2 particles, (c) Fe_3O_4@SiO_2@dNDs particles, and (d) Fe_3O_4@SiO_2@$(dNDs)_n$ particles.

Fig. 2. TEM images of core-shell structured magnetic silica microspheres before (a) and after (b), (c), (d) functionalized with dNDs.

Fig. 3. Enrichment isotherm of the tryptic BSA peptides pretreated by Fe_3O_4@SiO_2@$(dNDs)_n$ particles. The m/z of tryptic BSA peptides are 927.5 (YLYEIAR), 1163.3 (LVNELTEFAK), 1479.6 (LGEYGFQNALIVR) and 1567.7 (DAFLGSFLYEYSR).

Fig. 4. MALDI-TOF mass spectrometry of 2 fmol/μL tryptic BSA peptides (1mL) in the presence of saturated NaCl solution (a) without any pretreatment and (b) pretreated by Fe_3O_4@SiO_2@$(dNDs)_n$ (10 μg). (The asterisks in mass spectra denote peptides which can be identified in database.)

Fig. 5. SDS-PAGE analysis of the mixture of standard protein (BSA, β-casein and Cyto *C*). (a) directly analysis of standard protein with a series of mixture protein 0.2S (L1), 0.4S (L2), 0.6S (L3)、0.8S (L4), 1.0S (L5), 2.0S (L6), 4.0S (L7), respectively. The amount of BSA, β-casein and Cyto C in 1S sample is 0.75 μg, 0.5 μg and 1.25 μg, respectively. (b) analysis of the mixture of proteins eluated from Fe_3O_4@SiO_2@$(dNDs)_n$ microsphere pretreated in the 1mL of 4.0S sample sequentially by 20% ACN aqueous solution containing 0.1% TFA and (c) 50% ACN aqueous solution containing 0.1% TFA. To evaluate the recovery and reproducibility of the novel enrichment approach, the process of the method repeated 5 times.

Fig. 6. Distribution of MW (a) and p *I* (b) of the secreted proteins in Huh-7 cells pretreated by Fe_3O_4@SiO_2@$(dNDs)_n$ particles.

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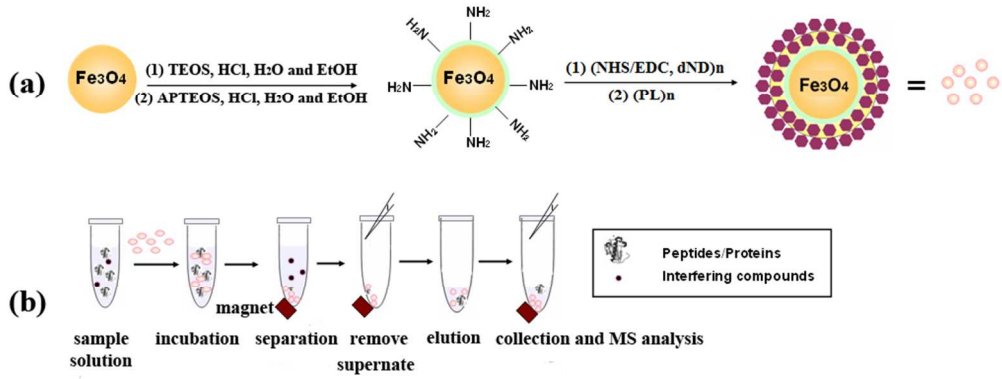

Schematic illustrating the facile synthesis of novel magnetic silica nanoparticles functionalized with dNDs by poly-L-lysine via a process of diimide-acivated amidation (a), and its application to the pretreatment of peptides and proteins in sample (b).
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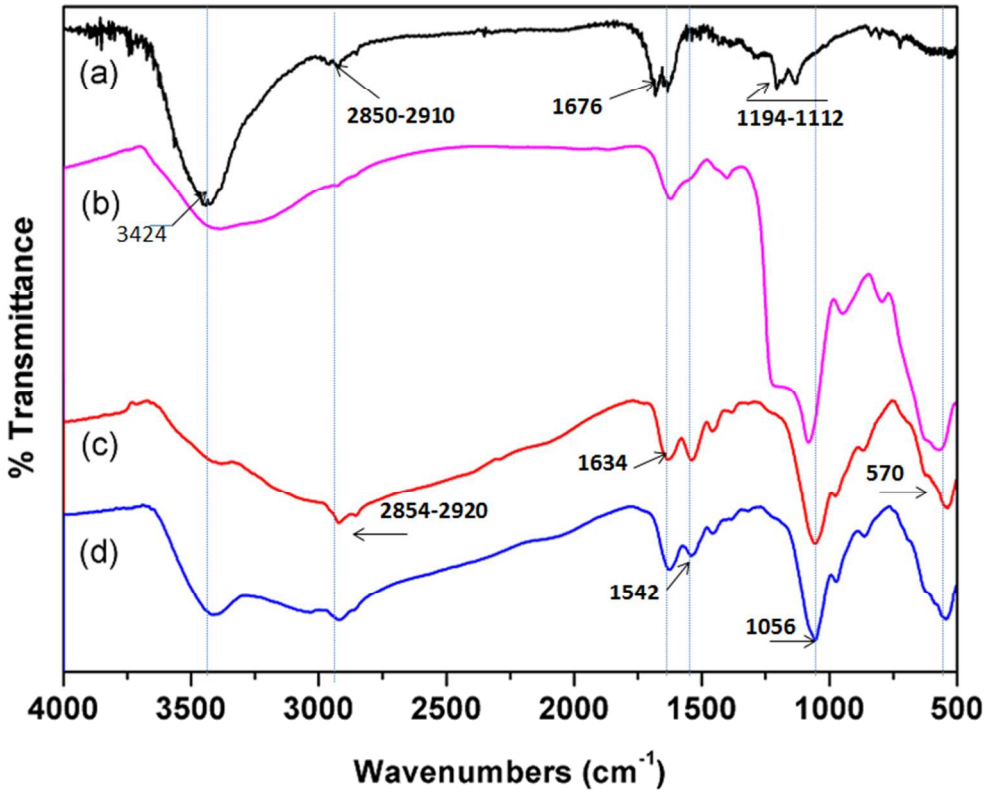

FT-IR spectra of (a) dND particles, (b) Fe3O4@SiO2 particles, (c) Fe3O4@SiO2@dNDs particles, and (d) Fe3O4@SiO2@(dNDs)n particles.
208x167mm (96 x 96 DPI)

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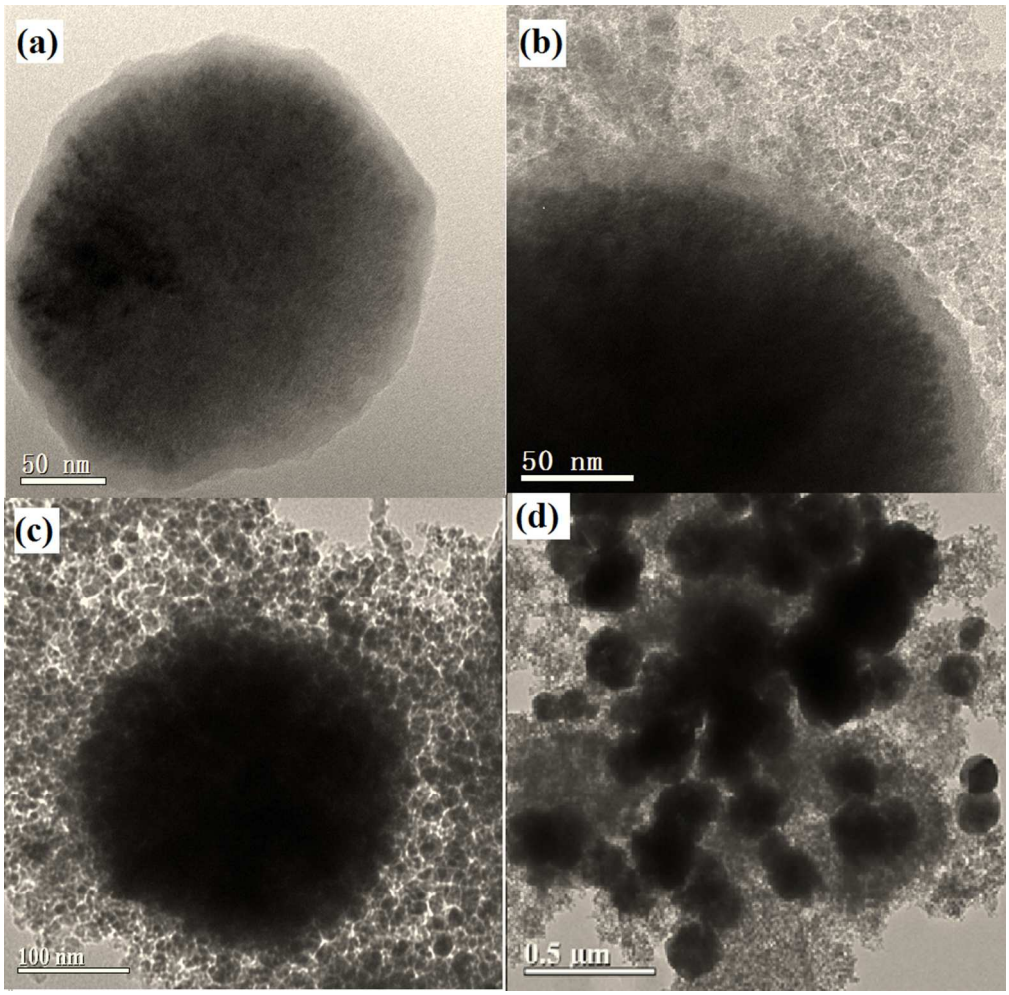

TEM images of core-shell structured magnetic silica microspheres before (a) and after (b), (c), (d) functionalized with dNDs.
270x266mm (96 x 96 DPI)

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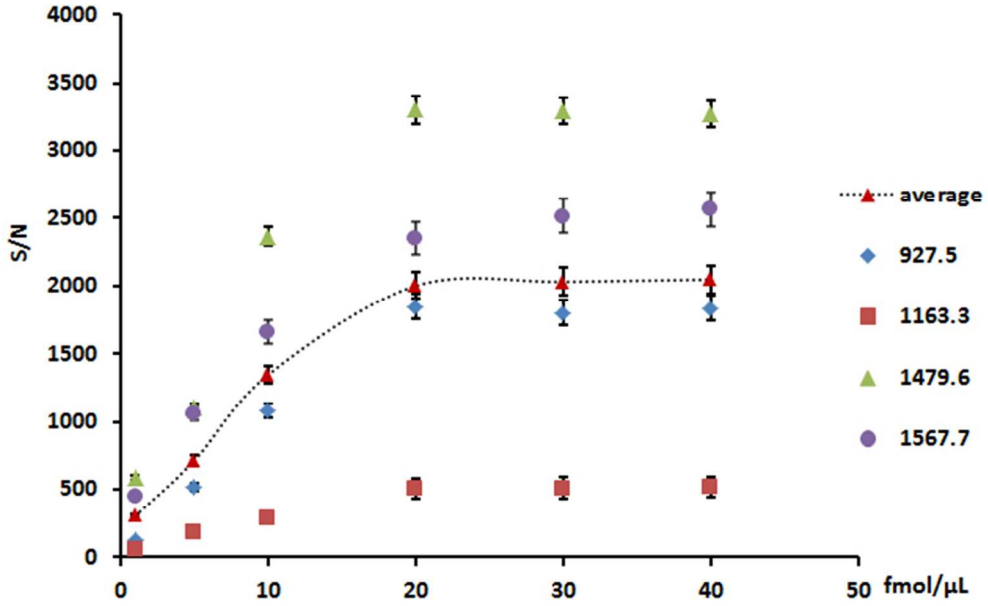

Enrichment isotherm of the tryptic BSA peptides pretreated by Fe3O4@SiO2@(dNDs)n particles. The m/z of tryptic BSA peptides are 927.5 (YLYEIAR), 1163.3 (LVNELTEFAK), 1479.6 (LGEYGFQNALIVR) and 1567.7 (DAFLGSFLYEYSR).
168x103mm (96 x 96 DPI)

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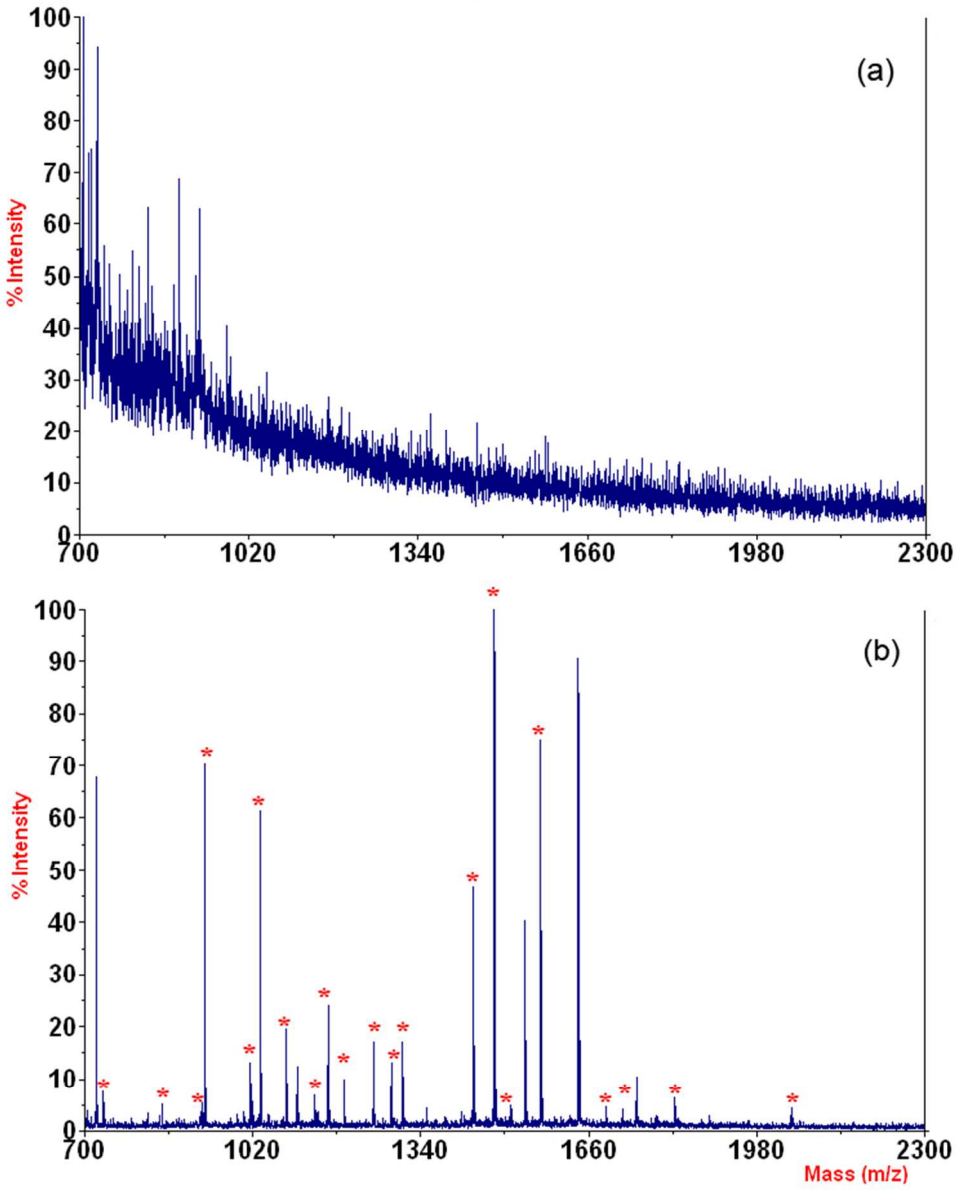

MALDI-TOF mass spectrometry of 2 fmol/μL tryptic BSA peptides (1mL) in the presence of saturated NaCl solution (a) without any pretreatment and (b) pretreated by Fe3O4@SiO2@(dNDs)n (10 μg). (The asterisks in mass spectra denote peptides which can be identified in database.)
198x246mm (96 x 96 DPI)

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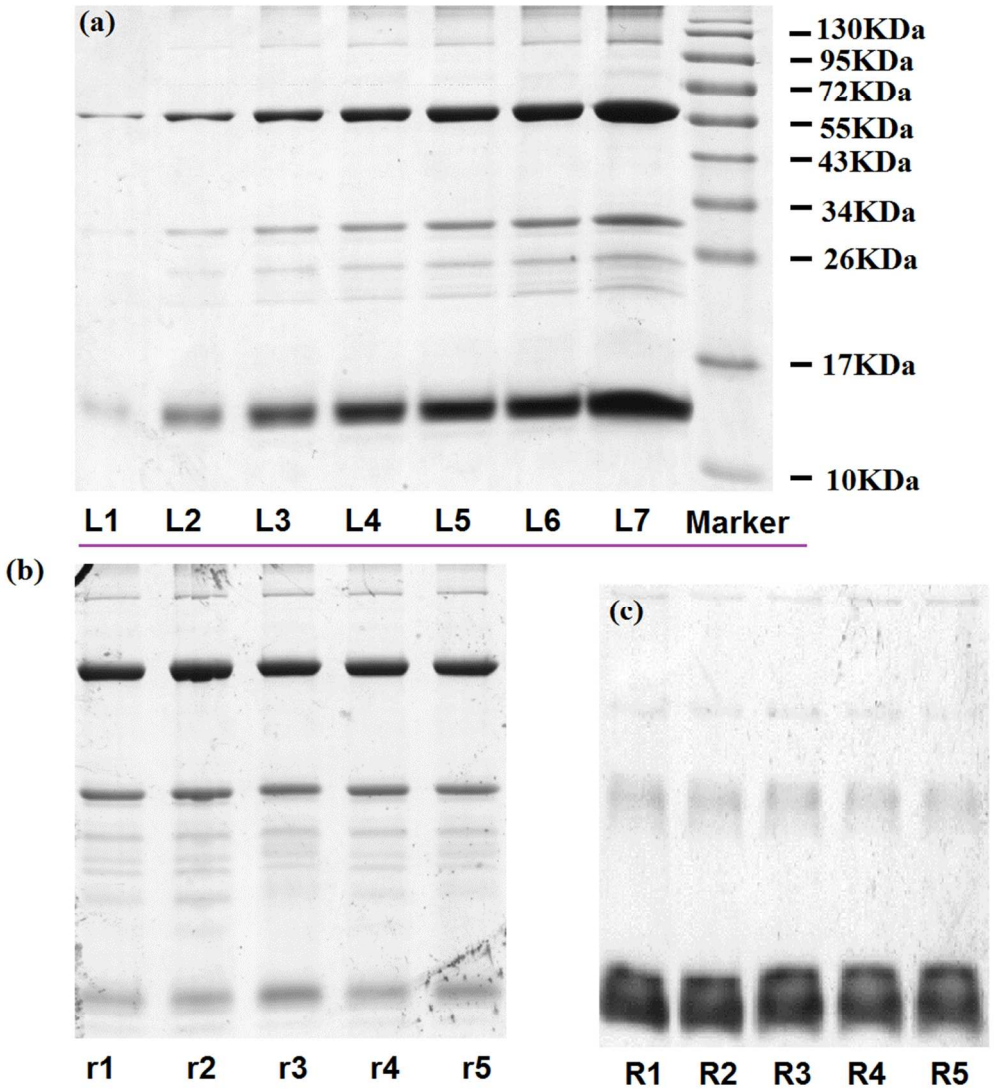

SDS-PAGE analysis of the mixture of standard protein (BSA, β-casein and Cyto C). (a) directly analysis of standard protein with a series of mixture protein 0.2S (L1), 0.4S (L2), 0.6S (L3)、0.8S (L4), 1.0S (L5), 2.0S (L6), 4.0S (L7), respectively. The amount of BSA, β-casein and Cyto C in 1S sample is 0.75 μg, 0.5 μg and 1.25 μg, respectively. (b) analysis of the mixture of proteins eluated from Fe3O4@SiO2@(dNDs)n microsphere pretreated in the 1mL of 4.0S sample sequentially by 20% ACN aqueous solution containing 0.1% TFA and (c) 50% ACN aqueous solution containing 0.1% TFA. To evaluate the recovery and reproducibility of the novel enrichment approach, the process of the method repeated 5 times.
271x304mm (96 x 96 DPI)

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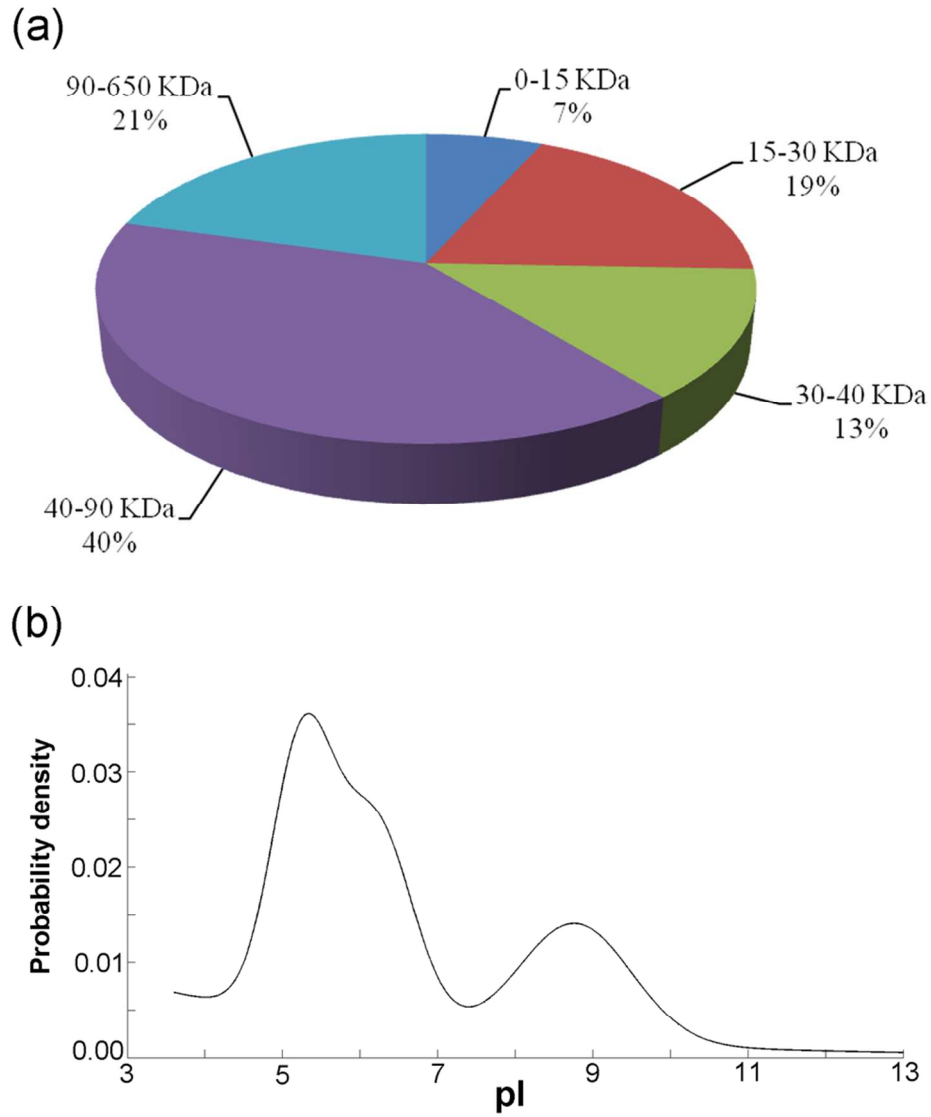

Distribution of MW (a) and p I (b) of the secreted proteins in Huh-7 cells pretreated by Fe3O4@SiO2@(dNDs)n particles.
90x101mm (300 x 300 DPI)

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