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Precisely designed rattle-type $mTiO_2$@P(NIPAM-co-MBA) microspheres with screening gel network for highly selective extraction of phosphopeptidome

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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Selective extraction of phosphopeptidome from complicated biological samples is of great importance towards the development of diagnostic and prognostic biomarkers, but still remains challenge. In this work, rattle-type $mTiO_2$@P(NIPAM-co-MBA) composite microspheres comprising a mesoporous crystalline $mTiO_2$ core, an intermediate hollow space and a crosslinked P(NIPAM-co-MBA) network shell were elaborately designed and fabricated *via* two-step reflux-precipitation polymerization followed by a hydrothermal process. Firstly, a non-crosslinked PMAA layer was directly coated onto the surface of TiO_2 core without any pretreatment. Then the formed TiO_2@PMAA was encapsulated with another crosslinked P(NIPAM-co-MBA) layer with the aid of the strong hydrogen bonding interaction between the two polymer layers. Finally, a hydrothermal process was adopted to convert the TiO_2 core into a crystalline and mesoporous counterpart. At the same time, non-crosslinked PMAA layer was selectively removed to form rattle-type structure. The crosslinked P(NIPAM-co-MBA) shell make the rattle-type $mTiO_2$@P(NIPAM-co-MBA) possess great size-exclusion effect against both high-molecular-weight nonphosphoproteins and high-molecular-weight phosphoproteins while the $mTiO_2$ core was in charge of the selective enrichment of low-molecular-weight phosphopeptides. With the help of these unique properties, the rattle-type $mTiO_2$@P(NIPAM-co-MBA) microspheres show excellent potential for one-step selective extraction of phosphopeptidome.

Introduction

Peptidome, the naturally existed peptides and low-molecular-weight (LMW) proteins (with molecular weight less than 20 kDa) in the biological samples, plays pivotal roles in regulation of diverse biological functions and activities of the proteins. Comprehensive analysis of the peptidome can contribute to a better elucidation of the biochemical functions as well as to the development of diagnostic and prognostic biomarkers.[1-5] To reveal the peptidome in complicated samples, several steps are usually involved, including sample collection, selective extraction of the peptidome, mass spectrometric (MS) detection and data mining. Of these four key steps, the selective extraction step has been recognized as the major obstacle for the successful identification of the peptidome.[6] There is fundamental difference between proteome and peptidome extraction. In proteome research, all the proteins are firstly digested into peptides mixture, and then the peptides are enriched by functional materials to avoid the interference of lipids and salts. In contrast, because extra peptides will be generated from the digestion of proteins, digestion process could not be executed for peptidome research. For this reason, large amount of high-molecular-weight (HMW) proteins (with molecular weight greater than 20 kDa) will exist in the test samples and their presence seriously hampers the mass spectrometric analysis of the peptidome.

Toward this end, three main methodologies are currently used to selective extraction of the peptidome. Organic solvent precipitation is a simple approach to discard highly abundant proteins.[7-9] However, the proteins may act as carriers for the low abundant peptides during the precipitation process. As a result, depletion of HMW proteins would lead to a concomitant loss of physiologically important peptides. Centrifugal ultrafiltration (UF) with an accurate molecular cutoff is considered to be a useful technology for the separation of proteins with different masses.[10-13] Nevertheless, when relative large amount of sample is applied, the ultrafiltration time will increase sharply. Moreover, other contaminants with low molecular weight, such as small molecules and salts, will also be concentrated and result in severe interference to the MS detection. Therefore, additional peptides enrichment and salt removal achieved by solid phase extraction, particularly using C18 sorbents, have to be employed before MS analysis. To simplify the extraction procedures, mesoporous nanomaterials endowed with size-exclusion effect have shed new light on the peptidome research. Ordered mesoporous nanomaterials, including mesoporous silica, mesoporous carbon and et al, with high in-pore surface areas, highly ordered channel and adjustable pore size facilitate the selective extraction of peptidome

while effective exclusion of HMW proteins.[14-25] And for modification-specific peptidome, such as glycopeptidome[26] and phosphopeptidome[27-29], except the size-exclusion effect, enrichment of specific glycopeptidome or phosphopeptidome from the whole peptidome is also indispensable. Further manipulating the surface chemistry of the pore channel has nicely solved this issue. Although this simple approach could block most proteins both in peptidome and modification-specific peptidome research, the outer surface of the mesoporous nanomaterials will also adsorb a few of specific proteins and this consequently brings unfavorable influence to the optimal identification of the peptidome and modification-specific peptidome.

To overcome the limitations of the state-of-the-art methods, we herein proposed a brand-new technique to implement the facile one-step extraction of peptidome and efficient exclusion of HMW proteins by using a composite microsphere with a rattle-type structure. The composite microspheres were deliberately designed to be constructed with a freely moveable core, an intermediate hollow space and a polymeric gel network shell. When this kind of microarchitecture was applied, HMW component, such as HMW proteins, could be effectively blocked by the gel network while peptidome and other composition with smaller size could pass through to get to the intermediate hollow space. Then the peptidome could be enrichment by the core and other LMW contaminants are eliminated. As a typical example, in this article, we designed and fabricated a rattle-type $mTiO_2$@P(NIPAM-co-MBA) microspheres for highly specific and facile extraction of the phosphopeptidome. The rattle-type $mTiO_2$@P(NIPAM-co-MBA) microspheres were designed to possess the following features: (1) a poly(*N*-isopropyl acrylamide-co-*N,N'*-methylenebisacrylamide) (P(NIPAM-co-MBA)) gel network shell showing effective exclusion of the HMW constitutes, including both HMW nonphosphoproteins and HMW phosphoproteins; (2) a mesoporous and highly crystalline $mTiO_2$ core possessing a remarkable selectivity and effectiveness toward enrichment of phosphopeptides and LMW phosphoproteins; (3) an intermediate hollow space offering interspace for the storage of LMW components and making the removal of the nonphosphopeptides and the LMW nonphosphoproteins easier through washing. These rattle type $mTiO_2$@P(NIPAM-co-MBA) microspheres with the aforementioned unique properties are anticipated to have high performance in the selective extraction of the phosphopeptidome from complicated biological samples.

Experimental methods

Materials

Tetrabutyl orthotitanate (TBOT) was bought from Jiangsu Qiang Sheng Chemical Reagent Co., Ltd. Anhydrous ethanol, potassium chloride, methylacrylic acid (MAA), Iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ammonium acetate (NH_4Ac), ethylene glycol (EG), trisodium citrate dehydrate and aqueous ammonia solution (25%) were purchased from Shanghai Chemical Reagents Company and used as received. *N,N'*-methylenebisacrylamide (MBA) was bought from Fluka and recrystallized from acetone. 2,2-azobisisobutyronitrile (AIBN) was supplied by Sinopharm Chemical Reagents Company. *N*-Isopropyl acrylamide (NIPAM, 97%) was obtained from Aldrich and recrystallized from hexane. β-Casein, asialofetuin (ASF), bovine serum albumin (BSA, 95%), cytochrome c from horse heart (Cyto C), 2,5-dihydroxybenzoic acid (2,5-DHB, 98%), ammonium bicarbonate (ABC, 99.5%) and 1-1-(tosylamido)-2-phenyl-ethyl chloromethyl ketone (TPCK)-treated trypsin (E.G 2.4.21.4) were purchased from Sigma (St.Louis, MO). Acetonitrile (ACN, 99.9%) and trifluoroacetic acid (TFA, 99.8%) were purchased from Merck (Darmstadt, Germany). Phosphoric acid (85%) was purchased from Shanghai Feida Chemical Reagents Ltd. (Shanghai, China). Matrix DHB was dissolved in acetonitrile (ACN)/water (50/50, v/v) solution containing 1% H_3PO_4 by keeping DHB at 10 mg/mL. Deionized water (18.4 MΩ cm) used for all experiments was obtained from a Milli-Q system (Millipore, Bedford, MA).

Preparation of monodisperse titania microspheres

Monodisperse spherical titania (TiO_2) microspheres were prepared *via* controlled hydrolysis of TBOT in ethanol/acetonitrile mixed solvent (ethanol/acetonitrile=1/1, v/v). For a typical experiment, an acetonitrile/ethanol volume of 100 mL was mixed with 0.50 mL of KCl aqueous solution (0.40 mol/L, containing 0.2 mmol KCl), followed by adding 4.0 mL of TBOT at ambient temperature with vigorous mechnical stirring. Reagents were completely mixed for the occurrence of the uniform nucleation throughout the whole solution. The initially homogeneous reaction mixtures became milky white after the addition of TBOT, which indicated the formation of visible titania particles as a uniform suspension. The controlled hydrolysis was ended after stirring the reaction system for 2 h at room temperature. The resultant titania microspheres were purified by three cycles of centrifugation, decantation and resuspension in acetonitrile. The resultant titania microspheres were dispersed in acetonitrile for further use.

Preparation of TiO_2@PMAA core/shell microspheres

The TiO_2@PMAA core/shell microspheres were synthesized by directly coating uncrosslinked polymethylacrylic acid (PMAA) onto the surface of titania microspheres without any modification. Coating PMAA layer onto TiO_2 microspheres was executed by reflux-precipitation polymerization[30] of MAA with AIBN as the initiator, in acetonitrile. Typically, about 50 mg of TiO_2 seed nanoparticles were dispersed in 50 mL acetonitrile in a dried 100 mL single-necked flask with the aid of ultrasonic. Then a mixture of 0.5 mL of MAA and 12.5 mg of AIBN were added to the flask to initiate the polymerization. The flask submerged in a heating oil bath was attached with a Liebig condenser. The reaction mixture was heated from ambient temperature to the boiling state within 30 min and the reaction was ended after 3 h. The obtained TiO_2@PMAA microspheres were collected by centrifugation and washed with acetonitrile in order to eliminate excess reactants. The final product was dispersed in acetonitrile for further use.

Preparation of TiO_2@PMAA@P(NIPAM-co-MBA) core/shell/shell microspheres

TiO_2@PMAA@P(NIPAM-co-MBA**)** core/shell/shell microspheres were synthesized by coating crosslinked P(NIPAM-co-MBA**)** shell onto the surface of TiO_2@PMAA *via* the second-step reflux-precipitation polymerization of NIPAM, with MBA as the cross-linker and AIBN as the initiator, in acetonitrile. Typically, about 100 mg of TiO_2@PMAA seed nanoparticles were dispersed in 80 mL acetonitrile in a dried 150 mL single-necked flask with the aid of ultrasonic. Then a mixture of 600 mg of NIPAM, 150 mg of MBA and 15 mg of AIBN were added to the flask to initiate the polymerization. The reaction mixture was heated from ambient temperature to the boiling state within 30 min and the reaction was ended after 3 h. The obtained TiO_2@PMAA@P(NIPAM-co-MBA**)** microspheres were collected by centrifugation and washed with acetonitrile in order to eliminate excess reactants.

Preparation of rattle-type $mTiO_2$@P(NIPAM-co-MBA) microspheres

The rattle-type $mTiO_2$@P(NIPAM-co-MBA) microspheres were achieved by hydrothermal treating the obtained TiO_2@PMAA@P(NIPAM-co-MBA) core/shell/shell microspheres. Typically, about 200 mg of the as-synthesized TiO_2@PMAA@P(NIPAM-co-MBA) microspheres was dispersed in 60 ml mixed solvent containing 40 mL of ethanol and 20 mL of deionized water, and then 3 mL of NH_3•H_2O was added to above suspension. The mixture was then transferred to a Teflon-lined stainless-steel autoclave (100 ml capacity). The autoclave was heated at 160 ^oC and maintained for 24 h. Then it was cooled to room temperature, the obtained rattle-type $mTiO_2$@P(NIPAM-co-MBA) microspheres were collected by centrifugation and washed with ethanol and deionized water to remove the uncrosslinked PMAA chains.

Preparation of tryptic digest of standard proteins

β-casein, BSA were each dissolved in 25 mM ABC at pH 8.0 (1mg/mL for each protein) and denatured by boiling for 10 min. Protein solutions were then incubated with trypsin at an enzyme/substrate ratio of 1:40 (w/w) for 12 h at 37 °C to produce proteolytic digests, respectively. The tryptic peptide mixtures were stored at -20 °C until further use.

Selective enrichment of phosphopeptides with $mTiO_2$@P(NIPAM-co-MBA)

The obtained rattle-type $mTiO_2$@P(NIPAM-co-MBA) was first washed with ethanol for three times and then suspended in deionized water with a concentration of 10 $mg{\cdot}mL^{-1}$. Tryptic digests of β-casein and BSA were dissolved in 100 μL of loading buffer (50% ACN containing 5% TFA), then 2 μL of $mTiO_2$@P(NIPAM-co-MBA) was added and incubated at room temperature. After that, $mTiO_2$@P(NIPAM-co-MBA) with captured phosphopeptides was separated from the mixed solutions by centrifugation. After washing with 200 μL of washing buffer (50% ethanol containing 5% TFA) to remove the nonspecifically adsorbed peptides, the trapped phosphopeptides were eluted with $NH_3{\cdot}H_2O$ (5%, 10 μL) for the further MS analysis. Enrichment of phosphopeptides from protein mixture was the same as described above, the protein mixture containing cyto-c (protein): BSA (protein): β-casein (protein): β-casein digest at mass ratio of 100:100:10:1.

MALDI mass spectrometry

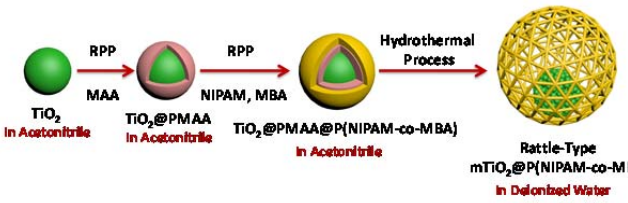
1 μL of the eluate was deposited on the MALDI probe, and then matrix solution DHB (1 μL) was deposited for MS analysis. MALDI-TOF mass spectrometry analysis was performed in positive reflection mode on a 5800 Proteomic Analyzer (Applied Biosystems, Framingham, MA, USA) with a Nd: YAG laser at 355 nm, a repetition rate of 400 Hz and an acceleration voltage of 20 kV. The range of laser energy was optimized to obtain good resolution and signal-to-noise ratio (S/N) and kept constant for further analysis. External mass calibration was performed by using standard peptides from myoglobin digests.

Characterization

Field-emission transmission electron microscopy (FE-TEM) images were taken on a JEM-2100F transmission electron microscope at an accelerating voltage of 200 kV. Samples dispersed at an appropriate concentration were cast onto a carbon-coated copper grid. Fourier transform infrared spectra (FT-IR) were determined on a NEXUS-470 FT-IR spectrometer over potassium bromide pellet and the diffuse reflectance spectra were scanned over the range of 400-4000 cm^{-1}. Thermogravimetric analysis (TGA) measurements were performed on a Pyris 1 TGA instrument. All measurements were taken under a constant flow of nitrogen of 40 mL/min. The temperature was first increased from room temperature to 100 °C and held until constant weight, and then increased from 100 °C to 800 °C at a rate of 20 °C /min. XRD patterns were collected on a X'Pert Pro (Panalytical, The Netherlands) diffraction meter with Cu KR radiation at λ =0.154 nmoperating at 40 kV and 40 mA. Nitrogen adsorption-desorption measurements were performed on an ASAP2020 (Micromeritics, USA) accelerated surface area analyzer at 77 K. Before measuring, the samples were degassed in a vacuum at 120 ^oC for at least 6 h.

Results and discussion

The overall synthetic route employed for the preparation of rattle-type $mTiO_2$@P(NIPAM-co-MBA) microspheres is schematically illustrated in scheme 1. Briefly, monodisperse spherical titania (TiO_2) microspheres were firstly synthesized *via* controlled hydrolysis of TBOT in a mixed solvent containing ethanol and acetonitrile with a volume ratio of 1:1. Then the as-prepared TiO_2 was directly coated with a non-crosslinked polymethylarylic acid (PMAA) interim layer by reflux-precipitation polymerization (RPP) of monomer MAA in the solvent of acetonitrile without any pretreatment of the titania seed microspheres. This interim layer is crucial for the formation of the ultimate rattle-type structure. On one hand, the strong hydrogen bonding interaction between the carboxyl group and the amide group of *N*-isopropyl acrylamide (NIPAM) facilitated the direct coating of P(NIPAM-co-MBA) layer over PMAA layer *via* the second-step RPP process. On the other hand, the PMAA layer could be selectively removed to form the rattle-type structure in order to make the surface of titania core available for anchoring phosphopeptidome. Subsequently, the TiO_2@PMAA microspheres were encapsulated with another crosslinked P(NIPAM-co-MBA) gel network shell *via* the second step RPP of monomer NIPAM and crosslinker MBA in the solvent of acetonitrile. Finally, the obtained TiO_2@PMAA@P(NIPAM-co-MBA) was subjected to a hydrothermal process for the sake of killing two birds with one stone: hydrothermal crystallization of the TiO_2 to form mesoporous crystalline $mTiO_2$ core and removal of the PMAA interim layer to shape into rattle-type structure.



Scheme 1 Schematic illustration of the synthetic procedure for preparation of rattle-type $mTiO_2$@P(NIPAM-co-MBA) microspheres.

Representative TEM images of TiO_2 and TiO_2@PMAA core/shell microspheres were shown in Fig. 1a and 1b. The spherical TiO_2 gave an average diameter of ca. 385 nm, and were uniform both in shape and size. After coating with non-crosslinked PMAA layer, the obtained TiO_2@PMAA microspheres possessed a distinctive core/shell structure and

the size of the composite microspheres increased to around 455 nm, indicating the shell thickness is about 35 nm. It is necessary to point out that the non-crosslinked PMAA layer is only stable in the solvent of acetonitrile. When the TiO_2@PMAA is transferred to the good solvent of PMAA, such as ethanol and deionized water, the PMAA layer will be removed. Therefore, the TiO_2@PMAA has to be stored in acetonitrile before further use and could not contact with the good solvent of PMAA. As seen in Fig. 1c, when the TiO_2@PMAA was encapsulated with the P(NIPAM-co-MBA) gel network shell, the boundary between the two polymer layers could not be clearly observed due to the low contrast between the PMAA and the P(NIPAM-co-MBA) layer. Nevertheless, the shell thickness was visibly increased from about 35 nm to around 95 nm, which suggested the thickness of the P(NIPAM-co-MBA) shell is about 60 nm. With a further hydrothermal treatment at the temperature of 160 ^oC for 24 h, the non-crosslinked PMAA interim layer could be completely removed to form the intermediate hollow space (Fig. 1d). At the same time, the TiO_2 core was no longer solid, but was porous instead and constructed with many small nanocrystals (Fig. 1e). The diameter of the final rattle-type product is about 950 nm.

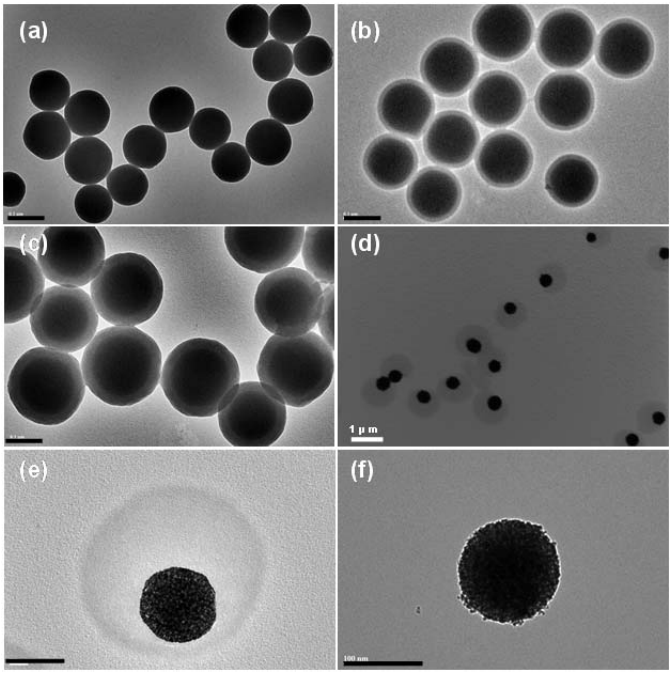


Fig. 1 Representative TEM images of (a) TiO_2, (b) TiO_2@PMAA, (c) TiO_2@PMAA@P(NIPAM-co-MBA), (d, e) rattle-type mTiO_2@P(NIPAM-co-MBA) and (f) mTiO_2. (The scale bars are 300 nm for a, b, c, e, f and 1 μm for d)

The whole fabrication process was continued to be investigated by FT-IR spectroscopy. As shown in Fig. 2a, in addition to the characteristic peaks of TiO_2, the new peak appearing at 1705 cm^{-1} attributed to the stretching vibration of C=O of carboxyl groups proved the presence of the PMAA layer in TiO_2@PMAA. After coating with another layer of P(NIPAM-co-MBA), besides the peak at 1705 cm^{-1}, the peaks corresponding to amide I band (C=O stretching) and amide II band (N–H bending) emerged at 1649 cm^{-1} and 1527 cm^{-1} respectively. Since the PMAA layer was almost completely removed after the hydrothermal process, the signal of the characteristic peak of carboxyl group (1705 cm^{-1}) could not be detected.

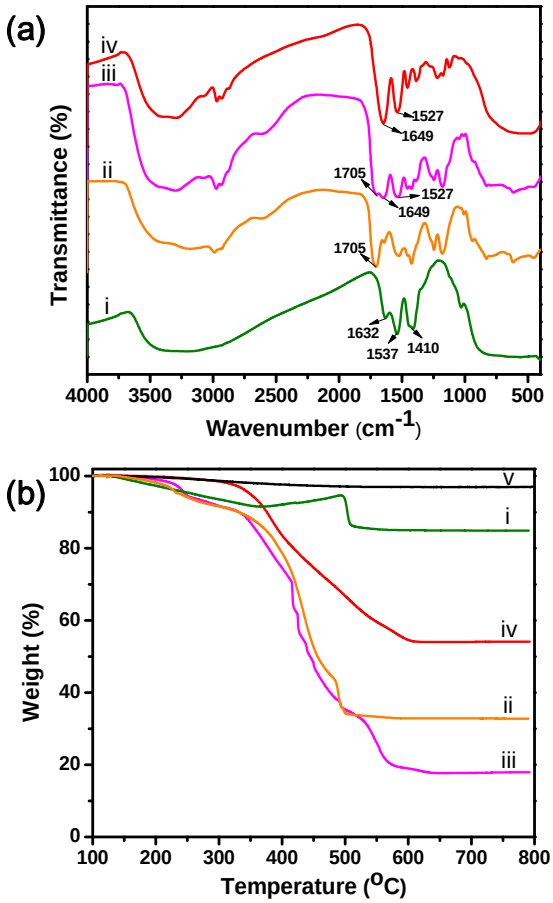


Fig. 2 (a) FT-IR spectra and (b) TGA curves of (i) TiO_2, (ii) TiO_2@PMAA, (iii) TiO_2@PMAA@P(NIPAM-co-MBA), (iv) rattle-type mTiO_2@P(NIPAM-co-MBA) and (v) mTiO_2.

To quantitatively determinate the composition of these composite microspheres, thermogravimetric analysis (TGA) was executed (Fig. 2b). The 15.1 wt% weight loss of TiO_2 is attributed to the dehydration reaction of hydroxyls, indicating that the TiO_2 contains a considerable amount of hydroxyls. The uncommon segment between 400 ^oC and 500 ^oC is caused by the phase transition.[31] After the coating by PMAA layer, the weight loss dramatically increased to 67.2 wt%. Through calculation, the weight percentage of PMAA layer is about 61.4% of the total weight of TiO_2@PMAA. When the P(NIPAM-co-MBA) shell was introduced to the system, the weight loss continuously increased to 82.2 wt%, which suggests that the TiO_2@PMAA@P(NIPAM-co-MBA) is composed of 21 wt% of TiO_2, 33.3 wt% of PMAA and 45.7 wt% of P(NIPAM-co-MBA). Besides the PMAA layer was eliminated, another important transition for rattle-type mTiO_2@P(NIPAM-co-MBA) is that the hydroxyls of TiO_2 has reacted with each other during the hydrothermal process. This fact could be powerfully proved by the TGA curve of mTiO_2, which was prepared by directly treating the TiO_2 microspheres under the same hydrothermal condition and used as a reference. The morphorlogy and structure of mTiO_2 (Fig. 1f) is same as the mTiO_2 core in rattle-type mTiO_2@P(NIPAM-co-MBA). Because the mTiO_2 core nearly has no weight loss, the 45.9 wt% weight loss of rattle-type mTiO_2@P(NIPAM-co-MBA) is ascribed to the weigh percentage of the P(NIPAM-co-MBA) shell. By comparing the weight ratio of the P(NIPAM-co-MBA) shell to the TiO_2 core in rattle-type mTiO_2@P(NIPAM-co-MBA) and that in

TiO_2@PMAA@P(NIPAM-co-MBA), it could be found that some non-crosslinked PNIPAM or PMBA chains were also simultaneously removed.

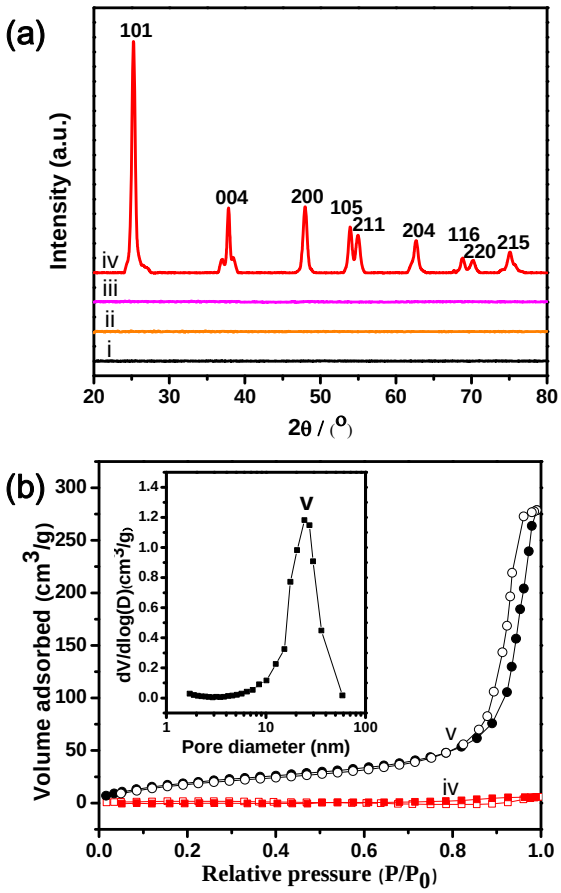
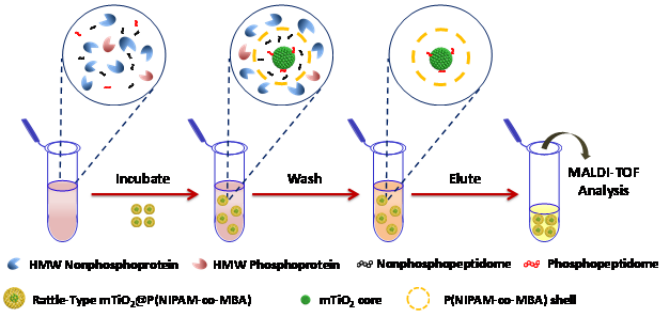


Fig. 3 (a) X-Ray diffraction patterns of (i) TiO_2, (ii) TiO_2@PMAA, (iii) TiO_2@PMAA@P(NIPAM-co-MBA) and (iv) rattle-type mTiO_2@P(NIPAM-co-MBA); (b) nitrogen adsorption–desorption isotherms (●=adsorption, ○=desorption) curves of (iv) rattle-type mTiO_2@P(NIPAM-co-MBA) and (v) mTiO_2 (the inset is the BJH pore-size distribution of mTiO_2).

The crystallization transition before and after the hydrothermal process was further studied by powder X-ray diffraction. As shown in Fig. 3a, prior to the hydrothermal process, TiO_2, TiO_2@PMAA and TiO_2@PMAA@P(NIPAM-co-MBA) all have no signal peak, which is indicative of the amorphous TiO_2 in all the three kinds of microspheres. However after hydrothermal process for 24 h, the PXRD pattern for the synthesized rattle-type mTiO_2@P(NIPAM-co-MBA) microspheres was noticeably different from the former patterns, distinct XRD peaks were clearly observed at 2θ values of 25.2°, 37.8°, 48.0°, 53.9°, 55.0°, 62.7°, 68.8°, 70.2°, and 75.1°, which are well assigned to the (101), (004), (200), (105), (211), (204), (116), (220), and (215) crystallographic planes of an anatase-phase TiO_2 (JCPDS card No. 21-1272). The rattle-type mTiO_2@P(NIPAM-co-MBA) microspheres were subjected to further study of the porosity of the mTiO_2 core, by means of nitrogen sorption analysis performed at 77 K. As shown in Fig. 3b, the specific surface area and the pore volume of the rattle-type mTiO_2@P(NIPAM-co-MBA) are all very low (only about 5 m^2/g and 0.009 cm^3/g respectively). The reason is that the polymer shell collapsed when the sample was dried, so the pore of the mTiO_2 core was blocked by the polymer shell. However, the mTiO_2 exhibited type-IV gas sorption isotherm, which were indicative of the mesoporous character. According to calculations made using the BET model, the mTiO_2 resulted in specific surface area of 77.3 m^2/g and pore volumes of 0.43 cm^3/g. The corresponding pore-size distribution was evaluated using the Barrett-Joyner-Halenda (BJH) model, and the average pore diameter is calculated to be about 19 nm (Fig. 3b inset). Considering the result together with TEM image as well as the PXRD patterns, the creation of the mesopore should be attributed to the tiny slit between the neighboring nanocrystals in both the mTiO_2 and mTiO_2 core. The crystalline and mesoporous structure will lead to a much better selectivity and capacity for the mTiO_2 in enrichment of phosphopeptidome than its solid counterpart.[32]

The requirements of the material used for extraction of phosphopeptidome can be summarized as the following two points.[27] One is that the material should have high selectivity toward phosphopeptides and LMW phosphoproteins enrichment. The other is that the material should have an excellent capability to achieve the size exclusion effect. The procedures of phosphopeptidome study using rattle-type mTiO_2@P(NIPAM-co-MBA) are illustrated in Scheme 2. When both proteins and peptides were mixed with rattle-type mTiO_2@P(NIPAM-co-MBA), HMW proteins were excluded by the crosslinking gel network due to the size exclusion effect, while peptidome were allowed to pass through the P(NIPAM-co-MBA) shell and reach the surface of the mTiO_2 core. With the aid of the strong interaction between the phosphoric acid groups and TiO_2, the phosphopeptidome was anchored onto the surface of the TiO_2 nanocrystals in the mTiO_2 core and other nonphosphopeptidome could be then washed away. Through centrifugal separation, the phosphopeptidome-captured microspheres could be well isolated from the mixture and then the adsorbed phosphopeptidome was desorbed for further mass spectrometric analysis.



Scheme 2 Schematic illustration of the typical process for selective extraction of phosphopeptidome by using rattle-type mTiO_2@P(NIPAM-co-MBA) microspheres.

To test the specificity of rattle-type mTiO_2@P(NIPAM-co-MBA) in phosphopeptidome enrichment, tryptic digest of standard phosphoprotein β-casein was mixed with digest of standard non-phosphoprotein BSA at a molar ratio of 1:100. The standard phosphoprotein β-casein harbors three phosphorylated sites, and it would generate three phosphopeptides after trypsin digestion, including m/z at 2061.83, 2556.09, and 3122.27 in the MALDI spectrum. In a typical enrichment procedure, the β-casein digest and BSA digest were first dissolved in a 100 μL loading buffer consisting of 50% acetonitrile containing 5% trifluoroacetic acid (TFA), and the solution was then mixed with rattle-type mTiO_2@P(NIPAM-co-MBA). The rattle-type mTiO_2@P(NIPAM-co-MBA) with captured phosphopeptides

were separated from the mixed solution by centrifugation and washed with the washing buffer (50% ethanol containing 5% TFA) several times to remove nonspecifically adsorbed nonphosphopeptides. Finally, the phosphopeptides were eluted from the rattle-type $mTiO_2$@P(NIPAM-co-MBA) with 10 μL of 5% $NH_3 \cdot H_2O$, and 4 μL of this solution was used for MALDI-TOF MS analysis. Before enrichment, the spectrum is dominated by nonphosphopeptides without the detection of any phosphopeptides (Fig. 4a). After selective enrichment, signals of the three phosphopeptides could be easily detected with a very clean background, shown in Fig. 4b. This result clearly proved that the peptides could pass through the P(NIPAM-co-MBA) gel network shell and the rattle-type $mTiO_2$@P(NIPAM-co-MBA) owns high enrichment selectivity toward phosphopeptides. As we known, the concentration of biological active peptides is always at an extremely low level. Therefore, the enrichment sensitivity of the rattle-type $mTiO_2$@P(NIPAM-co-MBA) was investigated, as illustrated in the MALDI mass spectrum shown in Fig. 4c. The targeted three phosphopeptides could be easily enriched and detected at a signal-to-noise ratio of 55, 164 and 42 for the phosphopeptide with m/z of 2061.83, 2556.09, and 3122.27 respectively, even when the total amount of β-casein was decreased to only 1 fmol·μL^{-1}.

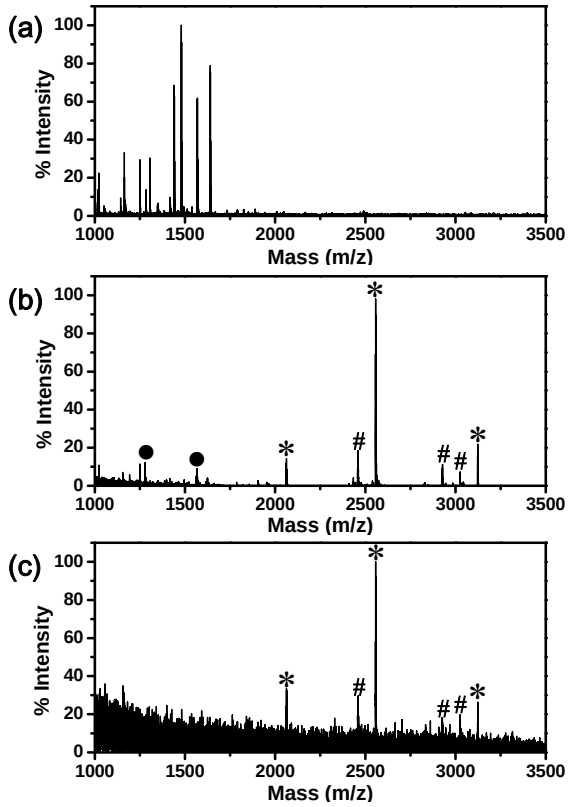


Fig. 4 MALDI mass spectra of the tryptic digest mixture of β-casein and BSA (with a molar ratio of β-casein to BSA of 1:100): (a) direct analysis, (b) analysis after enrichment using rattle-type $mTiO_2$@P(NIPAM-co-MBA). (c) MALDI mass spectrum of tryptic digests of β-casein (1 nM, 2 mL), after enrichment. "*" indicates phosphorylated peptides, "#" indicates their dephosphorylated counterparts and "●" indicates their doubly charged phosphorylated peptides, respectively.

To investigate the size-exclusion capability of P(NIPAM-co-MBA) gel network shell, tryptic digest of β-casein was mixed into a protein mixture including a standard phosphoprotein of β-casein (the molecular weight is 24 kDa), a standard nonphosphoprotein of BSA (the molecular weight is 66 kDa) and a standard nonphosphoprotein of cytochrome c (the molecular weight is 11 kDa) with a mass ratio of 1:10:100:100. Before enrichment, the mixture was directly analyzed with MALDI-MS, and the signals of peptides were too weak to be detected because of the interference of large amount of proteins (Fig. 5a). After enrichment, the phosphopeptides were eluted from rattle-type $mTiO_2$@P(NIPAM-co-MBA) and subjected to mass spectrometric analysis. As the mass spectrum (Fig. 5b) revealed, low-abundant phosphopeptides were efficiently fished out from the mixture with a prominent signal to noise ratio. The size-exclusion effect definitely helped the sensitive detection of phosphopeptides from complex sample.

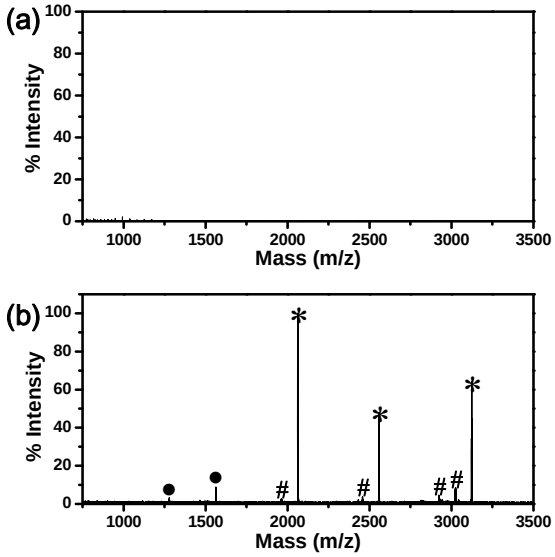


Fig. 5 MALDI mass spectra of tryptic digest of β-casein in the mixture of proteins containing β-casein, BSA and Cyto C (mass ratio of 1:10:100:100): (a) before and (b) after enrichment with rattle-type $mTiO_2$@P(NIPAM-co-MBA). "*", "#" and "●" indicate phosphorylated peptides, their dephosphorylated counterparts and double charged counterparts, respectively.

In order to further verify the size-exclusion effect against proteins and identify the molecular range at which the analyte could pass through the P(NIPAM-co-MBA) gel network shell freely, we measured the adsorption capability of rattle-type $mTiO_2$@P(NIPAM-co-MBA) toward both nonphosphoproteins and phosphoproteins with different molecular weight by SDS-PAGE analysis. Before adding the rattle-type $mTiO_2$@P(NIPAM-co-MBA), BSA, Cyto C, β-casein and ASF (phosphoprotein with a molecular weight of 38 kDa) were mixed with a mass ratio of nearly 1 : 1 : 1 : 1. After enrichment by rattle-type $mTiO_2$@P(NIPAM-co-MBA), the stock solution, supernatant, eluate as well as residue on material were collected and lyophilized for SDS-PAGE analysis. The protein adsorption capability of rattle-type $mTiO_2$@P(NIPAM-co-MBA) was evaluated by comparing the difference in densities of each protein collected from the above-mentioned four steps. As shown in Fig. 6, BSA, Cyto C and ASF were effectively excluded by rattle-type $mTiO_2$@P(NIPAM-co-MBA). Although the molecular weight of Cyto C is smaller than β-casein, rattle-type $mTiO_2$@P(NIPAM-co-MBA) did not adsorb Cyto C due to $mTiO_2$ core only selectively capture phosphoproteins. It is worth noting that small amount of β-

casein was found in the eluate, which indicates that these β-casein could partially pass through the P(NIPAM-co-MBA) shell. The partial adsorption of β-casein suggests that the critical exclusion molecular weight of the P(NIPAM-co-MBA) network shell is around 24 kDa, which perfectly meet the requirement of molecular weight range in the peptidome research (<20 kDa). All these results above clearly demonstrate that the rattle-type $mTiO_2$@P(NIPAM-co-MBA) possesses the desired great size-exclusion effect against both HMW nonphosphoproteins and HMW phosphoproteins.

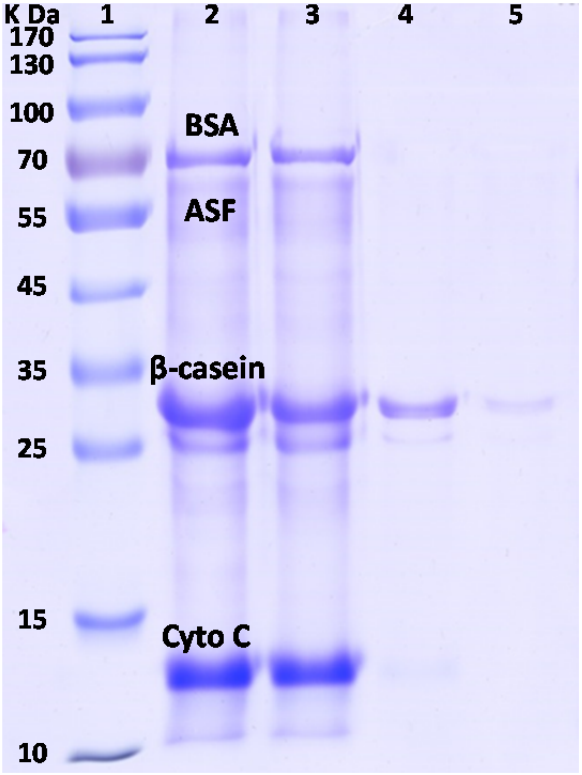


Fig. 6 SDS-PAGE analysis of standard protein mixtures (BSA + ASF + β-casein + Cyto C) before and after treatment with rattle-type $mTiO_2$@P(NIPAM-co-MBA). (Lane 1: marker; Lane 2: protein mixture (BSA + ASF + β-casein + Cyto C) before treatment; Lane 3: the supernatant of protein mixture (BSA + ASF + β-casein + Cyto C) after treatment by rattle-type $mTiO_2$@P(NIPAM-co-MBA); Lane 4: the eluate from rattle-type $mTiO_2$@P(NIPAM-co-MBA); Lane 5: the residual on the rattle-type $mTiO_2$@P(NIPAM-co-MBA).

Conclusions

In summary, we have presented a facile and repeatable synthetic route for the preparation of rattle-type $mTiO_2$@P(NIPAM-co-MBA) with well-defined core/void/shell structure and desired functionality. The non-crosslinked PMAA sacrificial layer plays a key role in the successful preparation of the target material. Taking advantages of the excellent size-exclusion effect against high-molecular-weight proteins offered by the polymer gel network shell as well as the remarkable enrichment capability toward phosphopeptides of the $mTiO_2$ core, the rattle-type $mTiO_2$@P(NIPAM-co-MBA) microspheres exhibited great capabilities for one-step selective extraction of phosphopeptidome from complicated samples containing large amount of high-molecular-weight proteins and nonphosphopeptides. In addition, we believe that our strategy could also be applied in selective extraction of peptidome and other modification-specific peptidome by simply alternating the $mTiO_2$ core with mesoporous carbon core or some other specific cores.

Acknowledgements

This work was supported by the National Science and Technology Key Project of China (2012AA020204 and 2012CB910602), National Science Foundation of China (Grant No. 21025519 and 21034003) and Shanghai Projects (Grants Eastern Scholar, 13520720200, 13JC1400500 and B109).

Notes and references

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† Wanfu Ma and Ying Zhang contributed equally to this work.

1 L. A. Liotta, M. Ferrari and E. Petricoin, *Nature*, 2003, **425**, 905-905.
2 E. F. Petricoin, C. Belluco, R. P. Araujo and L. A. Liotta, *Nat. Rev. Cancer*, 2006, **6**, 961-967.
3 E. P. Diamandis, *J. Proteome Res.*, 2006, **5**, 2079-2082.
4 G. Menschaert, T. T. M. Vandekerckhove, G. Baggerman, L. Schoofs, W. Luyten and W. Van Criekinge, *J. Proteome Res.*, 2010, **9**, 2051-2061.
5 W. H. Robinson and L. Steinman, *Nat. Biotechnol.*, 2011, **29**, 500-502.
6 L. Zhao, H. Q. Qin, R. A. Wu and H. F. Zou, *J. Chromatogr. A*, 2012, **1228**, 193-204.
7 J. Z. Chen, M. Anderson, D. E. Misek, D. M. Simeone and D. M. Lubman, *J. Chromatogr. A*, 2007, **1162**, 117-125.
8 R. Vitorino, A. S. Barros, A. Caseiro, R. Ferreira and F. Amado, *Talanta*, 2012, **94**, 209-215.
9 T. C. Li, S. P. Dai, Z. M. Wang and H. Q. Zhang, *Electrophoresis*, 2010, **31**, 1090-1096.
10 R. S. Tirumalai, K. C. Chan, D. A. Prieto, H. J. Issaq, T. P. Conrads and T. D. Veenstra, *Mol. Cell. Proteomics*, 2003, **2**, 1096-1103.
11 K. L. Johnson, C. J. Mason, D. C. Muddiman and J. E. Eckel, *Anal. Chem.*, 2004, **76**, 5097-5103.
12 X. Y. Zheng, H. Baker and W. S. Hancock, *J. Chromatogr. A*, 2006, **1120**, 173-184.
13 D. W. Greening and R. J. Simpson, *J. Proteomics*, 2010, **73**, 637-648.
14 R. J. Tian, H. Zhang, M. L. Ye, X. G. Jiang, L. H. Hu, X. Li, X. H. Bao and H. F. Zou, *Angew. Chem. Int. Ed.*, 2007, **46**, 962-965.
15 R. J. Tian, L. B. Ren, H. J. Ma, X. Li, L. H. Hu, M. L. Ye, R. Wu, Z. J. Tian, Z. Liu and H. F. Zou, *J. Chromatogr. A*, 2009, **1216**, 1270-1278.
16 A. Bouamrani, Y. Hu, E. Tasciotti, L. Li, C. Chiappini, X. W. Liu and M. Ferrari, *Proteomics*, 2010, **10**, 496-505.

17 S. S. Liu, Y. Li, C. H. Deng, Y. Mao, X. M. Zhang and P. Y. Yang, *Proteomics*, 2011, **11**, 4503-4513.
18 H. Q. Qin, P. Gao, F. J. Wang, L. Zhao, J. Zhu, A. Q. Wang, T. Zhang, R. Wu and H. F. Zou, *Angew. Chem. Int. Ed.*, 2011, **50**, 12218-12221.
19 F. Li, B. Dever, H. Q. Zhang, X. F. Li and X. C. Le, *Angew. Chem. Int. Ed.*, 2012, **51**, 3518-3519.
20 K. Qian, W. Y. Gu, P. Yuan, F. Liu, Y. H. Wang, M. Monteiro and C. Z. Yu, *Small*, 2012, **8**, 231-236.
21 P. Yin, Y. H. Wang, Y. Li, C. H. Deng, X. M. Zhang and P. Y. Yang, *Proteomics*, 2012, **12**, 2784-2791.
22 L. Zhang, S. B. Wu, C. Lia and Q. H. Yang, *Chem. Commun.*, 2012, **48**, 4190-4192.
23 G. T. Zhu, X. S. Li, Q. Gao, N. W. Zhao, B. F. Yuan and Y. Q. Feng, *J. Chromatogr. A*, 2012, **1224**, 11-18.
24 H. Wan, H. Q. Qin, Z. C. Xiong, W. B. Zhang and H. F. Zou, *Nanoscale*, 2013, **5**, 10936-10944.
25 G. T. Zhu, X. M. He, X. S. Li, S. T. Wang, Y. B. Luo, B. F. Yuan and Y. Q. Feng, *J. Chromatogr. A*, 2013, **1316**, 23-28.
26 L. T. Liu, Y. Zhang, L. Zhang, G. Q. Yan, J. Yao, P. Y. Yang and H. J. Lu, *Anal. Chim. Acta*, 2012, **753**, 64-72.
27 L. H. Hu, H. J. Zhou, Y. H. Li, S. T. Sun, L. H. Guo, M. L. Ye, X. F. Tian, J. R. Gu, S. L. Yang and H. F. Zou, *Anal. Chem.*, 2009, **81**, 94-104.
28 H. Q. Qin, F. J. Wang, P. Y. Wang, L. Zhao, J. Zhu, Q. H. Yang, R. A. Wu, M. L. Ye and H. F. Zou, *Chem. Commun.*, 2012, **48**, 961-963.
29 X. S. Li, Y. N. Pan, Y. Zhao, B. F. Yuan, L. Guo and Y. Q. Feng, *J. Chromatogr. A*, 2013, **1315**, 61-69.
30 S. Jin, Y. J. Pan and C. C. Wang, *Acta Chim. Sinica*, 2013, **71**, 1500-1504.
31 S. Eiden-Assmann, J. Widoniak and G. Maret, *Chem. Mater.*, 2004, **16**, 6-11.
32 W. F. Ma, Y. Zhang, L. L. Li, L. J. You, P. Zhang, Y. T. Zhang, J. M. Li, M. Yu, J. Guo, H. J. Lu and C. C. Wang, *Acs Nano*, 2012, **6**, 3179-3188.

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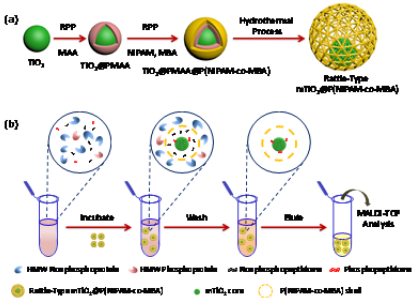

Rattle-type $mTiO_2$@P(NIPAM-co-MBA) microspheres with a screening gel network shell were elaborately designed and fabricated for highly extraction of endogenous phosphopeptidome.