Dendritic Mesoporous Silica Nanoparticles with Abundant Ti$^{4+}$ for Phosphopeptide Enrichment from Cancer Cells with 96% Specificity

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ABSTRACT: Selective enrichment and sensitive detection of phosphopeptides are of great significance in many bioapplications. In this work, dendritic mesoporous silica nanoparticles modified with polydopamine and chelated Ti$^{4+}$ (denoted DMSNs@PDA-Ti$^{4+}$) were developed to improve the enrichment selectivity of phosphopeptides. The unique central-radial pore structures endowed DMSNs@PDA-Ti$^{4+}$ with a high surface area (362 m$^2$ g$^{-1}$), a large pore volume (1.37 cm$^3$ g$^{-1}$), and a high amount of chelated Ti$^{4+}$ (75 μg mg$^{-1}$). Compared with conventional mesoporous silica-based materials with the same functionalization (denoted mSiO$_2$@PDA-Ti$^{4+}$) and commercial TiO$_2$, DMSNs@PDA-Ti$^{4+}$ showed better selectivity and a lower detection limit (0.2 fmol/μL). Moreover, 2422 unique phosphopeptides were identified from HeLa cell extracts with a high specificity (>95%) enabled by DMSNs@PDA-Ti$^{4+}$, better than those in previous reports.

Phosphorylation is a widely found protein post-translational modification, involved in many important cellular activities including signaling transduction, molecular recognition, and metabolic processes.2–5 Nearly one-third of all proteins in eukaryotes are phosphoproteins, which make potential biomarkers with clinical significance.6–8 Investigation of protein phosphorylation under different physiological conditions is important in understanding the signaling pathways, the mechanism of disease generation, and diagnosis,9,10 where sensitive detection of phosphorylated proteins is the key step.11–13 Currently, mass spectrometry (MS)-based technologies are the primary analytical tools for accurately identifying and locating phosphorylation sites due to their high sensitivity and strong sequence analysis capability.14,15 However, the low abundance and low ionization efficiency of phosphopeptides make them difficult to be identified by direct MS analysis.16–19 Therefore, separation and enrichment of phosphopeptides from complicated samples prior to MS analysis is necessary.

To date, many materials and techniques have been developed for phosphopeptide enrichment. Immobilized metal ion affinity chromatography (IMAC) is one of the most widely used enrichment techniques in phosphoproteomics analysis. Nevertheless, the major limitation of this method is the high level of nonspecific binding of acidic peptides.20–23 Although much effort has been devoted to improving the enrichment selectivity of IMAC materials, the results are still unsatisfactory. The enrichment selectivity of IMAC materials mainly depends on the hydrophilicity of materials and the amount of the immobilized metal ions. On one hand, phosphopeptides show higher hydrophilicity than other peptides;24–27 thus, increasing the hydrophilicity of the materials can improve the enrichment selectivity of phosphopeptides. On the other hand, IMAC materials primarily rely on the electrostatic interactions between phosphate groups and metal ions (e.g., Ti$^{4+}$, Fe$^{3+}$, Zn$^{2+}$, Ga$^{3+}$, and Zr$^{4+}$);28–30 thus, the more the immobilized metal ions, the better the enrichment selectivity. Therefore, an IMAC material with the above properties is anticipated to have excellent performance for phosphopeptide enrichment.

In order to increase the amount of the immobilized metal ions and subsequent phosphopeptide entrapment, high surface area and suitable pore structures are both important for candidate materials. Although mesoporous silica materials with high surface areas have been widely used in the synthesis of IMAC materials, most of them have relatively small pore sizes.

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Small mesopores could be easily blocked in the following modification process. In addition, for small pores with long pore length, the so-called “shadow effect” would hamper the diffusion and release of target phosphopeptides. In this regard, dendritic mesoporous silica nanoparticles (DMSNs), a new type of mesoporous silica material, are considered as an optimal alternative to conventional mesoporous silica. DMSNs have a unique central-radial pore structure, large pore sizes, and highly accessible surface areas; thus, polymers can be easily modified onto the inner wall of the pores without significant pore blocking, thereby increasing the binding amount of chelating ligands. Simultaneously, the large pore size and short pore length can prevent the “shadow effect” and provide efficient mass transportation.

Herein, a novel IMAC material, dendritic mesoporous silica nanoparticles modified with PDA and chelated Ti^{4+} (denoted as DMSNs@PDA-Ti^{4+}), has been synthesized for phosphopeptide enrichment. DMSNs@PDA-Ti^{4+} exhibit a large pore size of 18.8 nm, a surface area of 362 m^{2}/g, a pore volume of 1.37 cm^{3}/g, and a high chelated Ti^{4+} amount of 75 μg mg^{-1}. The efficacy of DMSNs@PDA-Ti^{4+} has been compared with conventional mesoporous silica-based materials (named mSiO_{2}@PDA-Ti^{4+}) and commercial TiO_{2} for phosphopeptide enrichment in various biological samples, including standard phosphoprotein, nonfat milk, human serum, and HeLa cell extracts. DMSNs@PDA-Ti^{4+} demonstrate a low phosphopeptide detection limit of 0.2 fmol/μL and an extremely high specificity (>95%) of phosphopeptides identified from HeLa cell extracts, showing great promise for phosphoproteome studies.

**EXPERIMENTAL SECTION**

**Materials and Chemicals.** Cetyltrimethylammonium bromide (CTAB), tetraethoxysilicate (TEOS), triethanolamine (TEA), sodium salicylate (NaSal), dopamine hydrochloride (DA-HCl), and Ti(SO_{4})_{2} were purchased from Aladdin. Ammonium bicarbonate (NH_{4}HCO_{3}), urea, protease inhibitor cocktail (components: AEBSF, Aprotinin, Bestatin, E-64, Leupeptin, Pepstatin A, Triton X-100, β-glycerophosphate disodium salt hydrate (C_{3}H_{7}Na_{2}PO_{6}), sodium pyrophosphate tetrasodium (Na_{2}P_{2}O_{7}), sodium fluoride (NaF), sodium orthovanadate (Na_{3}VO_{4}), α-casein (from bovine milk), 2,5-dihydroxybenzoic acid (DHB), bovine serum albumin (BSA), and trypsin were obtained from Sigma-Aldrich. Acetonitrile (ACN) and trifluoroacetic acid (TFA) were chromatographic grade. Commercial TiO_{2} (5 μm) was produced by Shimagdu. Nonfat milk was bought from a local shop. Water used in experiments was purified using a Milli-Q system (Millipore, Bedford, MA). All other reagents used in this research were of analytical grade without further purification.

**Preparation of DMSNs@PDA-Ti^{4+} and mSiO_{2}@PDA-Ti^{4+}**. DMSNs were synthesized according to a literature method. Typically, 0.136 g of TEA was added to 50 mL of deionized water. The solution was stirred at 80 °C in an oil bath for 0.5 h. Afterward, 0.76 g of CTAB and 0.336 g of NaSal were added and kept under stirring for another 1 h. Then, 8 mL of TEOS was added to the water—CTAB NaSal—TEA solution under a gentle stirring rate of 200 rpm for 2 h. The products were collected by centrifugation and washed with ethanol three times. Finally, the obtained products were extracted with HCl and methanol solution at 65 °C three times to remove the template and dried in vacuum at room temperature overnight. The synthesis of mSiO_{2} was similar to that of DMSNs, except NaSal was not added during the reaction.

The dried DMSNs and mSiO_{2} were modified with PDA by oxidative self-polymerization of DA in a mild condition. Briefly, 0.4 g of DA-HCl was dissolved in 200 mL of Tris buffer (10 mM, pH 8.5) by 5 min ultrasonication, and then 0.2 g of DMSNs or mSiO_{2} was added and sonicated for another 5 min. The above solution was transferred into a 500 mL flask and stirred for 12 h at 30 °C. The products were collected by centrifugation and washed with water several times.

Finally, the DMSNs@PDA or mSiO_{2}@PDA were incubated in Ti(SO_{4})_{2} solution (100 mM) for 2 h to immobilize Ti^{4+} cations. The products were washed with deionized water several times and then lyophilized to dryness.

**Characterization.** Transmission electron microscopy (TEM) images were carried out by JEOL JEM-1400 transmission electron microscope (JEOL, Japan). Field emission scanning electron microscope (FE-SEM) images were obtained with Nova NanoSEM 450 field emission scanning electron microscope (FEI, USA). The elemental mapping patterns were obtained using a XM-2 EDS (EDAX, USA) equipped with a Tecnai G2 F30 S-TWIN field emission transmission electron microscope (FEI, USA). Fourier transform infrared (FT-IR) spectra were performed on a Nicolet 6700 using KBr pellets (Thermo Fisher Scientific, USA). Thermogravimetric analysis (TGA, Pyris 1 TGA, PerkinElmer, USA) was carried out with a Varian ICP-710ES instrument (Varian, USA). Nitrogen adsorption−desorption isotherms were measured on an ASAP 2020 apparatus (Micrometritics, USA). The specific surface area and pore volume were determined by the Brunauer−Emmett−Teller (BET) method and the Barrett−Joyner−Halenda (BJH) model, respectively.

**Preparation of Protein Digestion.** α-Casein (1 mg) was dissolved in 1.0 mL of ammonium bicarbonate buffer (50 mM, pH 8.2) and digested by trypsin for 17 h at 37 °C with an enzyme/substrate ratio of 1:25 (w/w).

BSA (1 mg) was dissolved in 1 mL of denaturing buffer (8 M urea, 50 mM NH_{4}HCO_{3}, pH 8.2). Afterward, the protein solution was incubated with 5 μL of dithiothreitol (200 mM) at 56 °C for 45 min. Then, 20 μL of iodoacetamide (200 mM) was added and incubated for another 30 min in the dark. The protein mixture was digested by trypsin at an enzyme/substrate ratio of 1:25 (w/w) for 17 h at 37 °C. The protein digests were lyophilized to dryness and stored in a cryogenic refrigerator at −80 °C before use.

Human serum samples were collected from healthy people and obtained from the Sixth People’s Hospital affiliated with Shanghai Jiaotong University according to the standard clinical procedures. The serum sample was stored at −80 °C and used for endogenous phosphopeptide enrichment without further treatment.

Nonfat milk (30 μL) was diluted with 1 mL of NH_{4}HCO_{3} (25 mM, pH = 8.0), and then the mixtures were centrifuged at 16 000 g for 15 min. The supernatant was collected and denatured at 100 °C for 10 min. Subsequently, the mixtures were treated with 40 μg of trypsin for 17 h at 37 °C. The mixtures were processed as follows:...
obtained proteolytic digests were stored at −80 °C for further use.

HeLa cells were cultured to 90% confluence in 15 cm diameter dishes. After being washed twice with ice-cold PBS buffer (pH 7.4), the cells were resuspended in lysis buffer (8 M urea, 50 mM Tris-HCl, pH 7.4) consisting of 2% protease inhibitor cocktail, 1% Triton X-100, 1 mM C6H12Na6PO7, 1 mM Na2PO4, 1 mM NaF, and 1 mM Na3VO4. The suspension was then sonicated for 180 s (3 s sonication was followed with 3 s interval) in ice. The cell debris was removed by centrifugation at 15 000 g at 4 °C for 20 min. The supernatant was collected, and five volumes of precipitation solution (acetonitrile/ethanol/acetic acid, 50/49.9/0.1, v/v/v) was added for precipitation the protein at −20 °C for at least 2 h and then centrifuged at 15 000 g at 4 °C for 20 min to collect the protein precipitate.

Subsequently, the obtained protein pellet was resuspended in 50 mM NH4HCO3 buffer, and the concentration of the protein was measured by Bradford assay. The procedure of the protein digestion was the same as that of BSA.

**Selective Enrichment of Phosphopeptides from Protein Digests and Tryptic Digests of HeLa Cell Extracts.** Twenty micrograms of DMSNs@PDA-Ti4+ were added into 200 μL of loading buffer (ACN/H2O/TFA, 50/49.9/0.1, v/v/v) containing α-casein tryptic digest (2 μL), human serum (2 μL), or proteins extracted from nonfat milk (2 μL), and the mixture was incubated at room temperature for 30 min. Subsequently, the materials were collected by centrifugation and rinsed with 100 μL of washing buffer (ACN/H2O/TFA, 50/49.9/0.1, v/v/v) three times. Then the phosphopeptides were eluted from the materials with 10 μL of NH4OH solution (0.4 M) for 20 min incubation. The eluent was collected by centrifugation and analyzed by MALDI-TOF MS.

For HeLa cell extracts enrichment, 200 μg HeLa cell extracts were diluted with 500 μL of loading buffer (DMSNs@PDA-Ti4+; 1% TFA in 1:1 ACN-H2O; commercial TiO2; 0.1% TFA, 2 M lactic acid solution in 1:1 ACN-H2O), and then 2 mg of DMSNs@PDA-Ti4+ or commercial TiO2 was respectively added for phosphopeptide enrichment. The following enrichment procedure was the same as above. The eluent was collected and lyophilized for nano-LC-MS/MS analysis.

**MALDI-TOF MS Analysis.** One microliter analytes were dropped on the plate, and then 1 μL of matrix (DHB, 25 mg/mL, ACN/H2O/Po43− 70:29:1, v/v/v) was dropped and analyzed by MALDI-TOF MS. All the MALDI-TOF MS analyses were performed on a 4800 plus MALDI-TOF MS (AB Sciex, USA) with a Nd:YAG laser at 355 nm, a laser pulse frequency of 200 Hz, and an acceleration voltage of 20 kV in reflect positive ion mode. The scan range was 1000−3500 m/z.

**Nano LC-MS/MS Analysis.** The nano LC-MS/MS analyses were performed on a Dionex UltiMate 3000 RSLCnano system (Thermo Scientific, USA) with a Q-Exactive mass spectrometer (Thermo Scientific, USA). The lyophilized peptides were resuspended in 1% FA/H2O and automatically loaded onto a 15 cm C18 trap column (200 μm i.d.) at a flow rate of 5 μL/min. Subsequently, the peptides were separated on a reverse C18 capillary analytical column (75 μm × 15 cm) under a linear gradient elution condition. For the capillary analytical column (75 μm i.d.), one end of the fused-silica capillary was first manually pulled to a fine point with a spray tip and then packed in-house with C18 AQ particles (5 μm, 120 Å). For RPLC separation, mobile phase A (0.1% FA in H2O) and mobile phase B (0.1% FA in 80% ACN) were used to establish the linear gradient elution method which was carried out as follows: 0−4% mobile phase B for 2 min; 4−35% B for 90 min; 35−45% B for 10 min; 45−90% B for 5 min; 90% B for 5 min; and finally equilibration with mobile phase A for 15 min. The flow rate was adjusted to ∼300 nL/min.

The Q-Exactive mass spectrometer was operated in data-dependent MS/MS acquisition mode. The electrospray voltage was set to 2.0 kV, and the temperature of the ion transfer capillary was set as 250 °C. The full MS scan acquired in the Orbitrap mass analyzer was from m/z 400 to 2000 with a mass resolution of 70 000 (m/z 200). The 12 most intense parent ions with charge states ≥2 from the full scan were fragmented by higher-energy collisional dissociation (HCD) with a normalized collision energy (NCE) of 27%. The MS/MS acquisitions were performed in Orbitrap with a resolution of 35 000 (m/z 200), and the automatic gain control (AGC) target was set to 1 × 105 with a max injection time of 120 ms. Dynamic exclusion was set as 30 s. System control and data collection were carried out by Xcalibur software.

**Database Retrieval and Data Analysis.** The MS raw data files were exported using the Proteome Discoverer software (Thermo Fisher Scientific, version 1.4.0.288), and the MS/MS results were searched by Mascot (Matrix Science, London, U.K.; version 2.3) against a Uniprot-SwissProt database (taxonomy: human, 20201 entries). The data retrieval parameters were as follows: precursor-ion mass tolerance, 10 ppm; fragment-ion mass tolerance, 0.05 Da. Two missed cleavages were allowed by trypsin digestion. Carbamidomethyl of cysteine (C) was set as fixed modification. Oxidation of methionine (M) and phosphorylation of serine/threonine/tyrosine (S/T/Y) were set as the variable modifications. Peptide level false discovery rates (FDR) were controlled lower than 1% by the percolator algorithm. Finally, the PhosphoRS 3.0 was used to determine the probability of phosphorylation sites.

## RESULTS AND DISCUSSION

### Synthesis and Characterization of DMSNs@PDA-Ti4+

The synthesis procedure for DMSNs@PDA-Ti4+ is illustrated in Scheme 1a. DMSNs with a central-radial pore structure were synthesized by a sodium salicylate assisted method.37 The pore surface with Si–OH groups was modified with PDA by oxidative self-polymerization of DA in a mild condition. Subsequently, Ti4+ was immobilized on the PDA-modified nanoparticles (DMSNs@PDA) by the chelation of PDA with Ti4+ to obtain DMSNs@PDA-Ti4+.

The morphology and size of the products were observed by TEM and SEM. TEM images (Figure 1a) reveal that DMSNs are monodispersed with a uniform diameter of ~150 nm and obvious central-radial dendritic mesopores. After PDA coating, both DMSNs@PDA and DMSNs@PDA-Ti4+ have no obvious change in particle sizes (Figure 1b, c). SEM images (Figure 1d–f) show that the all three samples possess large pore openings, indicating that the PDA coating and Ti4+ chelating procedures did not block the large mesopores. Scanning TEM (STEM) image (Figure 1g) of DMSNs@PDA-Ti4+ combined with EDS elemental mapping of a single particle (Figure 1h–m) clearly reveal that Si, C, N, O, and Ti elements are homogeneously distributed in DMSNs@PDA-Ti4+. In addition, TEM images of mSiO2 synthesized without NaSal, mSiO2@PDA, and mSiO2@PDA-Ti4+ show typical small mesopores (Figure S1), in accordance with the literature report.47,48

FTIR spectra of DMSNs, DMSNs@PDA, and DMSNs@PDA-Ti4+ were recorded to provide qualitative information for
PDA coating and Ti$^{4+}$ chelating (Figure 2a). Compared to DMSNs, DMSNs@PDA display new characteristic adsorption peaks: the band at 1619 cm$^{-1}$ is attributed to the N–H stretching, and the peaks at 1503 and 1447 cm$^{-1}$ are assigned to benzene ring C–C vibration. After chelating Ti$^{4+}$, a new adsorption peak appearing at 606 cm$^{-1}$ is attributed to Ti–O stretching.

TGA was further utilized to confirm the large capacity of DMSNs for PDA coating. As shown in Figure 2b, the weight ratio of PDA in DMSNs@PDA is 37.04%, while in mSiO$_2$@PDA only 25.49%, indicating that the large and open dendritic mesopores of DMSNs can provide larger capacity for PDA coating than mSiO$_2$. In addition, the mass ratio of DMSNs to DA was optimized during the PDA modification process (see Supporting Information Figure S2), reaching a saturated PDA loading at a mass ratio (DMSN to DA) of 1:2.

In theory, the amount of immobilized Ti$^{4+}$ is proportional to the amount of coated PDA. Thus, ICP-AES was used to quantify the amount of immobilized Ti$^{4+}$. For DMSNs@PDA-Ti$^{4+}$, the amount of Ti$^{4+}$ was as high as 75 $\mu$g g$^{-1}$, which is higher than that of mSiO$_2$@PDA-Ti$^{4+}$ (48 $\mu$g g$^{-1}$) and many previous IMAC nanomaterials, such as Fe$_3$O$_4$@SiO$_2$@PEG-Ti$^{4+}$ (32.98 $\mu$g g$^{-1}$), MCNC@PMAA@PEGMP-Ti$^{4+}$ (41 $\mu$g g$^{-1}$), and Fe$_3$O$_4$@SiO$_2$@(HA/CS)$_{10}$-Ti$^{4+}$ (44.38 $\mu$g g$^{-1}$). These results suggest that the DMSNs@PDA possess strong ability for Ti$^{4+}$ chelating due to the unique structure of DMSNs.

Furthermore, N$_2$ adsorption–desorption was performed to study the texture properties. The surface area of DMSNs was calculated to be 679 m$^2$ g$^{-1}$, and the pore volume was 2.91 cm$^3$ g$^{-1}$, indicating a highly accessible surface area for PDA and Ti$^{4+}$ loading. The pore size of DMSNs was calculated to be 23.2 nm (Figure 2c inset), which could provide enough space to load PDA without significant pore blocking. After modification with PDA and Ti$^{4+}$ (Figure 2d), the DMSNs@PDA-Ti$^{4+}$ still have a high surface area (361 m$^2$ g$^{-1}$) and large pore volume (1.37 cm$^3$ g$^{-1}$) that could provide a large number of affinity sites for phosphopeptide enrichment. In addition, the large pore size of DMSNs@PDA-Ti$^{4+}$ (18.8 nm) could effectively prevent the “shadow effect” from the small and deep pores. For
The surface area of mSiO$_2$ is 436 m$^2$ g$^{-1}$, and the pore volume is 0.89 cm$^3$ g$^{-1}$. After PDA and Ti$^{4+}$ coating, the surface area and pore volume decreased to 174 m$^2$ g$^{-1}$ and 0.36 cm$^3$ g$^{-1}$, respectively, which are lower than that of DMSNs@PDA-Ti$^{4+}$. Selective Enrichment of Phosphopeptides from $\alpha$-Casein Tryptic Digests. To demonstrate the phosphopeptide enrichment efficacy of DMSNs@PDA-Ti$^{4+}$, $\alpha$-casein tryptic digests were selected as the model sample. The procedure for the enrichment of phosphopeptides from protein digestion is shown in Scheme 1b. For comparison, mSiO$_2$@PDA-Ti$^{4+}$ and commercial TiO$_2$ were also used to enrich phosphopeptides from $\alpha$-casein tryptic digests. As shown in Figure 3a, for direct analysis of $\alpha$-casein tryptic digests at a concentration of $4 \times 10^{-7}$ M, merely eight phosphopeptides were detected with weak MS intensity due to the severe interference of the abundant nonphosphopeptides. However, after enrichment by DMSNs@PDA-Ti$^{4+}$ (Figure 3b), almost all of the nonphosphopeptides were removed, and 36 phosphopeptides (the detailed information on the identified phosphopeptides is given in Table S1) were identified with greatly improved signal-to-noise (S/N) ratio. Simultaneously, a majority of the corresponding dephosphorylated peptides with mass loss of 98 Da were also observed. For mSiO$_2$@PDA-Ti$^{4+}$ and commercial TiO$_2$, the number of identified phosphopeptides was reduced to 24 and 18 phosphopeptides, respectively (Figure S4). The numbers of phosphopeptides enriched by the three materials from $\alpha$-casein tryptic digests are directly compared in Figure 4a. The enrichment capacity of the three materials was also investigated using different amounts of nanoparticles to enrich phosphopeptides from a fixed amount of $\alpha$-casein tryptic digests (1 $\mu$g). As shown in Figure 4b, the phosphopeptide loading capacities of DMSNs@PDA-Ti$^{4+}$, mSiO$_2$@PDA-Ti$^{4+}$, and commercial TiO$_2$ were calculated to be about 100, 67, and 40 mg g$^{-1}$, respectively. These results demonstrate that the DMSNs@PDA-Ti$^{4+}$ have an outstanding phosphopeptide enrichment performance and large phosphopeptide loading capacity due to the excellent hydrophilicity, highly accessible surface area, and large amount of immobilized Ti$^{4+}$. The selectivity of the three materials for phosphopeptide enrichment was also compared by MALDI-TOF MS. As shown in Figure 5, when the molar ratio of $\alpha$-casein and BSA was 1:5000 (loading buffer: 50% ACN, 6% TFA), 20 phosphopeptides were identified with a clean background after enrichment by DMSNs@PDA-Ti$^{4+}$ (Figure 5a). However, for mSiO$_2$@PDA-Ti$^{4+}$ (Figure 5b), only eight phosphopeptides with a greatly reduced signal intensity were observed together with some nonphosphopeptides. For commercial TiO$_2$, the spectrum was dominated by nonphosphopeptides, and only four phosphopeptides could be identified (Figure 5c). The number and signal intensity of enriched phosphopeptides by three materials are plotted in Figure 6 for direct comparison, which clearly show that DMSNs@PDA-Ti$^{4+}$ have significantly higher phosphopeptide enrichment selectivity and efficiency than commercial TiO$_2$. 

Figure 3. MALDI-TOF mass spectra of the tryptic digest of $\alpha$-casein ($4 \times 10^{-7}$ M, 200 $\mu$L) (a) before and (b) after enrichment by DMSNs@PDA-Ti$^{4+}$. # indicates dephosphorylated peptides.

Figure 4. (a) Comparison of the number of identified phosphopeptides enriched from $\alpha$-casein and nonfat milk by DMSNs@PDA-Ti$^{4+}$, mSiO$_2$@PDA-Ti$^{4+}$, and commercial TiO$_2$; (b) Comparison of phosphopeptide loading capacity among DMSNs@PDA-Ti$^{4+}$, mSiO$_2$@PDA-Ti$^{4+}$, and commercial TiO$_2$.

Figure 5. MALDI-TOF mass spectra of the tryptic digest mixtures of $\alpha$-casein and BSA after enrichment by (a) DMSNs@PDA-Ti$^{4+}$, (b) mSiO$_2$@PDA-Ti$^{4+}$, and (c) commercial TiO$_2$ at a molar ratio of 1:5000 (loading buffer: 50% ACN, 6% TFA).
mSiO\textsubscript{2}@PDA-Ti\textsuperscript{4+} and commercial TiO\textsubscript{2}, in terms of both the number and signal intensity of captured phosphopeptides. This trend is further confirmed when the acidity of loading buffer was decreased from 6\% to 3\% TFA (see Supporting Information Figure S5).

For sensitivity measurement, different concentrations of α-casein tryptic digests were used to determine the detection limit. As shown in Figure 7, 12 phosphopeptides could be captured by DMSNs@PDA-Ti\textsuperscript{4+} in 1 fmol/μL α-casein tryptic digest, and the minimum detection limit of DMSNs@PDA-Ti\textsuperscript{4+} for phosphopeptide enrichment was as low as 0.2 fmol/μL. However, the minimum detection limits of mSiO\textsubscript{2}@PDA-Ti\textsuperscript{4+} and commercial TiO\textsubscript{2} were 1 and 2 fmol/μL, respectively (Figure S6). Obviously, the DMSNs@PDA-Ti\textsuperscript{4+} have a better selectivity and lower detection limit for phosphopeptide enrichment than the other two materials, which is attributed to the excellent hydrophilicity and high density of immobilized Ti\textsuperscript{4+} as well as the large and accessible mesopores.

Selective Enrichment of Phosphopeptides from Real Biological Samples. DMSNs@PDA-Ti\textsuperscript{4+} with outstanding enrichment efficiency were further used to capture phosphopeptides from complex biological samples. Nonfat milk, human serum, and HeLa cell extracts were selected as real biological samples. For tryptic digests of nonfat milk, 30 phosphopeptides were observed with significantly enhanced S/N ratio (Figure 8b) after enrichment by DMSNs@PDA-Ti\textsuperscript{4+} (peptide sequences are listed in Table S1), whereas the number of phosphopeptides enriched by the other two materials was significantly reduced (Figure 4a). The number of the phosphopeptides identified by DMSNs@PDA-Ti\textsuperscript{4+} was also higher than those in many previously reported IMAC nanomaterials.\textsuperscript{53−55} For healthy human serum enrichment (Figure 8d and Figure S7c, d), since human serum contained only four endogenous phosphopeptides, the phosphopeptides identified by the three materials had no significant difference in

Figure 6. Comparison of the number and signal intensity of identified phosphopeptides enriched by DMSNs@PDA-Ti\textsuperscript{4+}, mSiO\textsubscript{2}@PDA-Ti\textsuperscript{4+}, and commercial TiO\textsubscript{2} from the mixture of α-casein and BSA digests at a molar ratio of 1:5000 (loading buffer: 50\% ACN, 6\% TFA).

Figure 7. MALDI-TOF mass spectra of phosphopeptides enriched from α-casein tryptic digest with different concentrations using DMSNs@PDA-Ti\textsuperscript{4+}.

Figure 8. MALDI-TOF mass spectra of the tryptic digest of nonfat milk (a) before and (b) after enrichment by DMSNs@PDA-Ti\textsuperscript{4+}. MALDI-TOF mass spectra of human serum (c) before and (d) after enrichment by DMSNs@PDA-Ti\textsuperscript{4+}. # indicates dephosphorylated peptides.
number but a slight difference in abundance. The detailed information on the four endogenous phosphopeptides from human serum is given in Table S2.

Furthermore, HeLa cell extracts, which contain a number of abundant phosphopeptides, were used to demonstrate the excellent enrichment performance of DMSNs@PDA-Ti4+. The enrichment experiments of DMSNs@PDA-Ti4+ and commercial TiO2 were performed under their respective optimal enrichment conditions (see details in the Experimental Section). Figure 9 gives an overview of the enrichment performance of DMSNs@PDA-Ti4+ and commercial TiO2 (the detailed enrichment results of three runs are listed in Table S3). After database searching, a total of 1707 phosphopeptides (860 monophosphopeptides and 847 multiphosphopeptides with the detailed information are given in Table S4) were identified after enrichment with commercial TiO2. The enrichment specificity (the ratio of the number of detected phosphopeptides to that of all identified peptides) was calculated to be 88.3%. However, for DMSNs@PDA-Ti4+, 2422 phosphopeptides were detected (1429 monophosphopeptides and 993 multiphosphopeptides with the detailed information are given in Table S5) with an ultrahigh specificity (96.3%), indicating that the DMSNs@PDA-Ti4+ could reduce the nonspecific adsorption to a large extent. This specificity is the highest among reported materials used for phosphopeptide enrichment from the lysate of real biological samples (see Table S6). Among the identified phosphopeptides, 1153 phosphopeptides were found in both materials (Figure 9c). In addition, the peak area values were also used to quantify the amount of identified phosphopeptides. As shown in Figure 9e, the amount of phosphopeptides captured by DMSNs@PDA-Ti4+ was 1.6 times that by commercial TiO2, indicating that the DMSNs@PDA-Ti4+ have a larger phosphopeptide loading capacity. Therefore, DMSNs@PDA-Ti4+ can be used as a powerful enrichment approach for phosphopeptide enrichment in cancer cells, which is useful in elucidating the regulatory mechanisms of disease-related signaling pathways.

In summary, DMSNs@PDA-Ti4+, a novel porous IMAC nanomaterial with high density of immobilized Ti4+ and excellent hydrophilicity, have been successfully synthesized. The large amount of immobilized Ti4+ could provide more affinity sites for phosphopeptide enrichment, and the excellent hydrophilicity could synergistically improve the selectivity of phosphopeptides. Besides, the large pore sizes could effectively prevent the "shadow effect", thereby facilitating the diffusion and release of phosphopeptides. Therefore, the DMSNs@PDA-Ti4+ performed better than mSiO2@PDA-Ti4+, commercial TiO2, and other reported materials in phosphopeptide enrichment. Furthermore, a total of 2422 phosphopeptides were identified from HeLa cell extracts with an ultrahigh specificity (96.3%). It is anticipated that the DMSNs@PDA-Ti4+ can be used as a promising IMAC material for high selective enrichment of phosphopeptides in phosphoproteome analysis.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b01369.

Optimization of PDA modification content; TEM images and N2 adsorption–desorption isotherms of mSiO2@PDA-Ti4+; MALDI-TOF mass spectra of the enrichment results of mSiO2@PDA-Ti4+ and commercial TiO2; detailed information on phosphopeptides obtained from α-casein digest, nonfat milk digests, human...
serum, and HeLa cell extracts; comparison of DMSNs@PDA-Ti4+ with the previous materials (PDF)

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The authors declare no competing financial interest.

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