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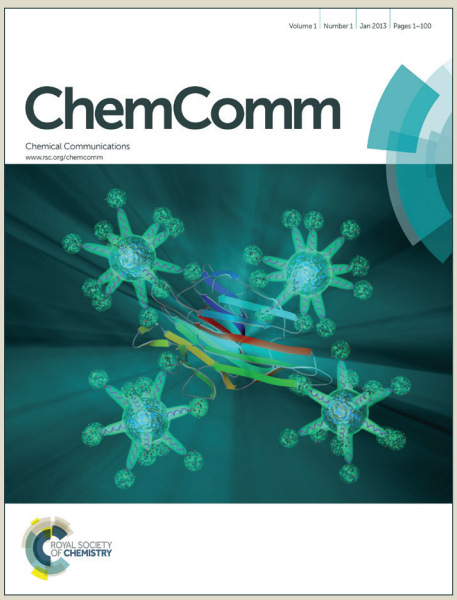

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Magnetic Cellulose/TiO_2 Nanocomposite Microspheres for Highly Selective Enrichment of Phosphopeptides

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A novel magnetic cellulose/TiO_2 nanocomposite microspheres with high surface area and magnetic susceptibility were fabricated, which exhibited remarkably selective enrichment of trace phosphopeptides from peptides mixture.

Magnetic nanocomposite microspheres with high surface area and tunable functions are widely applied in the fields of heterogeneous catalysis, separation, adsorption and drug delivery.[1] In recent years, regenerated cellulose materials with micro-nano porous structure are considered to be excellent substrates for facilely constructing nanocomposite microspheres via in-situ synthesizing metal or metallic oxide nanoparticles in the pores of cellulose matrix.[2] Meanwhile, cellulose-based materials have been adopted in biological experiments for long time due to their native advantages of biocompatibility and biodegradability. Therefore, magnetic cellulose nanocomposite microspheres have great application potentials.[3]

Reversible protein phosphorylation plays a vital role in regulating many complex biological processes such as cellular growth, division, and signaling transduction, and the investigations into this process are of keen interest of proteomics.[4] However, the identification and characterization of trace phosphopeptides from complex peptide mixtures by direct mass spectrometry (MS) analysis remains challenges because of a few amount and low signal-to-noise ratio. Therefore, the enrichment of phosphopeptides from complex samples becomes a required step prior to MS analysis. Recently, it has been reported that TiO_2 with positively charged surface at acidic pH can selectively adsorb phosphorylated species based on Lewis acid-base interaction,[5] and TiO_2 with high surface area possesses higher binding capacity on phosphopeptides.[6]

Here, the novel magnetic cellulose/TiO_2 nanocomposite microspheres (MCTiMs) were constructed via two-step procedure, for the first time. The first step, magnetic cellulose microspheres (MCMs) were prepared by efficiently microwave heating inducing sol-gel transition of cellulose/Fe_3O_4 colloidal micro-droplets (see ESI†). The second step, MCTiMs were prepared by dispersing MCMs into TiO_2 precursor solution, and then in-situ synthesizing TiO_2 nanoparticles in the micro-nano pores of MCMs (see ESI†for details). Meanwhile, the contents of TiO_2 in MCTiMs were controllable by changing the feeding volume of precursor solution to prepare series of MCTiMs, coded as MCTiMs-1, MCTiMs-2, MCTiMs-3, MCTiMs-5 (Fig. S1, ESI†). The morphology and structure of MCMs and MCTiMs were investigated by optical microscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM) and N_2 adsorption–desorption. The MCMs exhibited regular spherical shape with a mean diameter of 7 μm (Fig. S2, ESI†) and micro-nano porous microstructures (Fig. 1a, c). The pore size of MCMs was measured to be about 200 nm from the SEM image, indicating that the micro-sized porous structure appeared on the surface of the microspheres. Thus, the surface area and corresponding nano-sized porous structure of the MCMs was determined further by using N_2 adsorption–desorption to be 222.5 cm^2/g and 22 nm, respectively (Fig. S3, ESI†). The MCTiMs exhibited the similar spherical shape and size distribution as MCMs (Fig. S2, ESI†). However, a compact surface occurred in MCTiMs (Fig.1b, d). These results confirmed that the TiO_2 nanoparticles were in-situ synthesized successfully in pores of MCMs. As shown in the TEM images of the ultrathin section of MCTiMs (Fig. 1e), the TiO_2 nanoparticles homogeneously dispersed in the MCTiMs, and its TEM-EDS elemental mapping further displayed a uniform dispersion of TiO_2 in the microsphere. The content of the TiO_2 nanoparticles on surface was higher than that in inner (Fig. S4, ESI*). This could be explained that the TiO_2 nanoparticles were freely synthesized and aggregated on the surface of MCTiMs to form a compact shell layer, whereas controllably synthesizing was in 3D-porous structures of inner of the microspheres. Furthermore, high resolution TEM image of individual TiO_2 nanoparticles with spindle shapes (mean size is 52 nm×13 nm) (Fig. S4, ESI*) showed that clear crystalline lattice (d=0.35 nm) attributed to the (101) phases of TiO_2.[7]

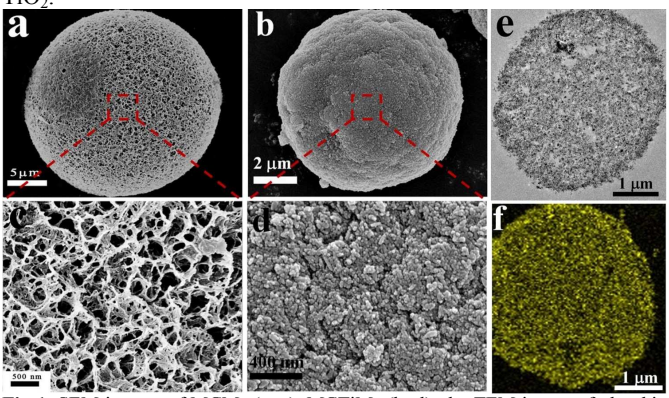


Fig 1. SEM images of MCMs (a, c), MCTiMs (b, d), the TEM image of ultrathin section of MCTiMs (e) and its TEM-EDS elemental mapping of Ti (f).

To study the influence of the structures of MCTiMs on the enrichment efficiency of phosphopeptides, the crystallinity, content of TiO_2 and its micro-environment in matrix were further characterized by thermogravimetric analysis (TG), X-ray diffraction (XRD) and Fourier transform infrared reflectance spectroscopy (FT-IR). The TG results indicated that the contents of Fe_3O_4 and TiO_2 in MCTiMs-2 were 12% and 40%, respectively (Fig. S5a, ESI†). It was worth nothing that the contents of TiO_2 in MCTiMs increased with an increase of the feeding amount of TBOT, and reach to the maximum in MCTiMs-2, and then slightly decreased in MCTiMs-3, MCTiMs-5. The results could be explained that pores in the cellulose microspheres limited the growth of TiO_2 nanoparticles. Meanwhile, the synthesis of TiO_2 nanoparticles on the surface of microspheres was unlimited, leading to the formation of a more compact shell layer at a higher content of TBOT, resulting in hindrance for TBOT diffusing into the microspheres. The XRD results showed that all the peaks of TiO_2 for MCTiMs-2 can be indexed to the anatase structure of TiO_2 (JCPDS card no. 21-1272) (Fig. S5b, ESI †). The average crystallite size of the TiO_2 nanoparticles was calculated by the strongest peak of (101) in the XRD spectrum using Scherrer's formula to be around 16.1 nm, which was consistent with that of nano-pore size of MCMs. FT-IR results revealed that the peaks at 3439 cm^{-1} corresponding to stretching vibrations of hydroxyl groups of cellulose shifted to 3416 cm^{-1} and 3347 cm^{-1} for MCMs and MCTiMs, with the increasing band width, indicating a strong interaction between the hydroxyl groups of cellulose matrix and TiO_2/Fe_3O_4 nanoparticles (Fig. S5c, ESI†). On the basis of the above results, the mono-dispersed TiO_2 nanoparticles were satisfactorily created in the pores of the MCM microspheres, and embedded tightly in cellulose matrix through strong physical interaction.

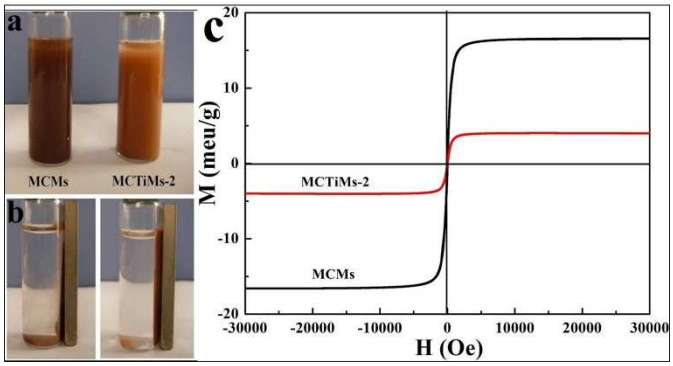
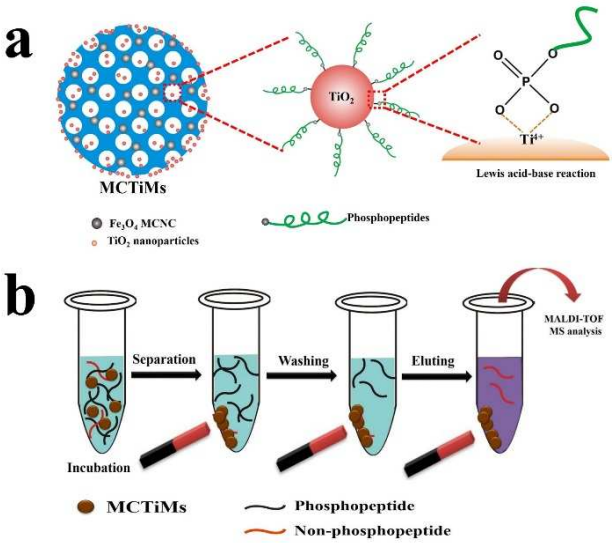


Fig 2. Photos of MCMs, MCTiMs-2 aqueous dispersion (a) and migration under a magnetic field (b), magnetic hysteresis curves of MCMs, MCTiMs-2 (c).

The excellent magnetic response properties of microspheres are essential for the successful application of phosphopeptides enrichment. Obviously, the colour of MCMs was dark brown, as a result of the existence of Fe_3O_4 in cellulose matrix. After introducing TiO_2 into MCMs, the colour of MCTiMs became light brown (Fig. 2a). Interestingly, The MCMs and MCTiMs-2 could quickly align into the magnetic field within 10 seconds (Fig. 2b). The magnetic properties of the nanocomposite microspheres were further studied using a vibrating sample magnetometer (VSM; Figure 4c). The results indicated that they all possessed a superparamagnetic character. From the TEM images of ultrathin section of MCTiMs-2 (Fig. S6a, ESI*), the Fe_3O_4 nanocrystal clusters (MCNC) were individually dispersed in the cellulose microspheres. The high resolution TEM images (Fig. S6b, c ESI*) further proven that MCNC was composed of Fe_3O_4 nanocrystals with a size of about 20 nm, maintaining the similar structure as native state. Clearly, the structures and magnetic properties of Fe_3O_4 were hardly affected by TiO_2 in pores of cellulose microspheres. Therefore, MCTiMs with a sensitively magnetic response could be easily separated out from the peptides solution by using an external magnetic field, leading to the easy recycling.

The selectivity of MCTiMs for phosphopeptides was demonstrated with a tryptic digest of β-casein, mixture of β-casein and BSA and fresh human serum samples. A schematic depiction of the enrichment mechanism and procedure of phosphopeptides by using MCTiMs is proposed in Scheme 1. As shown in scheme 1a, the TiO_2 nanoparticles were immobilized firmly in the pores of MCTiMs, and their structure and reaction activity were protected by the pore wall of the magnetic cellulose matrix. During the incubation process, phosphopeptides were diffused rapidly into the magnetic cellulose microspheres to contact with the TiO_2 nanoparticles, and anchored on the TiO_2 surface through Lewis reaction immediately. In a typical enrichment procedure (scheme 1b), β-casein digest was first dissolved in a 100 μL loading buffer consisting of 50% acetonitrile containing 3% trifluoroacetic acid (TFA), and then was incubated with MCTiMs for 30 min. The phosphopeptides captured with MCTiMs were separated from the solution using an external magnetic field. The collected phosphopeptide products were thoroughly washed with loading buffer to remove the non-specifically adsorbed peptides. Finally, the trapped phosphopeptides were eluted completely from MCTiMs with 50 μL 5% $NH_3 \bullet H_2O$, and 1 μL eluent was used for MALDI-TOF MS analysis. For comparison, the direct analysis of the β-casein digest was performed by MS analysis.



Scheme 1. Scheme illustration of enrichment mechanism of phosphopeptides by using MCTiMs (a) and the typical process for selective enrichment of phosphorylated peptides by using MCTiMs and magnetic separation (b).

Fig. 3a, b shows MS spectra of the tryptic digests of β-casein analyzed before and after enrichment by using MCTiMs-2. For without the pre-treatment procedure, the predominant component was non-phosphopeptides in the spectrum (Fig. 3a), and their presence led to a low signal-noise ratio for the phosphopeptides. However, after selective enrichment with MCTiMs-2 (Fig. 3b), the signal was clearly observed for all three phosphopeptides of β-casein (at m/z 2061.83, 2556.09, and 3122.27), along with their dephosphorylated counterparts. As control, the selective enrichment of phosphopeptides from β-casein digest by using MCMs was also investigated, and the signal of phosphopeptides was hardly detected

in its spectrum (Fig. S7a, ESI†). This confirmed that TiO_2 rather than Fe_3O_4 and cellulose played a key role for enrichment phosphopeptides. Furthermore, other three samples (MCTiMs-1, MCTiMs-3 and MCTiMs-5) were also used for enriching phosphopeptides of β-casein (Fig. S7b-d, ESI†), and exhibited slightly lower enrichment selectivity than MCTiMs-2. The results could be explained that the MCTiMs-1 had less affinity sites with phosphopeptides due to its lower content of TiO_2, whereas MCTiMs-3, MCTiMs-5 had more compact shell layers, leading to the difficulty of phosphopeptides to diffuse into the microspheres. Thus, MCTiMs-2 with a high content of TiO_2 and a relatively loose surface exhibited the best selectivity for phosphopeptides. The human serum sample was also used for testing the specificity of CMTiMs-2 in enrichment of endogenous low-abundant phosphopeptides. The dramatically non-phosphopeptides peaks appeared as direct analysis of the human serum (Fig. 3c). After selective enrichment with MCTiMs-2, four peaks of phosphopeptides could be distinctly observed (Fig. 3d).

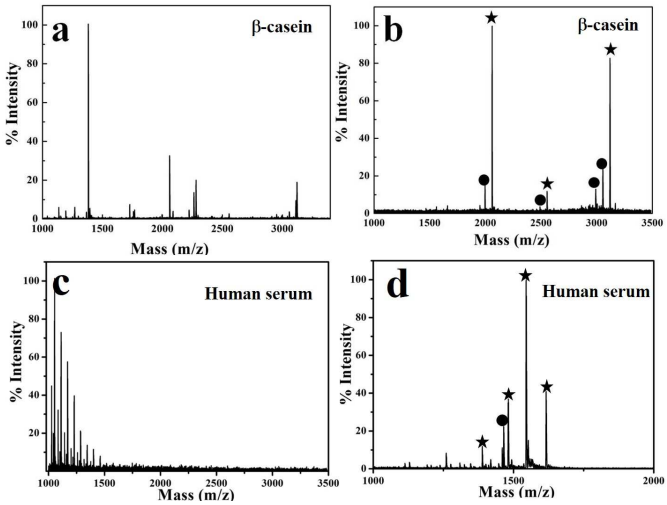


Figure 3. MALDI mass spectra of the tryptic digests of β-casein and human serum sample: direct analysis (a, c) and analysis after enrichment by using MCTiMs-2 (b, d). ★ phosphorylated peptides, ● their dephosphorylated counterparts. β-casein was at concentration of 1.0×10^{-7} M (3 pmol), human serum sample (2 μL).

As the level of phosphopeptide in a complex biological sample is much lower, the ability to enrich phosphopeptide from highly dilute solution is a key performance criterion to evaluate the enrichment efficiency of MCTiMs. Thus, β-casein digests with low amount of 100 fmol and 50 fmol were used for the examination of phosphopeptide enrichment by MCTiMs-2. As shown in Fig. 4a, b, the limit of detection for β-casein is 50 fmol by using MCTiMs-2. These results led us to believe that MCTiMs can be used for the selective enrichment of trace phosphopeptides. To further evaluate the ability of capture phosphopeptides in the complex peptide samples, MCTiMs-2 was applied to enrich phosphopeptides in series of mixtures of β-casein and non-phosphorylated BSA with different molar ratio. Interestingly, when the molar ratio of β-casein and BSA was 1:1, 1:10 and 1:100, phosphopeptides could be selectively enriched from the peptides mixtures (Fig. S8, ESI†). Furthermore, enrichment of phosphopeptides with MCTiMs-2 was also available when the molar ratio was 1:1000. As mentioned above, phosphopeptides were detected hardly by using direct analysis method (Fig. 4c). Amazingly, after enriching by using MCTiMs-2, the three phosphopeptides were clearly observed with a clean background in the mass spectrum (Fig. 4d). To the best of our knowledge, the enrichment selectivity of MCTiMs was much better than that of the enrichment materials previously reported in literatures.[8] To compare, commercial TiO_2 nanoparticles were used to enrich phosphopeptides from the mixtures of β-casein and BSA. When the molar ratio of β-casein and BSA was 1:1, the phosphopeptides were observed with a relative clean background in the mass spectrum (Fig. S9a, ESI*). However, a lot of non-phosphopeptide peaks were observed in the mass spectrum of a mixture of β-casein and BSA with a molar ratio of 1:10 (Fig. S9b, ESI*), indicating a much lower selectivity of the commercial TiO_2 nanoparticles than MCTiMs. This could be explained that the activity and high surface area of TiO_2 nanoparticles immobilized in the pores of cellulose microspheres was protected by cellulose matrix at a harsh environment. The results strongly supported that the magnetic cellulose/TiO_2 nanocomposite microspheres were an excellent adsorbent for trace phosphopeptides, showing remarkable selectivity, extreme sensitivity and sustainable enrichment recovery.

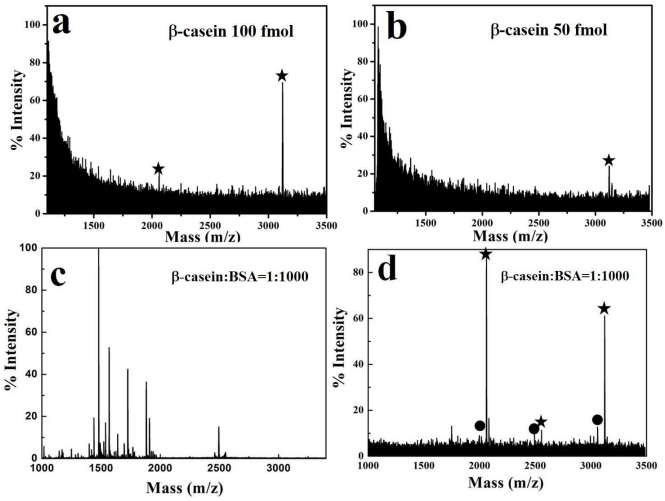


Figure 4. MALDI mass spectra of the tryptic digests of β-casein and a mixture of β-casein and BSA sample (with a molar ratio of β-casein to BSA of 1:1000). Low contents of β-casein with amount of 100 fmol (a), 50 fmol (b). The mixture directly analysed (c) and analysed after enrichment by using MCTiMs-2 (d). ★ phosphorylated peptides, ● their dephosphorylated counterparts.

In summary, a new facile synthetic route to fabricate magnetic cellulose/TiO_2 nanocomposite microspheres by using cellulose as matrix was successfully realized. The micro-nano porous structures of the cellulose matrix played an important role to in-situ synthesize TiO_2 nanoparticles, and to protect the native structure and character of TiO_2, leading to the process of enrichment at a harsh environment. The MCTiMs exhibited high efficiency for selective enrichment trace phosphopeptides from tryptic digest of β-casein and human serum samples, as a result of the strong capturing capability. It is worth noting that the selective enrichment of phosphopeptides from mixtures of β-casein and BSA with a molar ratio of 1:1000 by using MCTiMs was available, which was much better than commercial TiO_2 nanoparticles. In our finding, the TiO_2 nanoparticles embedded in the magnetic cellulose microspheres had high contents and specific surface areas, as well as good adsorbing nature, leading to a strong Lewis acid-base interaction. Therefore, MCTiMs will be an excellent candidate for enrichment of phosphopeptides in the complex systems with MS analysis.

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Notes and references

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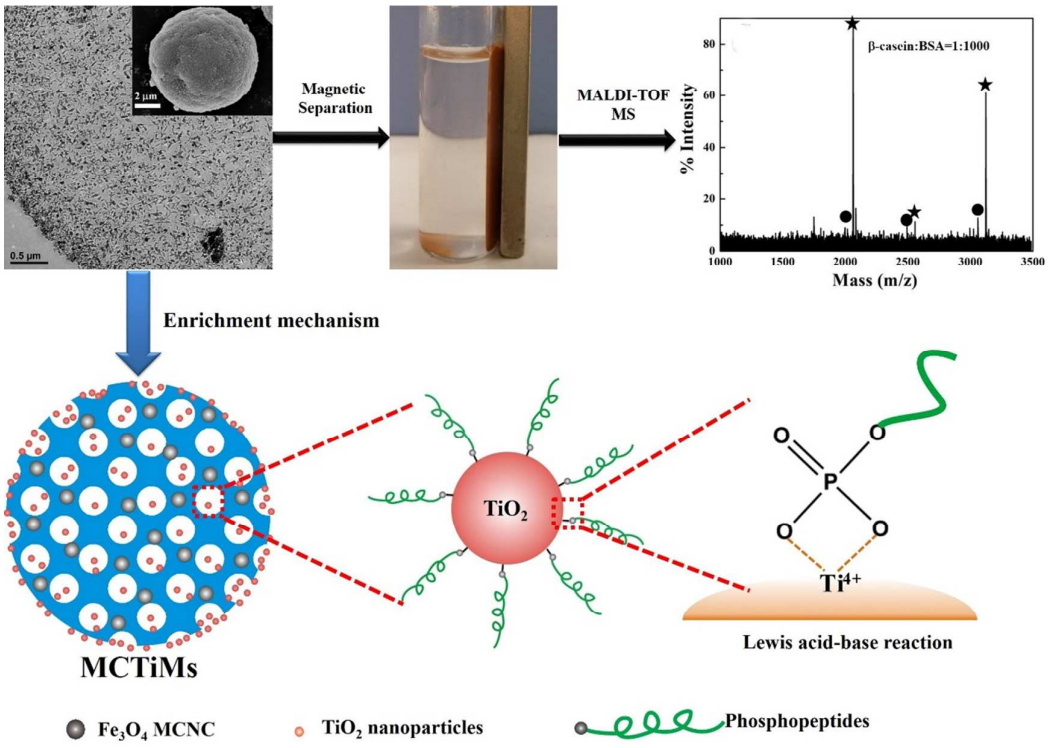

A novel magnetic cellulose nanocomposite microspheres with highly selective enrichment of traces of phosphopeptides were prepared by in-situ synthesis of TiO_2 nanoparticles in the micro-nano pores of magnetic cellulose microspheres. The selectivity of phosphopeptides on the nanocomposite microspheres were significantly higher than other reports as a result of the high-efficiency Lewis acid-base reaction.