Pore size-optimized periodic mesoporous organosilicas for the enrichment of peptides and polymers†

Kun Qian,a Fang Liu,a Jie Yang,a Xiaodan Huang,a Wenyi Gu,a Siddharth Jambhrunkar,a Pei Yuan3 and Chengzhong Yu*a

The enrichment of peptides is a key technique in mass spectrometry based proteomics and peptidomics. The tailored design of mesoporous materials with an optimum pore size for highly-efficient enrichment of target molecules is a challenging issue. Herein, a series of periodic mesoporous organosilicas (PMOs) are synthesized with the same structural symmetry (p6mm) and similar morphology, while the pore sizes are finely adjusted from 2.6 to 7.3 nm. Their enrichment performance for a standard E7 peptide (molecular weight 1120.6 Da) is investigated via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). It is found that PMO with the mesopore size of 5.8 nm exhibits the highest enrichment performance towards the E7 peptide. Moreover, a block copolymer (Brij 78) with the similar molecular weight (1150.8 Da) to E7 is also used to further study the influence of mesopore size upon the enrichment efficiency. It is shown that PMO with the pore size of 5.8 nm still holds the best enriching ability towards Brij 78 at low concentrations. The adsorption capacity of the PMOs for Brij 78 are further studied at high concentrations, showing a dependence on both the pore volume and pore size. This research may shed light on advanced enrichment and analysis of various peptides and polymers using designed nanoporous materials, an important topic in both material and biological science.

Introduction

Nowadays, the enrichment of peptides is a key bio-technique and a hot topic in mass spectrometry based proteomics1–4 and peptidomics,5–9 which allows the accurate and efficient detection and identification of diverse proteins and peptides in a bottom-up approach. Currently different methods can be employed for peptide enrichment, depending on the specific type of peptides. The peptides with specific functional groups can be isolated by chemo-affinity based methods, for example, metal oxide affinity chromatography (MOAC) for phosphorylated peptides10–14 and boronic acid affinity chromatography for glycosylated peptides.15–17 Even if it has enjoyed recent success, a lot of important peptides do not have such natural modifications and thus become very difficult to be enriched through chemo-affinity.18–23 which may limit the efficient detection and identification. Therefore, efficient materials for general non-specific peptide enrichment are desired in order to promote the development of this bio-technique and genetic engineering.

Mesoporous materials have received intense interests over the past few decades and have found applications in diverse fields, mainly due to their unique porous structure and huge surface area.24–28 In particular, ordered porous materials with a uniform and adjustable pore size are able to achieve series of size-dependent applications by the state-of-the-art nano-techniques, e.g. nanoreactors, nanodevices for separation and adsorption.29–32 Furthermore, owing to the size-selective/exclusive effects of meso-channels of a specific size,29–31,33–36 ordered mesoporous silica materials are able to serve as nano-traps or nano-blocks for peptide enrichment.30,31,35,36

The structural parameters of mesoporous materials are fundamentally important for peptide enrichment. Compared to the bare siliceous materials, periodic mesoporous organosilicas (PMOs) are proved to be more effective due to the relatively higher hydrophobicity induced by the organic functional groups homogeneously dispersed in the mesoporous framework.37–41 Previously we have also tested the enrichment performance of different mesoporous structures (such as hexagonal and cubic)34 and morphologies (such as nanoparticles and microparticles).39 It is worth noting that the pore size is an important factor, especially for the size-selective applications. Therefore, it is of key importance to tune the pore size of porous materials to provide the nanospace in a designed way.39,40 It has been reported that MCM-41 type silica materials with the pore size of 2 nm showed a higher immobilization ability for peptides compared with ~8 nm
mesopores in size-selective extractions. However, there is no systematic study about the effect of the finely tunable pore size from 2 to 8 nm on the enrichment performance. Till now it is interesting, but still challenging to identify the optimum pore size for the enrichment of one target molecule.

In order to study the effect of pore size on the peptide enrichment performance while excluding other possible influencing factors, the design of a series of experiments to controllably synthesize ordered mesoporous materials with the same symmetry and morphology but finely tunable pore sizes is necessary. Herein, combined strategies, including the rational selection of ionic or block copolymer surfactants as the structure-directing agents, and the addition of swelling agents or additives, and the adjustment of hydrothermal treatment temperatures, etc., are employed to synthesize a series of PMO materials with the same structural symmetry (p6mm) and a similar rod-like morphology, but with different pore sizes finely tuned from 2.6 to 7.3 nm. The cytotoxic lymphocyte (CTL) epitope of E7 (sequence RAHYNIVTF, molecular weight 1120.6 Da), a preventive therapeutic vaccine to generate immune response and inhibit tumor growth in clinical phase trials, is employed to investigate the enrichment ability of these materials through matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). It is shown that PMO with the mesopore size of 5.8 nm exhibits the highest enrichment performance towards the E7 peptide at a low concentration of 0.01 ng μL\(^{-1}\). In addition, a polymer molecule Brij 78 (Mw = 1150.8 Da) with the similar molecular weight as E7 is also used to further investigate the influence of mesopore size upon the enrichment efficiency. It is found that PMO with the pore size of 5.8 nm still holds the best enriching ability towards Brij 78 at low concentrations. The adsorption capacity of the PMOs for Brij 78 is further studied at high concentrations based on the thermo-gravimetric (TG) analysis, showing a dependence on both the pore volume and pore size. Our research sheds light on advanced peptide (or other target molecules) enrichment and analysis using designed nanoporous materials with optimised structural parameters.

Results and discussion

Structural and morphological characterization

The XRD analysis of ethane-bridged organosilicas with different pore sizes were performed after ethanol extraction (Fig. 1), where highly ordered mesoporous structures can be obtained for S1–S5. As shown in Fig. 1, three well-resolved peaks can be clearly observed for S1 and S2, which can be assigned to 100, 110, and 200 diffractions, reflecting the two-dimensional hexagonal mesostructure with a space group of p6mm. Four characteristic diffraction peaks in the 2θ range of 1–3° with a reciprocal d-spacing ratio close to 1 : 3 : 2 : 7 are generally observed in S4 and S5 (Fig. 1), which can be indexed as the 100, 110, 200, and 210 reflections based on a p6mm symmetry. The unit cell parameters (a) for all the samples were calculated based on the first reflection peak and the values are summarized in Table 1. S1 and S2 have a value of 4.6 and 5.8 nm, respectively, and S3–5 possess the similar a in the range of 11–12 nm.

Fig. 2 displays the nitrogen adsorption–desorption isotherms and the corresponding pore size distribution (PSD) curves calculated from adsorption branches using the Barrett–Joyner–Halenda (BJH) method of samples S1–S5 after extraction. The pore size, pore volume and Brunauer–Emmett–Teller (BET) surface areas are listed in Table 1. S1 and S2 show type IV isotherms with capillary condensation steps occurring at a relative pressure (P/P\(_0\)) of 0.3 and 0.4, respectively. S3–S5 prepared using a similar recipe but with the increasing hydrothermal treatment temperatures (See Experimental Section), exhibit type IV isotherms with type H1 hysteresis loops, indicating that the extracted mesoporous materials have 1-D pore structures. The relative pressures (P/P\(_0\)) where capillary condensation step takes place are shifted from ~0.6 (S3) toward higher values of 0.7 (S3), illustrating that the pore size is gradually enlarged with the increase of the

<table>
<thead>
<tr>
<th>Samples</th>
<th>a (nm)(^a)</th>
<th>P (nm)(^b)</th>
<th>V(_p) (cc g(^{-1}))(^c)</th>
<th>S(_{\text{BET}}) (m(^2) g(^{-1}))(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>4.6</td>
<td>2.6</td>
<td>0.70</td>
<td>896</td>
</tr>
<tr>
<td>S2</td>
<td>5.8</td>
<td>3.5</td>
<td>0.63</td>
<td>563</td>
</tr>
<tr>
<td>S3</td>
<td>11.8</td>
<td>5.8</td>
<td>0.49</td>
<td>485</td>
</tr>
<tr>
<td>S4</td>
<td>11.5</td>
<td>6.7</td>
<td>0.70</td>
<td>614</td>
</tr>
<tr>
<td>S5</td>
<td>11.2</td>
<td>7.3</td>
<td>0.77</td>
<td>671</td>
</tr>
</tbody>
</table>

\(^{a}\) a is the cell parameter. \(^{b}\) Pore size is calculated using adsorption branch by the BJH method. \(^{c}\) Calculated by the N\(_2\) amount adsorbed at the highest P/P\(_0\) (~0.99). \(^{d}\) BET surface area calculated using experimental points at a relative pressure of P/P\(_0\) = 0.05–0.25.
hydrothermal temperature in accordance with literature reports elsewhere. The narrow PSD curves confirm the capillary condensation of nitrogen in uniform mesopores (Fig. 2f) and the pore sizes are calculated to be 2.6, 3.5, 5.8, 6.7, and 7.3 nm for S1–S5, respectively (Table 1).

SEM images display the typical morphologies of S1–S5 shown in Fig. S1, ESI. S1 has a uniform rod-like morphology with the rod diameter of 500–750 nm and length of 0.3–2 μm (Fig. S1a, ESI). While large and irregular particles are observed with a size over 1 μm for S2 (Fig. S1b, ESI). In the case of S3–S5, similar rod-like morphologies can be seen (Fig. S1c–e, ESI), which reveals that the change of hydrothermal temperature does not affect the morphologies of the PMO materials.

TEM analysis was carried out to determine the mesostructures of S1–S5 (Fig. 3). The ordered straight one-dimensional channels can be observed to be parallel to the long axis of the rods in all the materials and the distances between two adjacent pore channels were measured to be consistent with d100 from the XRD patterns. The above characterizations suggest that a series of mesoporous organosilicas have been successfully prepared with ordered mesostructures and a finely tunable pore size ranging from 2 to 8 nm. These PMO materials possess the same pore symmetry, and similar morphology but different pore sizes and pore properties, making them as the ideal candidates for the systematic study on the role of pore size and properties in small molecule enrichment performance.

**Enrichment tests at low concentrations**

In order to evaluate the enrichment ability of S1–S5, the standard E7 peptide (isoelectric point of 8.75) was employed as a target molecule and MALDI-TOF MS was carried out. Fig. 4a displays the MS spectrum directly obtained from an E7 peptide solution (0.01 ng μL⁻¹). No signals can be found due to the very low peptide concentration. After being enriched by the synthesized PMO materials, the signal of the E7 peptides can only be seen in S3 with the pore size of 5.8 nm among all the materials (Fig. 4b–f for S1–S5, respectively) at m/z 1120.6 with the intensity of 27 and signal-to-noise ratio (S/N) of 2.8 (Fig. 4d). To validate this phenomenon, the laser intensity was raised to 45% for further tests. As shown in Fig. S2a, ESI, still no peptide peaks can be observed in the bulk E7 solution. For comparison, the E7 peptides can be identified at m/z 1120.6 and enriched by S1–S4 (not S5), suggesting that the optimum pore size is in the range of 2.6–6.7 nm to trap the E7 molecule.

When further analyzing the performance of S1–S4, the peptide signal is enhanced for S3 with the peak intensity of 194 and S/N of 5.4 compared to the former tests under 36% laser intensity. Moreover, the enrichment ability of S3 is also superior to all the other materials (the S/N is less than 2 for S1, S2 and S4) under the similar condition and could be advantageous in the MS/MS analysis for the sequence identification. In addition, a non-mesoporous organosilica material was also prepared for the peptide enrichment (see Materials synthesis in Experimental) and no peptide signal can...
be found in the spectrum (Fig. S2g, ESI), suggesting that the peptide is enriched inside the pores but not at the outer surface and the size of the pores should be the key parameter for the enhanced enrichment ability. Thus we deduce that the optimum pore size for E7 peptide enrichment is 5.8 nm.

To further confirm the enrichment effect of mesopores with different sizes, another polymer with a similar molecular weight to E7 peptides (Brij 78, Mw = 1150.8 Da) was used to examine the performance of the materials in the following experiments. Only noise signals can be observed in the MS spectrum from the bulk Brij 78 solution at a concentration of 1 ng μL⁻¹ (Fig. 5a). In contrast, a series of polymer ions can be probed during the MS after enrichment by the PMO materials (Fig. 5b–f for S1–S5, respectively). Typically the MS spectrum yields a series of partially mass-resolved signals centered within m/z 1100–1200 which is in agreement with the average molecular weight of Brij 78. Peak clusters were separated by a nominal value of 44 Da (ethane group), which is consistent with the (OCH₂CH₂)ₙ repeating unit of Brij 78. It is found that S3 and S4 have better enrichment ability towards the Brij 78 with enhanced polymer ion peak number/intensity compared to the other PMO materials (Fig. 5b–f and Fig. S3a, ESI), while the highest intensity (~250) and most polymer ion peaks (16) are obtained in S3 indicating the similar enrichment behavior of the Brij 78 polymer to the E7 peptide. The above results demonstrate that the 5.8 nm mesopores could serve as the optimum nano-traps for the chained peptides/polymers with a certain molecular weight (1120–1150 Da) in low abundance. Meanwhile, considering the structure of the polymers and the high price of the synthetic peptides, polymers can be used as cheap substitutes for the evaluation of the enrichment performance.

The adsorption capacity of these materials was also investigated towards the Brij 78 polymer at high concentrations through TGA as displayed in Fig. S3b, ESI and Fig. 6. The weight loss of the polymer loaded PMO materials is derived from the decomposition of the polymers and organo-groups of PMO, which can be calculated by the former reported method as shown in Fig. 6a based on the following equation:

\[
\frac{W_1 - B}{100 - T} = \frac{W_2 - W}{100 - T}
\]

Where \(W_1\) stands for the weight loss ratio from the organo-groups of bulk PMO, \(W_2\) presents the weight loss ratio of loaded PMO from polymers and organo-groups of bulk PMO, \(B\) and \(T\) are the total weight loss ratio of the extracted PMO and the polymer loaded PMO including the physically adsorbed water (<100 °C) and \(W\) is the weight loss ratio from the polymers. Thus the loaded Brij 78 ratio is measured to be 9.2%, 26.8%, 24.0%, 22.7% and 18.8% for S1–S5, respectively (Fig. S3b, ESI and Fig. 6).

It can be observed that samples with the pore size of 2.6 and 3.5 nm have the lowest (9.2%) and highest (26.8%) immobilization capacity towards Brij 78, while the absorption amounts gradually decrease from 26.8% to 18.8% as the pore size increases from 3.5 to 7.3 nm. The lowest capacity of S1 (2.6 nm) can be attributed to the small pore size obstructing the entrance of the polymers. When the pore size is increased to 3.5 nm, on the one hand the polymer is able to enter the pores easily; on the other hand, the Brij 78 molecules can be trapped more effectively compared to the larger opening pore size (e.g. 5.8 nm) considering the 24 h adsorption time for equilibrium. When the pore size is larger than 3.5 nm, the decreasing trend in the absorbed volume can be explained as the increased pore size not fit for immobilization of the molecules and the Brij 78 molecules can escape from the pores easily. Meanwhile, it should be mentioned that S2 has a much larger pore volume (0.626 cc g⁻¹) than S3 (0.489 cc g⁻¹), which contributes to the enhanced enrichment capacity for high concentration tests as
well. Thus it is concluded that the total adsorption volume of the PMO materials relies not only on the suitable pore size, but the pore volume as well.

**Experimental**

**Chemicals**

Triblock copolymer EO_{12}PO_{70}EO_{12} [denoted P123, where EO is poly(ethylene oxide) and PO is poly(propylene oxide)], potassium chloride (KCl, 99.5%, AnaLAR, Australia), fuming hydrochloric acid (36%, Lab-Scan, Analytical Science, Thailand), ethanol (99.5%, Asia Pacific Speciality, Australia), ammonium bicarbonate, dry toluene, sodium perfluorooctanoate (PFONa), 1,2-bis(triethoxysilyl)ethane (BTEE, 96%) and 1,2-bis-(trimethoxysilyl)ethane (BTME, 96%) were purchased from Aldrich. Octadecyltrimethylammonium bromide (OTAB), cetyltrimethylammonium bromide (CTAB), Brij 78 polymer, E7 peptide (99%) and ammonium aqueous solution (25%) were obtained from Sigma. Acetonitrile (ACN, 99.9%), α-cyano-4-hydroxycinnamic acid (CHCA, 99%) and trifluoroacetic acid (TFA, 99.8%) were purchased from Merck. All reagents were used as received without further purification. Deionized water (18.2 MΩ cm) used for all experiments was obtained from a Milli-Q system (Millipore, Bedford, MA).

**Materials synthesis**

Five PMO samples were successfully synthesized using literature methods with adjustable pore sizes (2–8 nm), and the obtained samples are named as S1, S2, S3, S4, and S5, respectively. The experimental details are shown separately as follows.

For S1: 0.40 g of CTAB was stirred and dissolved in 100 mL of 20% ammonium aqueous solution at 40 °C. 0.01 g of PFONa was dispersed in the reaction system and then 0.667 mL of BTEE was added. After continuously stirring for another 3 h at 40 °C, the resulting powder was filtered, washed and dried at room temperature.\(^{34}\)

For S2: 0.56 g of OTAB and 0.236 g of NaOH were dissolved in 15.9 g of water at 25 °C, then 0.63 mL of BTME was added and stirred for 24 h at 25 °C. The mixture was transferred to an autoclave and aged at 95 °C for 21 h. The resulting powder was filtered, washed and dried at room temperature.\(^{35}\)

For S3: 0.33 g of P123 and 1 g KCl was dissolved in 20 g of 0.167 M HCl solution at 38 °C. Then 0.47 g of BTME was added and stirred for 10 min. The above reaction system was aged at 38 °C for 24 h and the resulting powder was filtered, washed and dried at room temperature.\(^{50}\)

For S4 and S5: the synthesis process was similar to S3, but the aging temperature was raised to 70 °C and 100 °C, respectively.

For non-porous the organosilica, the synthesis process was similar to S3, but no template (P123) was added to the reaction system.

All the above as-made PMO products were extracted by refluxing with ethanol/HCl (3.8 g of 36% HCl in 150 mL of 98% ethanol) for 6 h (2 times) to remove the templates. The final products were dried at room temperature.

**Materials characterization**

X-Ray diffraction (XRD) patterns were recorded on a German Bruker D8 advanced X-ray diffractometer with Ni-filtered Cu-Kα radiation (wavelength of 0.154 nm) at a voltage of 40 mV and a current of 40 mA. The powder samples were pressed and loaded onto the sample plate. The XRD patterns were recorded and analyzed by the Bruker software DIFFRAXRD Commander. Nitrogen-sorption isotherms of the samples were obtained by a Quantachrome’s Quadrasorb SI analyzer at 77 K. Before the measurements, the samples were degassed at 383 K for at least 8 h in vacuum. The TGA curves were recorded on a Perkin-Elmer TGA7/DTA7 device. The starting temperature and ending temperature were set to be 25 °C and 900 °C, respectively, with a heating rate of 10 °C min\(^{-1}\) under air flow. Scanning electron microscopy (SEM) images were recorded on a JEOL Philips XL30 microscope operating at 20 kV. The samples were coated with gold before the observations. Transmission electron microscopy (TEM) images were directly taken with a JEOL 2011 microscope operated at 200 kV by dispersing the samples on a Cu grid covered with carbon films.

**Enrichment tests**

The peptide solution was prepared using a stepwise dilution method and the obtained PMO materials were dispersed in water at a concentration of 10 mg mL\(^{-1}\). In the analysis of E7 peptide, 1 µL material slurry was directly dispersed in the peptide solution after stepwise dilutions (500 µL, 0.01 ng µL\(^{-1}\)) and no buffer was used during the dilution process. After being stirred for 5 min, the mixture was centrifuged to remove the supernatant. Then 0.5 µL of a CHCA matrix solution (10 mg mL\(^{-1}\), in ACN/water/TFA, 50 : 49.9 : 0.1%, v/v/v) was added to the precipitation to elute the peptides, and the mixtures were deposited on a MALDI MPT 384 plate. The samples were analyzed on a Bruker Autoflex TOF/TOF III Smart beam. All peptide mass spectra were obtained in the RP-HPC-Proteomics mode via an accumulation of 500 laser shots at 10 different sites under a laser intensity of 36%/45% instrument for data collection and calibrated using an auto calibration method. Two standard peptides, Angiotensin II (Mw = 1046.5 Da) and ACTH-Clip (Mw = 2465.7 Da), were used for calibration purposes to reduce variability.

The polymer solution was prepared using a stepwise dilution method for the Brij 78 polymer. For low concentration tests, 1 µL material slurry was dispersed in the polymer solution (500 µL, 1 ng µL\(^{-1}\)) and these mixtures were centrifuged to remove the supernatant after being stirred for 5 min. Then 0.5 µL of a CHCA matrix solution (10 mg mL\(^{-1}\), in ACN/water/TFA, 50 : 49.9 : 0.1%, v/v/v) was added to the precipitation, and the mixtures were deposited on the MALDI MPT 384 plate for the following MS analysis. For high concentration tests, 50 mg of the different PMO materials was added to 20 mL Brij 78 aqueous solutions (5 mmol L\(^{-1}\)), respectively. The above slurries were shaken at 25 °C for 24 h (120 rpm/min). The mixtures were centrifuged to remove the supernatant. Then the precipitation was washed and dried at 50 °C before the TG analysis.
Conclusions

In summary, a series of PMO materials have been successfully synthesized with pore sizes that are finely tunable from 2.6 nm to 7.3 nm. These PMO materials have the same pore symmetry and similar morphology and are ideal candidates for the study on the role of pore size in small molecule enrichment performance. PMO with the pore size of 5.8 nm is proved to be the most effective host to enrich the standard E7 peptide (1120.6 Da) and Brij 78 polymer (1150.8 Da) with similar molecular weights at low concentrations, whereas PMO with the 3.5 nm pore size has the highest saturated adsorption capacity at high concentration due to the suitable pore size and high pore volume. This systematic study provides insights into the rational design of nanoporous materials with optimised structural parameters for the advanced enrichment and analysis of various molecules.

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Notes and references