VIP Mesoporous Materials

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Highly Efficient Extraction of Serum Peptides by Ordered Mesoporous Carbon**

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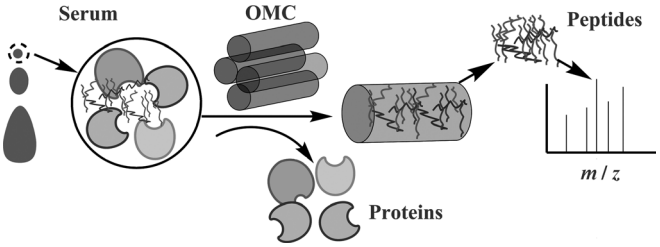
Human serum is of great importance for organ function and the potential diagnosis of diseases. Serum biomarkers have been widely applied in clinical diagnosis and disease therapies.[1] Endogenous serum peptides are an important class of potential biomarkers for the elucidation of biological and pathological variations in the serum.[2] However, owing to the complexity and the highly dynamic range of protein concentrations in serum, the discovery of serum peptides, especially those at low abundance levels from minuscule blood samples, is still a challenge.[3] With regard to obtaining serum peptides, organic solvent precipitation is a simple method for the removal of highly abundant proteins.[4] However, the difficulty in discriminating between peptides and proteins together with the inevitable loss of peptides during the removal of proteins by solvent precipitation makes this method inefficient for the extraction of peptides. Centrifugal ultrafiltration (UF) with an accurate molecular weight cutoff is considered to be a very useful technology for the separation of proteins with low and high masses. By using UF coupled with a nanospray ionization hybrid ion trap/Fourier transform mass spectrometer, 300 unique peptides were identified from a 60 μL of serum sample.[3,5] However, the coconcentration of small molecules and salts result in inefficient peptide extraction and severe interference to the MS detection. Therefore, additional peptide enrichment as well as salt removal by solid phase extraction (SPE), particularly using hydrophobic C18 adsorbents, has to be adopted before MS analysis.[3,6]

To simplify the extraction of peptides from serum, ordered mesoporous silica materials have been applied to enable the selective extraction of serum peptides rather than large proteins by the size-exclusion effect of the mesopores.[7] Unfortunately, owing to the inherently insufficient hydrophobicity of silica some peptides are not extracted, thus resulting in a low number of unique peptides being identified. Surface modifications to mesoporous silica and titanium oxide materials have been developed for the enrichment of some specific posttranslational peptides, such as phosphopeptides and glycopeptides in serum.[8] So far, a highly efficient general method for the extraction of a broad spectrum of serum peptides rather than for specific peptides is still absent; such a method would be a crucial technology for the discovery of serum biomarkers from very small blood samples. Herein, we describe the synthesis of an ordered mesoporous carbon material (OMC) and its use for the enrichment of a broad spectrum of endogenous peptides from serum. The expected high efficiency of this method for peptide enrichment is due to the distinct hydrophobicity of carbon[9] as well as the size exclusion of the mesopores against serum proteins. The procedure for the extraction of a broad spectrum of endogenous serum peptides using OMC is illustrated in Scheme 1.



Scheme 1. Enrichment of serum endogenous peptides by OMC.

The OMC was synthesized using a soft-template method.[10] The TEM and nitrogen sorption measurements (see the Supporting Information, Figures S1 a and S1 b) show that it has a well-ordered hexagonal pore structure with a narrow pore-size distribution centered at 4.8 nm, and a specific surface area of about 639 $m^2 g^{-1}$. A pore size of 4.8 nm is an ideal cutoff size to exclude most highly abundant serum proteins (such as HSA; 67 kDa, 5 nm × 7 nm × 7 nm). First, the hydrophobicity of OMC and MCM-41 were characterized by the contact angles of water on their surfaces (see the Supporting Information, Figure S1 c & S1 d). Angles of 58° and 13° for OMC and MCM-41, respectively, indicate the higher hydrophobicity of mesoporous carbon materials than silica materials. Additionally, a water vapor adsorption of 0.085 $mg m^{-2}$ for OMC and 0.126 $mg m^{-2}$ for MCM-41 as well as the toluene adsorption of 217.0 $mg g^{-1}$ for OMC and

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150.8 mg g^{-1} for MCM-41 also indicate the stronger hydrophobicity of OMC versus MCM-41.

To confirm whether the higher hydrophobicity of OMC would result in a stronger retention of the peptides on the adsorbent, an examination was carried out to evaluate the adsorption of the tryptic digests of standard bovine serum albumin (BSA) on silica (MCM-41) and carbon materials (OMC) using loading buffers with acetonitrile (ACN) concentrations of 0%, 10% and 50% (Figure 1). It was found that the number of peptides detected and the peptide peak

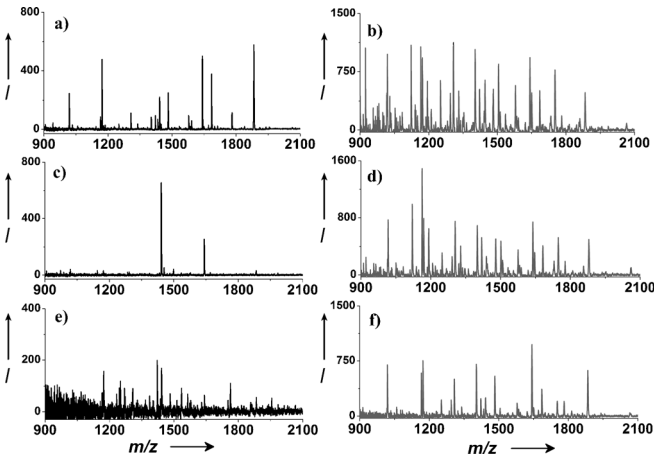

Figure 1. Enrichment of BSA tryptic peptides by MCM-41 and OMC with different concentrations of ACN. a) MCM-41 with 0% ACN, b) OMC with 0% ACN, c) MCM-41 with 10% ACN d) OMC with 10% ACN, e) MCM-41 with 50% ACN, and f) OMC with 50% ACN. The loading amount of peptides was 100 fmol of tryptic digest of BSA.

intensities were much higher for OMC than for MCM-41 when using loading buffers with ACN concentrations of either 0% or 10% and even 50%. With an ACN concentration of 50% almost no peptides were detected on the MCM-41 silica material. In contrast, there were still a reasonable number of peptides retained and detected by MALDI-TOF MS on OMC, thus indicating the stronger retention of peptides on the OMC material.

The other important issue concerns the molecular weight (MW) cutoff of OMC. With regard to the adsorption of BSA proteins and tryptic peptides on OMC it was shown that the adsorption capacity of OMC for peptides ($\leq$ 6 kDa) from the BSA tryptic peptide mixture is about 300 mg g^{-1}, while the adsorption capacity is only 30 mg g^{-1} for BSA ($\geq$ 67 kDa; see the Supporting Information, Figure S2a). Measurements of the extent of N_2 adsorption on OMC indicated that the pore volume of OMC decreased by about 30% after the adsorption of the BSA tryptic peptide mixture, but that after the adsorption of BSA there is almost no change in pore volume (see the Supporting Information, Figure S2b). Analysis of the mass distribution of the endogeneous peptides that had been enriched using OMC (see the Supporting Information, Figure S3), revealed that it was only possible to detect peptides with a MW of less than or equal to 10 kDa. A sample was prepared by adding cytochrome C into the peptide mixture extracted from the serum, and enriching this mixture using OMC again; this sample was then analyzed by MALDI-TOF MS, and the result obtained also indicated that the MW cutoff for OMC is around 10 kDa (9–12 kDa; see the Supporting Information, Figure S4). All the above results indicate that peptides with a MW of less than or equal to 10 kDa can enter into the OMC pores and thus be adsorbed, while proteins were too large to enter into the pores owing to the size-exclusion effect.

The tryptic digest of a mixture of six standard proteins was selected as the standard peptide sample to verify the enrichment efficiency of OMC in comparison to MCM-41 and C18 SPE beads (see the Supporting Information, Table S1). MCM-41 with pore sizes of 2 nm and 4 nm were also used to varify the effect of pore size. It was observed that 156 and 146 different peptides were identified using OMC and C18 materials, respectively, and 102 and 107 different peptides were detected using MCM-41 with pore sizes of 2 nm and 4 nm, respectively. It is clear that the OMC was more efficient than the MCM-41 for the extraction of peptides from the tryptic digest of a mixture of six standard proteins. In comparison to the C18 bead, OMC was just as efficient for the identification of different peptides for all proteins tested. MCM-41 with pore sizes of 2 nm and 4 nm gave very similar results in the extraction and identification of peptides, therefore only MCM-41 with a pore size of 2 nm was used for further investigations. The above results indeed indicate that OMC and C18 materials were more efficient for peptide enrichment than the silica material MCM-41 because of the better hydrophobicity of the carbon material and the long alkyl carbon chain of the C18 material. However, the preremoval of highly abundant large proteins is required for complex samples when using C18 extraction because of the lack of the size-exclusion mechanism possessed by the mesoporous materials.

To further investigate the size-exclusion mechanism of OMC for the peptide enrichment of complex samples, OMC, MCM-41, and UF were used to extract endogenous peptides from human serum samples. Serum samples before and after pretreatment were analyzed by MALDI-TOF MS. Few peptides were detected in the serum before the removal of highly abundant proteins (Figure 2a), however a large number of peptides were detected by MALDI-TOF MS after enrichment with MCM-41, OMC, and UF (see the Supporting Information, Figure S5). Compared to UF and MCM-41, OMC gave the best results for the extraction of serum peptides as the most peptides were identified (Figure 2d). This result can be explained by the following points: 1) although UF can provide the size-selective filtration of highly abundant proteins in the serum, the sample loss through filtration as well as the subsequent coconcentration of other small molecules and salts in the serum made MS detection difficult; 2) the mesoporous materials MCM-41 and OMC not only work by size exclusion of the highly abundant serum proteins but also offer hydrophobic interactions for the adsorption of serum peptides, which made the removal of salts possible by tandem washing steps of the materials containing the adsorbed peptides; 3) by comparing OMC with MCM-41, it is shown that many more peptides with stronger peak intensities were detected by OMC rather than MCM-41.

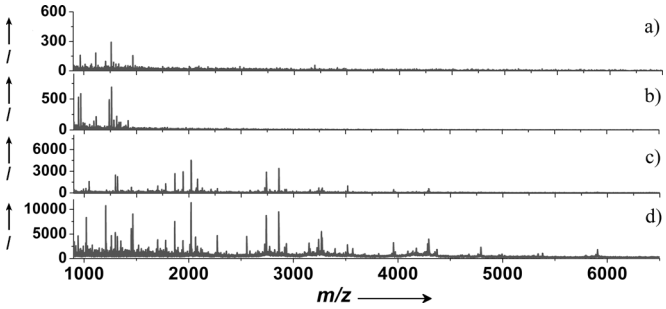

Figure 2. MALDI-TOF MS analysis of human serum peptides by a) direct analysis, and after treatment by b) UF/SPE, c) MCM-41, and d) OMC.

This result could be attributed to the stronger hydrophobicity of OMC over MCM-41, as previously discussed.

The aim of this work is to develop a technology for the efficient extraction of peptides; such a technology is a crucial requirement for the discovery of peptide biomarkers in serum, especially from very small samples. Thus, a 3 μL serum sample was used to evaluate the extraction ability of MCM-41 and OMC. Based on 1D LC-MS/MS analysis, 309 and 790 different peptides were identified using MCM-41 and OMC, respectively (see the Supporting Information, Figure S6). In contrast, only 249 different peptides were identified from 20 μL of serum using UF coupled with C18 SPE extraction (Figure 3, right inset). Among these three extraction methods, OMC was the best for the extraction of endogenous peptides from very small samples of serum. The number of different peptides identified from the serum by OMC extraction was about 2.6 and 3.2 fold more than those extracted using MCM-41 and UF-SPE extraction, respectively (peptide sequences in the Supporting Information, Tables S3 and S4). The mass distribution of the identified peptides (Figure 3) revealed that many more peptides with a MW of less than or equal to 4 kDa or 2 kDa were identified by OMC extraction than by UF/C18 SPE or MCM-41 extraction, respectively. Thus, OMC was shown to be better for the extraction of peptides, especially from small amounts of highly complex samples, because of the combination of the hydrophobic adsorption and size-exclusion mechanism of OMC (see the Supporting Information, Table S2).

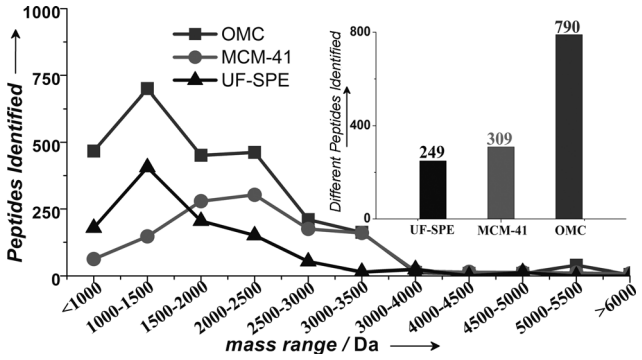

Figure 3. The mass distribution of peptides and the number of different peptides (right inset) identified from human serum by MCM-41, UF-SPE, and OMC extraction.

The limitations of MCM-41 for the extraction of low mass peptides (< 2 kDa) could be attributed to its insufficient hydrophobicity for retaining peptides. Because the extraction of endogeneous peptides was performed at pH 7.4, the peptides with a pI of 3.5–7.4 would be negatively charged and could not be well retained on MCM-41 because of its lower hydrophobicity. However, these peptides might still be retained on OMC because of its stronger hydrophobicity. The identified peptides can be classified into two groups in acidic and basic pH ranges (see the Supporting Information, Figure S7 a). There were many more different peptides identified using OMC than MCM-41 in the acidic pH range (with low pI values), especially for peptides with a pI of 3.5–7.4 and a mass of less than 2 kDa (see the Supporting Information, Figure S7 b).

The peptides extracted by OMC were further analyzed by 2D liquid chromatography coupled with tandem mass spectrometry (2D LC-MS/MS), and a total of 3402 unique peptides were identified from only 20 μL of human serum (peptide sequences in the Supporting Information, Table S5). This number could be the largest number of peptides identified from such a small amount of serum, as compared with previously reported approaches, including silica mesoporous materials of MCM-41,[7] ultrafiltration[11] and adsorption using magnetic nanoparticles[12] (see the Supporting Information, Figure S8), and even the combination of ultrafiltration and online C18 solid phase extraction. To the best of our knowledge, this method using OMC is the most powerful method to determine the endogenous peptides contained in a very small serum sample (20 μL) in 16 h analysis.

In summary, a highly ordered mesoporous carbon material (OMC) was synthesized by a soft-template approach and used for the enrichment of endogenous peptides from a very small serum sample. The OMC material possesses the characteristic hydrophobicity of carbon as well as the size-exclusion (against serum proteins) properties of the mesopores, and thus, performs better than the other reported methods in the extraction and recovery of peptides from serum. By a combination of OMC extraction and 2D LC-MS/MS analysis, a total of 3402 different endogenous peptides were identified from only 20 μL (smaller than 1 droplet) of human serum; this could be the largest number of peptides identified from such a small amount of serum. We believe that the highly efficient extraction of endogenous peptides from human serum by OMC is a very promising technology for the high throughput discovery of serum biomarkers for disease diagnosis.

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