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Biomolecule separation using large pore mesoporous SBA-15 as a substrate in high performance liquid chromatography

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Functionalized large-pore mesoporous SBA-15 is utilized for the first time as a good substrate in high performance liquid chromatography (HPLC) to separate biomolecules including peptides and proteins.

SBA-15, a new type of mesoporous silica with large pore diameter (pore size between 2 and 50 nm), showing excellent homogeneity and stability,[1] has received substantial attention. The morphology of SBA-15 can be well controlled by using block copolymers, co-surfactants or co-solvents,[2] which makes such uniform materials useful for catalysis[3] and adsorption/desorption processes.[4] Furthermore, SBA-15 exhibits excellent characteristics for absorption and desorption of biomolecules such as proteins.[5–8] However, the application of large pore SBA-15 as a substrate in high performance liquid chromatography (HPLC) to separate proteins has not been explored yet. Much effort has been made to exploit mesoporous materials such as MCM-41 as useful column substrates for separation due to their large surface area,[9,10] but such work has revealed problems for biomolecules other than small organic molecules. Here we demonstrate, for the first time, that the functionalized large-pore mesoporous SBA-15 can be used as a satisfactory substrate in HPLC to separate large biomolecules, especially peptides and proteins.

Mesoporous SBA-15 with uniform particle size was synthesized according to a previously reported method,[1,2]† by using cetyltrimethylammonium bromide (CTAB) as a cosurfactant; dimethyloctadecylchlorosilane ($CH_3(CH_2)_{17}Si(CH_3)_2Cl$) ($C_{18}$) was used to produce the C_{18} modified SBA-15 (C_{18}-SBA-15). We manufactured a 250 μm i.d. capillary column (10 cm) including a frit-end fabricated in a manner similar to ref. 11. Capillary LC was conducted using a PE series 200 quaternary pump in a splitter low-flow mode with 1.0 μL injection volume, and an end-column wall-jet amperometric detector with a three-electrode system[12] (homemade) was connected to a CHI potentiostat (Jiangsu Electroanalytical Instruments Works, China). Phosphorate buffer (pH 3.0) was typically applied as the mobile phase. In peptide and protein separation, a 2 mm i.d. steel column was also packed using a common slurry method, and an Agilent HP 1100 Chemstation was used.

The XRD patterns for SBA-15 products in this work exhibit characteristic peaks of highly ordered two-dimensional hexagonal mesostructure,[1] and their functionalization with the C_{18} group leads to a relatively large decrease in the intensities of these peaks, which can be well explained by the fact that the presence of the organic component can decrease the intensity of the XRD patterns,[13] implying a successful surface modification. Scanning electron microscopy (SEM) images for the SBA-15 product show spherical particles with a relatively uniform size of 2 μm. N_2 adsorption/desorption measurements show a well-defined step associated with the filling of the mesopores due to capillary condensation and give a pore size profile. This pore size profile displays a peak at 8.1 nm, with a full width of half maximum ($W_{1/2}$) of no more than 1 nm, indicating a narrow pore size distribution. The surface area and pore volume of SBA-15 were determined to be 800 $m^2\ g^{-1}$ and 1.05 $cm^3\ g^{-1}$ by applying the Barrett–Emmett–Teller (BET) equation and Barrett–Joyner–Halanda (BJH) formula,[14,15] respectively. The surface area of SBA-15 is much higher than that of chromatographic-grade silica of the same pore volume, which could be beneficial for chromatographic separation.

Fig. 1 shows the separation results of four small biomolecules using C_{18}-SBA-15 as a substrate in capillary HPLC. From left to right, the four peaks are the current responses of cysteine (Cys), glutathione (GSH), 6-thiopurine (6-TP) and dopamine (DA), respectively. Each component of the mixture shows a symmetric eluted peak following closely a Gaussian distribution, proving that our homemade capillary column is packed well. These results show that these four small biomolecules can be entirely separated using the functionalized C_{18}-SBA-15 packed capillary HPLC. Compared with the results on a commercial C_{18} column (inset of Fig. 1), the peaks using the C_{18}-SBA-15 packed column are narrower and more symmetric, especially for the DA peak. The plate number for DA is calculated to be as high as 1000, much higher than that (500) for a commercial column; plate numbers of *ca.* 1000 are obtained also for the other three components. Furthermore, the resolution between Cys and GSH ($R_s \sim 1.7$) in C_{18}-SBA-15 is higher than that in a commercial column ($R_s \sim 1.4$), and is capable of discriminating them completely, though their profiles (first two peaks) appear only close to a baseline separation. The longer retention time in the C_{18}-SBA-15 column compared to that in the commercial column may be a result of the higher surface area as well as the uniform inner pores of SBA-15, which should also enhance the LC resolution by increasing the molecular capacity factors. These results indicate that functionalized C_{18}-SBA-15 with high surface area and homogenous pores is a good HPLC substrate.

The intrinsic performance of C_{18}-SBA-15 as a HPLC substrate can also be illustrated in separation of relatively large biomolecules such as peptides. Fig. 2(a) shows the peptide map of myoglobin (horse) digested by trypsin. The molecular weights (MW) of myoglobin peptides are in the range 280–1900 daltons (Da), and the numbers of amino acids range from 2 to

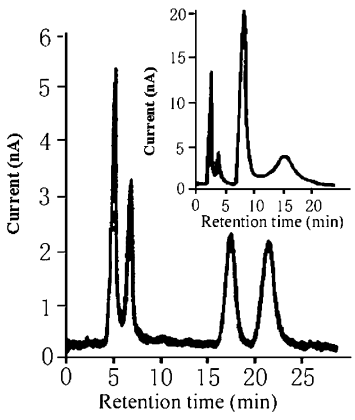

Fig. 1 Chromatogram of four small biomolecules with a C_{18}-SBA-15 capillary column. The inset shows a separation on a commercial C_{18} column. The four peaks represent Cys, GSH, 6-TP and DA, respectively, from left to right. Mobile phase: 0.1 M phosphorate buffer (pH = 3).

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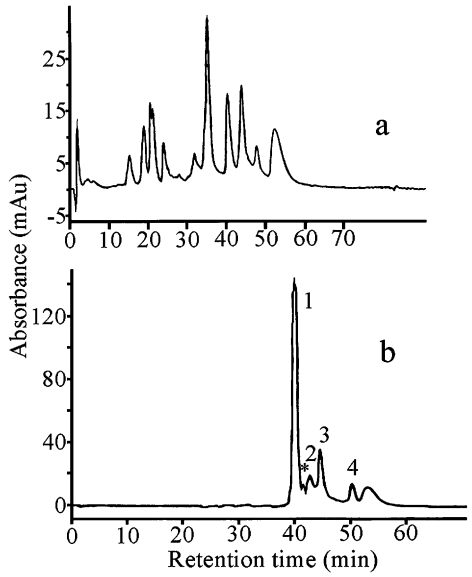

Fig. 2 Chromatogram of myoglobin peptides and four proteins with a C_{18}-SBA-15 2 mm i.d. steel column; mobile phase: H_2O (0.1% trifluoroacetic acid, TFA) and acetonitrile (0.1% TFA). (a) Peptide separation gradient from 5–80 min with acetonitrile (0.1% TFA) changed from 0 to 80%; (b) protein separation.

16. Ten well-resolved peaks in the chromatographic trace are observed (Fig. 2(a)), suggesting that the digested peptide mixture has been separated quite well in the C_{18}-SBA-15 capillary column. As is well known, there are at most 18 peptides from myoglobin under suitable digestion conditions, with five small peptides consisting of only two or three amino acids. The polarity of these small peptides is strong so that they are eluted by water quickly together with salts. The first peak (retention time 4 min) in Fig. 2(a) displays the UV absorption of these small peptides and salts in solution. It seems that the hydrophilic/hydrophobic character of the analytes is the most important factor in these experiments. The well symmetric and baseline separated peaks verify clearly the excellent chromatographic ability of C_{18}-SBA-15 for peptides with relatively large molecular weight.

Large-biomolecule separation has also been investigated for four proteins using the C_{18}-SBA-15 column, and its chromatogram is depicted in Fig. 2(b). These four proteins studied lysozyme, bovine serum albumin (BSA), myoglobin (horse) and ovalbumin (chicken), are different in MW and spatial structure. Table 1 summaries the retention time (R_t) and $W_{1/2}$ for each protein. Six peaks are observed in the chromatographic spectrum, four of which (labeled 1–4) are very symmetric representing lysozyme, BSA, myoglobin and ovalbumin (Fig. 2(b)), respectively. The first shoulder peak (labeled* shown in Fig. 2(b)) is the hemo-group dissociated from myoglobin, which

Table 1 Separation results and information of protein analytes

	Lysozyme	BSA	Myoglobin	Ovalbumin
R_t[a]/$W_{1/2}$ (min)	40.0/0.9	42.7/1.0	44.6/1.0	50.3/0.9
MW (Da)	14 300	66 400	16 900	42 700
Hydrophobic index[b]	65.12	74.14	89.35	90.18

[a] R_t is the retention time, obtained with conditions of a gradient of acetonitrile (0.1% TFA) ranging from 0 to 80% between 5 and 70 min. [b] Hydrophobic indexes taken from an internet Compute pI/MW search on the web site http://expasy.hcuge.ch/

is confirmed by the means of an impurity test using HPLC and electrospray mass spectrometry for a sole myoglobin sample (data not shown). The last wide peak is verified as an unknown impurity present in the commercial ovalbumin sample. The relatively asymmetric peak of BSA can be attributed to the non-uniform interspaces among the substrate particles since the SBA-15 particles used in HPLC are not perfect spheres, which causes the eluted path of the large BSA to be inhomogeneous. These results clearly show that the main components of the protein mixture have been efficiently separated using funtionalized large pore C_{18}-SBA-15 as a substrate. It is noted that the retention time for BSA is shorter than ovalbumin, though the MW of the former is larger. This shorter retention time might be directly attributed to the smaller hydrophilic index of BSA (see Table 1) in addition to the unfavorable effect of molecular sieving on BSA separation. After an auto-integration of each peak, the plate number for each protein is calculated to be $>10\,000$ indicating that C_{18}-SBA-15 is an excellent HPLC packing material for protein separation.

In conclusion, a large pore mesoporous SBA-15 containing a C_{18} functional group is a promising chromatographic substrate for protein/peptide separation, and is expected to have potential use in the separation of various biomolecules.

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Notes and references

† *Characteriation* of SBA-15 products: a low-angle (2θ = 0.7–6°) powder X-ray diffraction pattern (XRD) was recorded on a Rigaku D/Max-IIA using filtered Cu-Kα radiation. The XRD pattern exhibited three characteristic peaks at 2θ = 0.9, 1.4 and 1.6°. The cell parameter (*a*) was calculated to be 11.3 nm. N_2 adsorption desorption isotherms were obtained at 77 K using a Micromeritics Tristar 3000 analyzer. The size and morphology of the products were investigated by SEM (Philips XL-30).

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