Different alkyl dimethacrylate mediated stearyl methacrylate monoliths for improving separation efficiency of typical alkylbenzenes and proteins

Zhendong Xu, Limin Yang, Qiuquan Wang

Department of Chemistry and the MOE Key Laboratory of Modern Analytical Sciences, Xiamen University, Xiamen 361005, China
State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen 361005, China

ARTICLE INFO
Article history:
Received 12 August 2008
Received in revised form 22 January 2009
Accepted 26 January 2009
Available online 31 January 2009

Keywords:
Monoliths
Cross-linker
Stearyl methacrylate
Capillary liquid chromatography
Protein
Alkylbenzene

ABSTRACT
Monoliths were prepared in 530 µm I.D. fused silica capillaries via in situ copolymerization of stearyl methacrylate (SMA) with a dimethacrylate cross-linker in the presence of a binary porogenic solvent containing tert.-butanol and 1,4-butanediol. Alkyl dimethacrylate cross-linkers other than the monomer were used to tune the monolith properties, and, as a result, an increase in the hydrophobicity of the final monoliths (the methylene selectivity αCH2 increased from 1.396 to 1.475) was observed through an increase in the molecular chain length between two methacrylate units from the 0.360 nm of ethylene glycol dimethacrylate to the 1.241 nm of 1.9-nonanediol dimethacrylate. Moreover, the hydrophobicity of the final monoliths was also greatly affected by the methyl group branch in the cross-linkers, among which the 2-methyl-1,8-octanediol dimethacrylate (2-Me-1,8-ODDMA) mediated monolith exhibited the highest hydrophobicity (αCH2 was 1.482) and fastest mass transfer kinetics (C-term was 9.14 ms). Besides the effective separation of six model proteins, the poly(SMA-co-2-Me-1,8-ODDMA) monolith also showed an improved performance in the separation of alkylbenzenes. The theoretical plate numbers reached 83 000 plates/m and 52 000 plates/m for thiourea (nonretained compound) and butylbenzene (retained compound), respectively, when using acetonitrile–water (70:30, v/v) as the mobile phase at a typical linear velocity of 1 mm/s. This improved performance towards small molecules was attributed to an increased mesopore proportion in the monolith and the faster dynamic process of mass transfer arising from novel tailoring of the monolith by choosing a suitable monomer/cross-linker pair.

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1. Introduction

As high-performance liquid chromatography (HPLC) advanced, extensive endeavours were devoted to making more efficient separation media. The most prevalent HPLC stationary phase now is a column packed with functional beads, which can offer good separation of analytes in a variety of samples. However, the large void volume between the packed beads significantly broadens the chromatographic zones and leads to a decrease in the separation efficiency, especially in the case of macromolecule separation, due to their slow diffusion between the streaming mobile phase and the stagnant liquid in both the large void volume between the beads and that inside the porous beads. On the other hand, decreasing the size of the beads can increase the separation efficiency, but high backpressure is encountered when operating at a high velocity. A monolithic column [1], which is an integrated continuous porous separation medium with relatively large size of through-pores compared to the size of skeletons [2], provides a satisfactory solution to the above-mentioned problems, and the mass transfer rate is also accelerated as a result of the small-sized skeletons compared to the particles producing a similar size of interparticle voids as the through-pores of the monolithic column [3–8]. Furthermore, its open channel structure results in high permeability, thus rapid and high throughput separations can be easily realized. The development of a monolithic stationary phase opened a new avenue in chromatographic separation science [2]. As a result of the availability of a wide variety of monomers and the ease of preparation, polymer-based monoliths have been developed very rapidly in recent years, and monoliths in the shape of disks, rods and tubes are already commercially available [9,10].

Generally, polymer monoliths are prepared by bulk one-step copolymerization of functional monomers with cross-linkers in the presence of porogenic reagents through thermal initiation or by UV irradiation. Tuning the porous backbone structure and functional surface chemistry of the monoliths are the main concerns in obtaining the desired chromatographic performance. The most frequently used method for tuning the porous structure involves altering the type or composition of the porogenic reagents, so as to affect the
salvation of the polymer chains during the early stages of polymerization [11]. However, this approach does not change the chemical composition of the final monoliths. Additionally, variation of the other parameters such as monomer, cross-linker or type of initiator can also change the porous property and surface chemistry [9]. One straightforward way to attain different surface chemistry involves employing different monomers, such as acrylamide [12], styrene [13], acrylic acid [14] and methacrylate [15–20] as well as their derivatives [21], and the resulting monoliths have been widely applied in capillary liquid chromatography (cLC) [13,18,19,21] and capillary electrophoresis (CE) [12–17] with different separation modes, as well as solid–phase microextraction [20]. Moreover, the polymerization temperature, the monomer to porogenic reagents ratio, the amount of cross-linker and the method of initiation are known to be effective parameters influencing the properties of monoliths [11]. Like a functional monomer, the cross-linker is also incorporated into the porous framework and remains as an integral part of the final monolithic backbone after polymerization, while the porogenic reagents are washed away. Thus, the surface properties of monoliths depend on the chemistry of both the monomer and the cross-linker. Monovinyl methacrylates with different alkyl substituents are reported to control the surface chemistry (hydrophobic properties) of monoliths [22–24]. However, most research on methacrylate monoliths adopts ethylene glycol dimethacrylate (EGDMA) as the cross-linker, and few other cross-linkers are mentioned in the literature [25,26]. Research concerning the effect of the nature of the cross-linker on the properties of the resulting monoliths has been more or less neglected.

In the present study, alkyl dimethacrylate with different alkyl chain lengths and/or isomeric alkyl chains were proposed as the cross-linkers. A series of stearyl methacrylate (JAVA) monoliths were synthesized in a large bore fused silica capillary (530 μm I.D.) to demonstrate the role of the cross-linkers on the chromatographic performance towards typical alkylbenzenes and proteins. Scanning electron microscopy (SEM), nitrogen adsorption measurement, mercury intrusion porosimetry (MIP) and inverse size-exclusion chromatography (ISEC) were used to characterize the resulting morphological features and structural properties. The influence of cross-linkers on the porous structure and surface chemistry will be discussed based on the characterizing data and chromatographic behavior of alkylbenzenes and proteins.

2. Experimental

2.1. Chemicals

SMA (95%) was purchased from TCI (Tokyo, Japan) for use as a monomer. The structures of the cross-linkers used in this study are shown in Fig. 1. EGDMA (97%) and 1,3-butanediol dimethacrylate (1,3-BDDMA, 95%) were obtained from TCI (Tokyo, Japan); 1,4-butanediol dimethacrylate (1,4-BDDMA, 95%) and 1,6-hexanediol dimethacrylate (HDDMA) from Aldrich (Milwaukee, WI, USA); and neopentyl glycol dimethacrylate (NPGDMA), 2-methyl-1,8-octanediol dimethacrylate (2-Me-1,8-ODDMA) and 1,9-nonanediol dimethacrylate (NDDMA) from Shin-Nakamura Chemical Co. (Wakayama, Japan). 3-(trimethoxysilyl)propyl methacrylate (γ-MAPS, 95%) from TCI was used as a silanization reagent. The polystyrene (PS) standards (Mn: 2500, 4000, 25 000, 50 000, 123 000, 200 000, 400 000 and 2000 000) used for SEC were obtained from Afia Aesar (Ward Hill, MA, USA); Azobisisobutyronitrile (AIBN 97%, recrystallized before use), thiourea, alkylbenzenes (benzene, toluene, ethylbenzene, propylbenzene and butylbenzene), HPLC-grade acetonitrile (ACN) and tetrahydrofuran (THF), tert.-butanol and 1,4-butanediol (distilled before use) were bought from Sinopharm Chemical Reagent (Shanghai, China). Trifluoroacetic acid (TFA) was obtained from Merck (Darmstadt, Germany). The proteins of ribonuclease A (bovine pancreas), cytochrome c (horse heart), bovine serum albumin (BSA), insulin (bovine pancreas), lysozyme (chicken egg white) and myoglobin (horse skeletal muscle) were obtained from Sigma (St. Louis, MO, USA). The ultra pure water used throughout this study was produced using a high-pressure reverse osmosis water purification system (18 MW, Pen-Tung Sah Micro-Electro-Mechanical Systems Research Center of Xiamen University).

2.2. Instrumentation

Fused silica capillary (690 μm O.D., 530 μm I.D.) was purchased from Yongnian Reafine Chromatography (Hebei, China) to prepare the capillary monoliths. The cLC system consisted of two LC-20AD pumps (Shimadzu, Kyoto, Japan), an SPD-20A UV detector with a 210-nL flow cell (Shimadzu, Kyoto, Japan), a CMB-20A system controller (Shimadzu, Kyoto, Japan) and a micro valve injector with a 50-nL inner sample loop (Valco, Houston, TX, USA). A Shimadzu LC-solution chromatography workstation was used for data acquisition. All experiments were performed at room temperature (typically 25 °C) and thiourea was used as the nonretained marker to determine the dwell volume of the system.

An XL30 SEM instrument (Philips, Amsterdam, The Netherlands) was used to study column morphology. The pore size distribution of the monoliths synthesized was measured on a Poremaster 60 mercury intrusion apparatus (QuantaChrome, Boynton Beach, FL, USA), and the surface area was determined on a Micromeritics Tristar 3000 (Norcross, GA, USA) through nitrogen adsorption/desorption, and the results were calculated using the BET equation.

2.3. Column pretreatment

Before use in cLC, monoliths must be firmly bonded to the inner capillary wall. There is some excellent discussion concerning surface modification in the literature [27–30], however, only small bore capillaries (I.D. ≤ 200 μm) or glass plates were investigated before, and no direct data relating to large bore (e.g. 530 μm I.D.) fused silica capillary could be used for reference. Therefore, the influence of surface modification efficiency on the strength of attachment was studied. The inner wall of the fused silica capillary was first rinsed with acetone for 30 min to clean out any organic materials, and the capillary was then activated with 1 mol/L NaOH for 2 h, and leached with 0.5 mol/L HCl for 2 h to remove trace metals, followed by washing with water and acetone. The capillary was dried under a stream of nitrogen at 160 °C overnight in a GC oven. A 30% (v/v) solution of γ-MAPS at pH 5–6 (adjusted using acetic acid) was pumped through the capillary at a flow rate of 20 μL/min for 30 min, then both ends

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Fig. 1. Structure of the cross-linkers used in this study. The length (l) between the two methacrylate units in the corresponding cross-linker was calculated using Chem3D software.
of the capillary were sealed with silicon rubber septa and it was left overnight. Finally, the reactants were purged out using a stream of nitrogen. To evaluate the quality of modification, the capillary rise method [28] was adopted using four commonly used solvents. The results are given in Table 1.

The contact angle, \( \theta \), can be calculated from the capillary rise, \( h_1 \), using the following equation:

\[
\cos \theta = \frac{1}{4} \frac{h_1 d (\rho - \rho_v) g}{\gamma}
\]

where \( h_1 \) is the height of the rise (cm), \( d \) is the internal diameter of the capillary (cm), \( \rho \) is the density of the liquid used (g/mL), \( \rho_v \) is the density of the surrounding air saturated liquid used (g/mL), \( g \) is the acceleration of gravity (cm/s^2), and \( \gamma \) is the surface tension of the solvent (dynes/cm). Water was used as the testing liquid and \( \theta \) was calculated using the following data [28]: \( \rho = 0.997 \) g/mL, \( \rho_v = 1.1845 \times 10^{-3} \) g/mL, \( \gamma = 71.97 \) dynes/cm, and \( g = 980.665 \) cm/s^2.

The higher the value of the contact angle \( \theta \), the more \( \gamma \)-MAPS was bonded on the inner wall surface, and thus the more vinyl anchor sites which could be used for polymerization and so enhance the attachment. The \( \theta \) value (23.5°) of the rinsed and activated capillary was similar to that reported for small bore (50 and 75 \( \mu \)m I.D.) capillaries [24, 28]. The distinct difference should be noted between whether or not the drying procedure under a stream of nitrogen at 160 °C overnight before silanization. Thus the \( \theta \) value is 82.0° and 105.7°, respectively. The residual water on the inner wall, which hinders silanization sites, was effectively removed by drying overnight at 160 °C. Although the contact angles of the other three solvents were also higher than the 70° proposed by Horváth et al. [13] as the threshold value of silanization, toluene was selected here as the solvent during the silanization procedure because it showed the most effective silanization with the lowest \( h_1 \) value (−1.50) and the highest \( \theta \) value (105.7°; Table 1).

### 2.4. In situ preparation of the capillary monolithic column

The preparation procedure for polymerization has been described in detail elsewhere [22]. Briefly, the polymerization mixture was composed of 0.68 g monomers (SMA-cross-linker in equal mole ratio of 2/1), 6.8 mg polymerization initiator (AIBN, 1%, w/w, with respect to monomers) and 1.32 g porogenic solvent (tert.-butanol and 1,4-butanediol) as detailed in Table 2. They were mixed ultrasonically into a homogenous solution followed by purging with nitrogen for 10 min to remove the dissolved oxygen. The polymerization solution was immediately introduced into the silanized fused silica capillary using a syringe. After both ends of the capillary were sealed with silicon rubber septa, the capillary and the remaining polymerization solution sealed in a glass vial were kept at 65 °C for 24 h in a GC oven.

After polymerization, the capillary ends (10–20 mm) were cut off for SEM and the monolithic column was connected to the HPLC pump, flushed with methanol at 10 \( \mu \)L/min to remove residual reagents for further use. The parallel polymerized bulk materials in the glass vial were taken out and ground into small pieces, then soxhlet extracted with methanol for 12 h to remove soluble components. After being kept in a vacuum at 60 °C overnight, these monolithic materials were used for surface area and porosimetric measurements.

### 3. Results and discussion

#### 3.1. Selection and optimization of porogenic solvent

In order to obtain uniform structure and good separation performance, the starting polymerization mixture should be a homogeneous solution. Although typical ternary porogenic solvent (1-propanol, 1,4-butanediol and water) were designed originally for CEC monoliths, in which water is necessary to introduce ionizable functionalities generating electroosmotic flow in CEC application [15], they are also frequently used for the preparation of methacrylate monoliths for cLC purposes [18,19,22]. Our preliminary experiments, however, indicated that the SMA and alkyl dimethacrylate cross-linkers used could dissolve only partly even in the mixture of 1-propanol and 1,4-butanediol without water, which might be attributed to less polarity of the monomer and cross-linkers used and thus limited solubility. This might be one reason why few reported methacrylate-based monoliths are based on long alkyl chain methacrylate monomers [2,23,24]. The correct solvent should be selected in order to obtain a homogeneous polymerization solution and thus the desired porous monoliths. Since porous structure and the copolymer feature depend on the solubility parameter (\( \delta \)) values of monomers and solvents [31], this is often used as a guideline in selecting the correct solvents for preparing macroporous copolymer beads [32]. Unfortunately, the most commonly and frequently used method in selecting and optimizing porogenic solvent for the synthesis of monoliths is the trial and error approach; and the \( \delta \) value is very rarely considered before selection of the porogenic solvent [33]. From the data listed in Table 2, the \( \delta \) values of SMA and alkyl dimethacrylates used are around 17.5 MPa^{1/2}. The \( \delta \) value of tert.-butanol (21.7 MPa^{1/2}) is closer to 17.5 MPa^{1/2} than those of 1,4-butanediol (24.8 MPa^{1/2}) and 1-propanol (24.3 MPa^{1/2}). In this study, therefore, a binary mixture of tert.-butanol (good solvent) and 1,4-butanediol (poor solvent) was used as the porogenic solvent. A homogeneous polymerization mixture of SMA and the corresponding cross-linkers can be obtained in a wide range ratio of monomers/porogenic solvent. In the case of the poly(SMA-co-NDDMA) monolith, increasing the proportion of tert.-butanol from 60% to 75% in the mixture of the porogenic solvent resulted in a decrease of the precursory globule size and the void between them, which arose from later phase separation during the polymerization process. As a consequence, the pore size of the monolith was smaller and the backpressure was slightly increased together with the increase in tert.-butanol proportion (Fig. 2). When the tert.-butanol proportion was 65%, the height equivalent to a theoretical plate (HETP) reached a minimum of 42 \( \mu \)m for butylbenzene. Considering both separation efficiency and permeability, 65% tert.-butanol was used in the porogenic solvent in the following studies although there might have

### Table 1

Comparison of the water meniscus (\( h_1 \)) and the contact angle (\( \theta \)) of water in the modified capillary.

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Unsilanizeda</th>
<th>Tolueneb</th>
<th>Tolueneb</th>
<th>Acetonec</th>
<th>Methanolc</th>
<th>Acetonitrilec</th>
</tr>
</thead>
<tbody>
<tr>
<td>( h_1 ) (cm)</td>
<td>5.10</td>
<td>0.77</td>
<td>−1.50</td>
<td>1.11</td>
<td>1.06</td>
<td>0.20</td>
</tr>
<tr>
<td>( \theta ) (°)</td>
<td>23.5</td>
<td>82.0</td>
<td>105.7</td>
<td>78.5</td>
<td>79.0</td>
<td>87.9</td>
</tr>
</tbody>
</table>

a Fused-silica capillary rinsed and activated.
b Fused-silica capillary rinsed and activated then silanized in toluene without first drying under a stream of nitrogen at 160 °C overnight.
c Fused-silica capillary rinsed and activated then dried under a stream of nitrogen at 160 °C overnight before silanization.
d Average values of triplicate measurements.
e The \( \theta \) value was calculated according to Eq. (1).
Table 2
The polymerization mixtures and properties of the different alkyl dimethacrylate mediated stearyl methacrylate monoliths.

<table>
<thead>
<tr>
<th>Monolith</th>
<th>Cross-linker</th>
<th>Solubility parameter (a ) (\text{MPa}^{1/2})</th>
<th>Monomer mixture</th>
<th>Solubility parameter (B ) (\text{MPa}^{1/2})</th>
<th>BET (\text{m}^2/\text{g})</th>
<th>Mean pore size (\mu\text{m})</th>
<th>HETP (\text{cm}^2/\text{m})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(SMA-co-EGDMA)</td>
<td>EGDMA</td>
<td>18.7</td>
<td>7.7</td>
<td>26.29</td>
<td>2.31</td>
<td>42.9</td>
<td>0.79</td>
</tr>
<tr>
<td>Poly(SMA-co-1,3-BDDMA)</td>
<td>1,3-BDDMA</td>
<td>18.0</td>
<td>8.53</td>
<td>25.47</td>
<td>2.31</td>
<td>42.9</td>
<td>0.78</td>
</tr>
<tr>
<td>Poly(SMA-co-NPGDMA)</td>
<td>NPGDMA</td>
<td>17.4</td>
<td>8.52</td>
<td>25.08</td>
<td>2.31</td>
<td>42.9</td>
<td>0.79</td>
</tr>
<tr>
<td>Poly(SMA-co-HDDMA)</td>
<td>HDDMA</td>
<td>16.0</td>
<td>9.23</td>
<td>24.71</td>
<td>2.31</td>
<td>42.9</td>
<td>0.76</td>
</tr>
<tr>
<td>Poly(SMA-co-2-Me-1,8-ODDMA)</td>
<td>2-Me-1,8-ODDMA</td>
<td>17.8</td>
<td>10.36</td>
<td>23.64</td>
<td>2.31</td>
<td>42.9</td>
<td>0.78</td>
</tr>
</tbody>
</table>

\(a\) Solubility parameter \(a\) of the cross-linker used was calculated applying Small's equation [30].

As shown in Fig. 1, the main difference between the cross-linkers used was the length of alkyl chain between the two methacrylate units. Besides this, methyl group branches exist in 1,3-BDDMA, NPGDMA and 2-Me-1,8-ODDMA. In order to investigate the effect of the cross-linker used on the monolith properties, a series of monoliths was prepared at an equal mole ratio of SMA to the cross-linker used and under identical conditions, except that the kind of cross-linker used was as detailed in Table 2.

3.2. Properties of the monoliths mediated with different cross-linkers

As shown in Fig. 1, the main difference between the cross-linkers used was the length of alkyl chain between the two methacrylate units. Besides this, methyl group branches exist in 1,3-BDDMA, NPGDMA and 2-Me-1,8-ODDMA. In order to investigate the effect of the cross-linker used on the monolith properties, a series of monoliths was prepared at an equal mole ratio of SMA to the cross-linker and under identical conditions, except that the kind of cross-linker used was as detailed in Table 2.

3.2.1. Porous structure

The morphology of the monoliths mediated with different cross-linkers is illustrated in Fig. 3. The SEM images show typical cauliflower like internal structures, which are formed from microglobules of relatively uniform size agglomerated into large clusters. Almost no obvious size difference can be found amongst the microglobules, clusters and channels within the monoliths. These observations can be easily understood because of the equal molar concentrations of all cross-linkers in the corresponding monomers. Moreover, their solubility parameter values are very close, thus the swelling ability of early formed cross-linked nuclei in the binary porogenic solvent does not significantly change, and the phase separation occurs at a similar stage during polymerization, leading to an apparently similar porous structure [11].

SEM provides visual images of the surface, but no quantitative characterization of the surface area or pore size distribution could be obtained. In order to further evaluate the porous properties, nitrogen adsorption and mercury porosimetry measurements were carried out. Data listed in Table 2 show relatively small specific surface areas in the range from 1.44 to 2.06 \text{m}^2/\text{g} of all the monoliths prepared when compared with those of silica based monoliths (170–370 \text{m}^2/\text{g}) [34], and mercury porosimetry revealed that the median pore size diameter was about 1.8 \text{µm} (Table 2), indicating a low percentage of micropores and mesopores in the structure. The majority are macropores, which contribute very little to the surface area. No noticeable porous structure differences can be concluded from the above results. It should be borne in mind that both nitrogen adsorption and mercury porosimetry measurements are performed in the dry state of monoliths and provide little informa-
Fig. 4. Chromatograms of the PS standards and toluene injected individually on the poly(SMA-co-2-Me-1,8-ODDMA) monolith. Column size: 20 cm × 530 μm; mobile phase: 100% THF; flow rate: 5 μL/min; UV detection at 254 nm; peaks: PS standards of (1) $M_r = 2000000$, (2) $M_r = 4000000$, (3) $M_r = 2000000$, (4) $M_r = 123000$, (5) $M_r = 50000$, (6) $M_r = 25000$, (7) $M_r = 4000$, (8) $M_r = 2500$, and (9) toluene ($M_r = 92$).

3.2.2. Improved surface chemistry towards small molecule separation

Besides porous structure properties, surface chemistry is another important factor during the separation process. To investigate the effect of the cross-linker on surface chemistry, a series of alkylbenzenes was separated under isocratic elution on the different cross-linker mediated monolithic columns prepared. Fig. 5a shows that all the columns (20 cm long) exhibit good chromatographic performance on the separation of alkylbenzenes when an ACN–water mixture (70:30, v/v) was used as the mobile phase at a flow rate of 13 μL/min (corresponding to a linear velocity of approximately 1 mm/s). The dependence of the log $k$ on the percent (v/v) ACN in the mobile phase for butylbenzene on those 7 monoliths is shown in Fig. 6, demonstrating that log $k$ linearly decreases with the increase in ACN concentration in the mobile phase. This phenomenon together with the fact that the elution order of the five
Fig. 5. Chromatograms of the typical alkylbenzenes (a) and proteins (b) on various cross-linker mediated monolithic columns. Column size: 20 cm × 530 μm; separation of alkylbenzenes: mobile phase: ACN–water (70:30, v/v); flow rate: 13 μL/min; UV detection at 254 nm; peaks: (1) thiourea, (2) benzene, (3) toluene, (4) ethylbenzene, (5) propylbenzene, (6) butylbenzene, separation of proteins: mobile phase: (A) water + 0.1% TFA, (B) ACN + 0.1% TFA; gradient elution program: 25–50% B in 10 min; flow rate: 22.8 μL/min; UV detection at 214 nm; peaks: (1) ribonuclease A, (2) insulin, (3) cytochrome c, (4) lysozyme, (5) BSA, and (6) myoglobin.
The hydrophobicity of the stationary phase can be conveniently characterized by the methylene selectivity, $\alpha_{CH2}$, which is calculated from the slope of log $k$ against the carbon number of $n$-alkylbenzenes. As listed in Table 2, the values of $\alpha_{CH2}$ were in the range of 1.369–1.485 when using ACN–water (70:30, v/v) as a mobile phase, which was higher than those of the butyl methacrylate monoliths under the same experimental conditions [38]. Such a phenomenon was mainly ascribed to the increase in the alkyl chain length from butyl to stearyl of the methacrylate monomer used in this study. On the other hand, an increasing hydrophobicity tendency together with the increase in alkyl bridge length ($l$ from 0.360 to 1.241 nm) of the cross-linkers was also found. For example, $\alpha_{CH2}$ of EGDMA ($l$= 0.360 nm), 1,4-BDDMA (0.609 nm), HDDMA (0.806 nm) and NDDMA (1.241 nm) mediated monoliths were 1.396, 1.416, 1.468 and 1.475, respectively. This was attributed to the difference in alkyl chain length of the cross-linkers used because the same monomer was used. Extending the alkyl bridge length resulted in an increase in the intrinsic hydrophobicity of the cross-linkers, and led to the increase of apparent hydrophobicity of the monolithic backbone due to the fact that the cross-linker remained as an integral part of the final monolith. It should also be noted that the $\alpha_{CH2}$ values of butylbenzenes on the monoliths mediated with 1,3-BDDMA, NPGDMA and 2-Me-1,8-ODDMA were somewhat higher than with their adjacent counterparts. Thus, although 1,3-BDDMA and 1,4-BDDMA are isomers, the $\alpha_{CH2}$ values on the poly(SMA-co-1,3-BDDMA) monolith and the poly(SMA-co-1,4-BDDMA) monolith were 1.477 and 1.416, respectively; similarly, those on another pair of isomers (2-Me-1,8-ODDMA and NDDMA) mediated monoliths were 1.482 and 1.475, respectively. This was ascribed to the branch methyl group in the cross-linkers used (Fig. 1). In the final monolith backbone, the methyl group branch in the cross-linker may have stood out at the monolith surface rather than merged in the backbone like the alkyl chain between the two methacrylate units. The brush-like methyl groups provided interaction sites in addition to the C18 functional groups of the monomer. On the other hand, the appearance of the methyl group branch in the cross-linker might have resulted in bigger cavities in the monolithic structure, which would have prompted the C18 functional groups to be more easily exposed on the monolith surface, and thus resulted in stronger interaction. This effect may have dominated due to the fact that the hydrophobicity of the C18 functional group is much higher than that of the methyl group. However, when there were two methyl groups located at the same carbon atom, as in the alkyl chain of NPGDMA, the $\alpha_{CH2}$ value of butylbenzene on poly(SMA-co-NPGDMA) was 1.436 between those on poly(SMA-co-1,4-BDDMA) (1.416) and poly(SMA-co-1,3-BDDMA) (1.477), suggesting that steric hindrance might have occurred when the second methyl group was introduced on the same carbon atom in the cross-linker, and the flexibility of the cross-linker was greatly decreased. As discussed above and based on the separation results obtained, 2-Me-1,8-ODDMA was found to be a more suitable cross-linker to the SMA monolith, and it was extensively studied as described in the following sections.

The separation of small molecules like alkylbenzenes is sensitive to porosity and the surface chemistry of the monolith due to their multiplicative distribution between the mobile and stationary phases. Because mobile phase gradient will compress the peaks, HETP must be determined under an isocratic condition [37]. Fig. 7 shows the plots of HETP against the linear velocity of eluent on different cross-linker mediated monoliths using butylbenzene as a test compound. As the increase of the alkyl bridge length of the cross-linkers from 0.360 nm in EGDMA to 1.241 nm in NDDMA, the HETP decreased from about 119–29 $\mu$m (Table 2). Furthermore, the HETPs of the monoliths mediated with the cross-linkers containing a methyl branch group such as 31 $\mu$m for poly(SMA-co-1,3-BDDMA) and 19 $\mu$m for poly(SMA-co-2-Me-1,8-ODDMA) were smaller than those of their isomer counterparts, 64 $\mu$m for poly(SMA-co-1,4-BDDMA) and 29 $\mu$m for poly(SMA-co-NDDMA). In general, the results clearly indicated again that the efficiency of poly(SMA-co-2-Me-1,8-ODDMA) monolith was higher than that of any other monolith prepared in this study.

In many cases concerning separation efficiency, HETP–$u$ curves are usually constructed with nonretained molecules; since if retained ones are selected, HETP should be considerably higher [38,39]. To characterize this aspect of the chromatographic performance of the poly(SMA-co-2-Me-1,8-ODDMA) monolith, HETP versus linear velocity were plotted using thiourea as the nonretained marker and the alkylbenzenes as the retained ones. As can be deduced from Fig. 8, HETPs around the 12 $\mu$m for thiourea and 19 $\mu$m for butylbenzene were found at the typical $u$ value of ca. 1 mm/s. The corresponding number of theoretical plates/m was calculated to be 83 000 and 52 000 for thiourea and butylbenzene, respectively; while the numbers of theoretical plates/m

![Fig. 6. Relationship between log $k$ for butylbenzene and % (v/v) ACN in the mobile phase on the monoliths mediated with different cross-linkers. Column size: 20 cm × 530 $\mu$m.](image)

![Fig. 7. HETP–$u$ curves for butylbenzene on monoliths mediated with different cross-linkers. Column size: 20 cm × 530 $\mu$m; mobile phase: ACN–water (70:30, v/v).](image)
for thiourea and butylbenzene under the same conditions on the poly(SMA-co-EGDMA) monolith prepared in this study were only 11 000 and 8300, respectively. This increased separation efficiency was attributed to the improved mass transfer characteristic (C-term). For example, C-term values (butylbenzene) of the poly(SMA-co-EGDMA) monolith and the poly(SMA-co-2-Me-1,8-ODDMA) monolith were 54.8 and 9.14 ms, respectively. Using 2-Me-1,8-ODDMA as the cross-linker led to a higher proportion of mesopores compared with that when EGDMA was used in the final monolith. These highly interconnected mesopores provided an improved surface area and a fast dynamic process for the small alkylbenzenes to go in and out of the stationary phase, and thus the effective thickness of the diffusion layer might be significantly decreased due to the fast kinetics [40]. Moreover, the alkyl chain lengths between the two methacrylate units of EGDMA and 2-Me-1,8-ODDMA were 0.360 nm and 1.241 nm, respectively, and this bridge length expansion increased the flexibility of both the cross-linker [41] and the C18 groups in the backbone. This increasing flexibility, together with the effect of the methyl group branch in 2-Me-1,8-ODDMA, might have prompted a more efficient stationary phase surface and thus a faster dynamic process of mass transfer. Thus, the mass transfer characteristics of different alkylbenzenes on the poly(SMA-co-2-Me-1,8-ODDMA) monolith were further investigated. The HETP-u curves were analyzed using the simplified van Deemter equation [13]. C-term values for thiourea, benzene, toluene, ethylbenzene, propylbenzene and butylbenzene were 2.97, 6.24, 7.22, 7.99, 9.46 and 9.14 ms, respectively. An increased tendency of C-terms together with the increase in the alkyl chain length of alkylbenzenes could be found (Fig. 8). This could be attributed to the mass transfer resistance in the mesopores, which is more significant for bulkier alkylbenzenes [36,42]. Nevertheless, the HETP-u curves of all the alkylbenzenes used are relatively flat in linear velocity up to approximately 5 mm/s, thus indicating the improved mass transfer properties of this monolith.

### 3.3. Separation of typical proteins on the poly(SMA-co-2-Me-1,8-ODDMA) monolith

Methacrylate-based monoliths have been extensively investigated and widely used due to their suitability for rapid separation and purification of large biomolecules. As expected, the poly(SMA-co-2-Me-1,8-ODDMA) monolith synthesized (biggest mean pore size 2.0 µm among all the seven monoliths synthesized) in this study also showed improved separation of a mixture of six model proteins (Fig. 5b) in the RP mode using a gradient elution. After flushing with about 1500 column volumes, the poly(SMA-co-2-Me-1,8-ODDMA) monolith exhibited a virtually identical performance. The relative standard deviations (RSDs) of the six model proteins were found to be from 0.3% to 0.7% for retention time, 2.7% to 5.7% for peak width at half height, and 0.63% to 4.7% for Rs of two adjacent peaks. The monolith showed good long-term stability, and was robust enough for routine daily use. Actually, the protein mixture could also be baseline separated on the other six monoliths using the same gradient program (Fig. 5b), although the separation efficiency towards alkylbenzenes of these monoliths were lower than that on the poly(SMA-co-2-Me-1,8-ODDMA) monolith. This observation indicated that macropores in the monoliths synthesized in this study were mainly responsible for effective separation of the proteins alongside the hydrophobic interactions, which were reachable by convective flow, and acted as active sites for the proteins.

### 4. Conclusion

The results obtained in this study suggest that it is possible to control the monolith properties by employing different alkyl dimethacrylate cross-linkers. Hydrophobicity of the stationary phase can be changed by the alkyl chain length between the two methacrylate units and the branch groups in the bridge of the cross-linkers. Besides the effective separation of macromolecular proteins, the mesopore characteristics and faster mass transfer dynamics in the swollen state of the poly (SMA-co-2-Me-1,8-ODDMA) monolith improved the separation performance towards small molecules. This strategy is informative for designing and synthesizing more efficient methacrylate polymer based monolithic columns. Different cross-linker mediated hydrophilic monoliths will be the subject of future studies.

### Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (20535020, 20775062), the National “863” Hi-Tech Project of China (2006AA06Z404) and the National Basic Research Program of China (2009CB921600). Professor John Hodgkiss of the University of Hong Kong is thanked for his help with English.

### References


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**Fig. 8.** HETP-u curves for thiourea and alkylbenzenes on the poly(SMA-co-2-Me-1,8-ODDMA) monolith. Column size: 20 cm × 530 µm; mobile phase: ACN–water (70:30, v/v).