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The effect of hydrothermal treatment on column performance for monolithic silica capillary columns

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ABSTRACT

Monolithic silica capillary columns with i.d. 100 μm and monolithic silica rods were prepared with tetramethoxysilane (TMOS) or a mixture of TMOS and metyltrimethoxysilane (MTMS) using different hydrothermal treatments at $T = 80\,^{\circ}C$ or 120 °C. Nitrogen physisorption was applied for the pore characterization of the rods and inverse size exclusion chromatography (ISEC) for that of the capillary columns. Using nitrogen physisorption, it was shown change of pore size and surface area corresponds to that of hydrothermal treatment and silica precursor. The results from ISEC agreed well with those from nitrogen physisorption regarding the pore size distribution (PSD). In addition, the retention factors for hexylbenzene with the ODS-modified capillary columns in methanol/water = 80/20 at $T = 30\,^{\circ}C$ could also support the results from nitrogen physisorption. Furthermore, column efficiency for the columns was evaluated with alkylbenzenes and three kinds of peptides, leucine-enkephalin, angiotensin II, and insulin. Column efficiency for alkylbenzenes was similar independently of the hydrothermal treatment at $T = 120\,^{\circ}C$. Even for TMOS columns, there was no significant difference in column efficiency for the peptides despite the difference in hydrothermal treatment. In contrast, for hybrid columns, it was possible to confirm the effect on hydrothermal treatment at $T = 120\,^{\circ}C$ resulting in a different column efficiency, especially for insulin. This difference supports the results from both nitrogen physisorption and ISEC, showing the presence of more small pores of ca. 3–6 nm for a hybrid silica without hydrothermal treatment at $T = 120\,^{\circ}C$. Consequently, the results suggest that hydrothermal treatment for a hybrid column with higher temperature or longer time is necessary, compared to that for a TMOS column, to provide higher column efficiency with increase in molecular size of solute.

1. Introduction

Monolithic silica materials have been of considerable concern in HPLC because of the continuous and bimodal porous structure (meso- and macroporosity). It has been demonstrated that the structure provides larger column permeability and higher column efficiency simultaneously, compared to particulate columns [1,2]. The conventional monolithic silica column (i.d. 4.6 mm × 100 mm) sealed with a poly(ether ether ketone) tube has been already commercialized by Merck (Darmstadt, Germany). Monolithic silica columns can also be prepared as capillary columns using tetramethoxysilane (TMOS) or a mixture of TMOS and methyltrimethoxysilane (MTMS) [3,4]. A monolithic silica capillary column, even longer ones, is accessible by a facile procedure, compared to a particulate column requiring frits to keep particles and high pressure to pack small particles in a long capillary column. Monolithic silica columns are of fundamental interest, because the porosity can be adjusted by variation of the synthesis formula [5–7]. For fast and high-efficiency separations, it was reported that a monolithic silica capillary column can provide a high column efficiency which is comparable with that of a particulate column packed with 2–2.5 μm particles, but the pressure is similar to that of a particulate one packed with particles at ca. 5 μm [5]. In addition, owing to the high column permeability, quite long monolithic silica capillaries can be used in HPLC [8]. As an application in proteome analysis, it was recently reported by Ishihama and co-workers that identification of more than 2600 proteins from *Escherichia coli* cells was carried out using a C18 long capillary column with 3.5 m in a μ-LC/MS/MS system, providing higher than 400,000 theoretical plates for hexylbenzene under less than 20 MPa [9]. Thus, a main advantage of monolithic silica columns is high separation efficiency combined with lower pressure compared to a particulate column.

In general, the elucidation of the relationship between the porosity and HPLC performance is of vital importance for the development or improvement of monolithic silica columns. However, the precise characterization of the porosity (pore volume, surface area, size distribution of meso- and macropores) is still a challenge by itself, in particular for capillaries, due to the very low amount of material available in one particular capillary.

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One of the main objectives of the present study is to relate HPLC properties to variations in porosity of monolithic silica capillaries, applying suitable methods for the characterization of meso- and macroporosity.

Important techniques to analyze the porosity are mercury sorption [10–13], but recently transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM) was introduced as a valuable characterization method for the macroporosity [14–18]. For the characterization of the mesoporosity, physisorption is usually utilized, mainly using nitrogen.

Several recent studies have already addressed the influence of porosity parameters on HPLC properties. Using nitrogen physisorption, it was reported that the average mesopore sizes or pore size distribution (PSD) of monolithic silica rods can be controlled by treatment with ammonia solution after the phase separation [19,20]. In that case, the formation of mesopores in monolithic silica strongly depends on the pH value, time and temperature for the immersion in ammonia solution, which is governed by Ostwald ripening [19–23]. To study column efficiency of conventional monolithic silica columns with different pore sizes, Guiochon and co-worker have recently reported the effect on mass transfer of solute within pores in HPLC [24]. For protein separation using a monolithic silica column, the influence of pore size on separation efficiency was already described [25]. Such a study is vital to understand the effect of hindrance of solute diffusion inside pores corresponding to the relationship between molecular size of solute and pore sizes in silica.

As a particularly suitable approach for characterization of column porosity in HPLC, inverse size exclusion chromatography (ISEC) can be utilized [26–30]. ISEC allows the determination of PSD of a porous material using precisely defined polystyrene standards with known molecular weight, dissolved in tetrahydrofuran (THF). This method is based on the relationship between the rotational coil diameter of polystyrene in a solvent and the corresponding pores of silica [27]. For conventional monolithic silica columns, the pore characterization has already been performed using ISEC [11,31]. It was recently demonstrated by Thommes and co-workers that there is a reasonable correlation for monolithic silica rods between the PSD mathematically estimated from ISEC and that obtained from using Non-Local Density Functional Theory (NLDFT) model by nitrogen physisorption [32].

However, such systematic analysis has not been carried out so far for monolithic silica capillaries, since the amount of material in one capillary is significantly too small for a meaningful physisorption analysis. ISEC can be regarded as a useful method to study the mesoporosity of a capillary column, because it is possible to characterize the mesoporosity directly from HPLC measurements. In the present study, ISEC is applied to monolithic silica capillaries with respect to different aspects.

First, the validity of the PSD determination by ISEC is studied by comparing ISEC (using polystyrene standards) with high-precision nitrogen physisorption applied to monolithic silica rods, which are prepared by the same recipe as the monolithic silica capillaries under question. So far, ISEC has only been rarely used to characterize monolithic columns, especially capillaries.

Second, ISEC is used to study the influence of hydrothermal treatment on the mesoporosity in capillaries. Hydrothermal treatment involves the generation of mesopores and is one of the few methods allowing a fine tuning of the mesoporosity in monolithic silica.

As example, the characterization for TMOS monolithic silica capillary columns using ISEC was reported by Demesmay and coworkers, to simplify the preparation process [33]. In this work, only small molecules like alkylbenzenes or PAHs were utilized to evaluate a capillary column in HPLC. A comprehensive understanding of the effect of hydrothermal treatment on column efficiency in HPLC requires the employment of solutes with different molecular sizes [24]. Demesmay and co-workers reported that a column without suitable hydrothermal treatment at $T = 120\,^{\circ}C$ results in significantly lower column efficiency with increase in linear velocity in HPLC, compared to a column exposed to the treatment. It should be emphasized that there is only a limited number of methods to modify the mesoporosity in monolithic silica and, thus, the HPLC performance without disturbing the peculiar meso- and macropore structure.

As third objective, our study is dedicated to elucidate the differences in the impact of hydrothermal treatment on pure monolithic silica columns compared to hybrid monolithic silica columns. It was recently shown that hybrid monolithic silica columns represent an interesting approach to study the HPLC performance, in particular using mixtures of TMOS/MTMS, e.g. by studying the influence of MTMS on column efficiency [7].

Monolithic silica columns were prepared in capillaries with an inner diameter (i.d.) of 100 μm, and monolithic silica rods using TMOS and a mixture of TMOS/MTMS according to a previous report [7]. The hydrothermal treatment with urea at $T = 80\,^{\circ}C$ or $120\,^{\circ}C$ was carried out for generating mesopores. For the characterization of the meso- and microporosity, ISEC was utilized for bare monolithic silica capillary columns using polystyrene standard samples in THF and nitrogen physisorption for the silica rods, to compare the porosity between them. The evaluation of monolithic capillary columns modified by octadecylsilylation was performed with alkylbenzenes, leucine-enkephalin, angiotensin II, and insulin in reverse-phase liquid chromatography (RPLC), to observe the effect of hydrothermal treatment on column efficiency due to mass transfer of solute inside pores.

2. Experimental

2.1. Materials and chemicals

Tetramethoxysilane (TMOS), octadecyldimethyl-*N,N*-diethylaminosilane (ODS-DEA), *N*-(trimethylsilyl)imidazole (TMSI), poly(ethylene glycol) (PEG; $M_n = 10{,}000$), urea, alkylbenzenes ($C_6H_5(CH_2)_nH$, $n = 1$–6) and acetonitrile of HPLC grade were obtained from Merck Company (Darmstadt, Germany). Polystyrene standards with a molecular weight (M_w) of 474, 890, 1820, 3470, 9730, 17,600, 28,000, 77,000, 100,000, 141,000, 229,000, 321,000, 633,000, 1,044,000, and 2,190,000 were purchased as well as leucine-enkephalin, angiotensin II, and insulin with HPLC grade from Sigma–Aldrich (Taufkirchen, Germany). Polystyrene standards with $M_w = 5858$, 13,648, 48,900 came from Pressured Chemical (Pittsburgh, PA, USA). Methanol and tetrahydrofuran (THF) of HPLC grade were purchased from Carl Roth (Karlsruhe, Germany), trifluoroacetic acid (TFA) (≥99%) from VWR international GmbH (Darmstadt, Germany), and methyltrimethoxysilane (MTMS) from Dow Chemical Company (Midland, Michigan, USA). Fused-silica capillaries of i.d. = 100 μm and o.d. = 375 μm were purchased from Polymicro Technologies (Phoenix, AZ, USA).

2.2. Monolithic silica synthesis

2.2.1. Preparation of monolithic silica capillary columns

The preparation conditions of the monolithic silica columns were similar to those reported previously [4,5,7,8]. Typical conditions are as follows. A fused-silica capillary tubing of 1.5 m in length was treated with a 1 M aqueous sodium hydroxide solution at $T = 40\,^{\circ}C$ for 3 h, washed with water and acetone, and then dried. A monolithic silica capillary column was prepared from TMOS. A TMOS (5.6 ml) was added to a solution of PEG (1.20 g) and urea (0.90 g) in 0.01 M acetic acid (10 ml) at $T = 0\,^{\circ}C$ and stirred for 30 min.

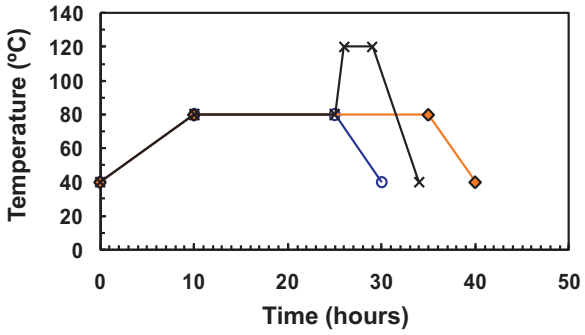

Fig. 1. Process of hydrothermal treatment for monolithic silica rods. Symbol: hydrothermal treatment (HT) at 80 °C for 15 h (○), HT at 80 °C for 25 h (◆), HT at 80 °C for 15 h + 120 °C for 3 h (✕).

The homogeneous solution was then stirred for 10 min at $T = 25$ °C, filtered with a 0.20 μm PTFE filter, charged into a fused-silica capillary tube, and allowed to react at $T = 25$ °C in a water bath. The resultant gel was subsequently aged in the capillary overnight at the same temperature.

Then, a hydrothermal treatment for a capillary column was performed in an oven to form mesopores by the ammonium carbonate generated by the hydrolysis of urea, as shown in Fig. 1.

(i) The temperature was raised slowly from $T = 40$ °C to $T = 80$ °C for 10 h for long capillary columns.
(ii) As the following process, the monolithic silica columns were treated for 15 h or 25 h at $T = 80$ °C and then cooled down to $T = 40$ °C for 5 h.
(iii) To confirm the effect of temperature for hydrothermal treatment on mesoporosity, the additional treatment at $T = 120$ °C for 3 h to a long column was carried out after the heat treatment at $T = 80$ °C for 15 h and then cooled down to $T = 40$ °C for 5 h.

After washing a capillary with methanol and drying, heat treatment was carried out at $T = 330$ °C for 24 h, resulting in the decomposition of the organic moieties in the capillary.

In addition, a hybrid capillary column was prepared using a mixture of TMOS/MTMS ($V_T/V_M = 85/15$) to form a hybrid structure [7]. Similar hydrothermal treatment for making mesopores was performed as well as that for a TMOS capillary column.

Table 1 shows the initial preparation conditions and identifiable information for the monolithic silica capillary columns.

2.2.2. Preparation of monolithic silica rods

Monolithic silica rods were obtained by the similar preparation conditions as shown in Fig. 1 and Table 1. Ca. 7 ml of a preparation feed solution was stored in a polypropylene plastic tube and then the gelation occurred in a water bath for a day. The hydrothermal treatment at $T = 80$ °C for a silica rod was carried out directly using a polypropylene plastic tube. For the hydrothermal treatment at $T = 120$ °C, a TMOS silica rod was stored in the solution of urea (0.09 g/ml) prepared with 0.01 M acetic acid using a glass vessel which can withstand the increased pressure at $T = 120$ °C, and a hybrid silica rod in the other solution of urea (0.10 g/ml). Urea in such a solution using the glass vessel was decomposed simultaneously during the hydrothermal treatment at $T = 80$ °C for a silica rod. Then, the treatment at $T = 120$ °C for a silica rod was carried out in 50 ml of that solution for 3 or 4 h. The silica rods were crushed to small pieces for nitrogen physisorption and washed with methanol in a glass vessel for 7 days, and heat treatment was carried out at $T = 330$ °C for 24 h in an oven.

2.2.3. Surface modification

Surface modification of a monolithic silica capillary column was carried out on-column with continuously feeding a solution of ODS-DEA (2 ml) in 8 ml of toluene driven at $T = 60$ °C by a syringe pump. At least, for the surface modification, the solution with 10 times larger volume than the tubular volume of a capillary was flowed into a column. Before RPLC measurement using the three kinds of peptides, TMS modification was carried out with a solution of TMSI (2 ml) in 8 ml of acetonitrile as well as octadecylsilylation with ODS-DEA. To show the endcapping with TMSI, the last abbreviation "e" was added for a column name, as changed from MS(100)-T120-3h to MS(100)-T120-3h-e.

2.3. Characterization

2.3.1. Nitrogen physisorption of monolithic silica rods

The nitrogen physisorption measurements were performed in an automated gas adsorption station (Autosorb-1-MP, Quantachrome Corporation, Boynton Beach, USA). The device was utilized for standard characterization measurements of nanostructured matter by nitrogen sorption isotherms at $T = 77$ K. The instrument software supported the standard data reduction algorithms such as Brunauer–Emmett–Teller (BET), as well as NLDFT kernels for typical pore geometries. The monolithic silica rods were filled in standard glass tubes and were stabilized at the measurement temperature with $T = 77$ K kept by liquid nitrogen in standard cryostats. The isotherms were measured up to 0.95 of the equilibrium nitrogen pressures p°. Before the measurements, the monolithic silica rods were evacuated for 6 h at $T = 120$ °C.

2.3.2. SEM observation

The morphology of the monolithic silica capillary columns was examined by a high resolution scanning electron microscope (HSEM: Leo Gemini 982, Leo (Zeiss), Oberkochen, Germany) using a fractured surface.

2.3.3. HPLC measurement for capillary columns

HPLC instrument was used for the characterization and evaluation of the monolithic silica capillary columns. The set was L-7100 pump (Hitachi, Tokyo, Japan) with split injection/flow using

Table 1
Preparation conditions for monolithic silica capillary columns.[a]

Column	TMOS (ml)	TMOS/MTMS = (85/15) (ml)	PEG (g)	Urea (g)	AcOH (ml)	Gelation temperature (°C)
MS(100)-T80-15h MS(100)-T80-25h MS(100)-T120-3h	5.6		1.20	0.90	10	25
MS(100)-Hy80-15h MS(100)-Hyl20-4h		5.5	0.48	1.01	10	35

[a] The abbreviation, MS, stands for monolithic silica followed by the capillary diameter in parentheses, and T or Hy for the silica support material, TMOS or hybrid. The following number is for temperature of hydrothermal treatment, and the last number for the treatment time. For example, the treatment at $T = 80$ °C for 15 h was carried to MS(100)-T80-15h, and the additional treatment at $T = 120$ °C for 3 h to MS(100)-T120-3h, as shown in Fig. 1.

a Rheodyne 7125 (Rheodyne, Cotati, CA, USA), UV detector K-2501 (Knauer, Berlin, Germany) for ISEC measurement. For the detection, a fused-silica capillary with i.d. 30 μm was utilized as a UV capillary cell. The cell length from column outlet to the detection window was always kept 3.4 cm to estimate column porosity exactly. The chromatographic measurements using split injection/flow mode were performed as previously described [4]. The chromatographic data were processed with D-7000 HSM software (Hitachi). The ISEC measurement was carried out with THF in order to characterize column properties of the bare monolithic silica columns at $T = 30\,^{\circ}C$ using the polystyrene standards. A linear velocity was set to 1.0 mm/s in the ISEC measurements.

In the ISEC measurements, we used a flowmeter attached to the outlet of UV capillary cell and always measured the elution times of an excluded peak and the peak of toluene corresponding to total permeation from one chromatographic run. The fraction of elution volume for polystyrene standards with a molecular weight of 3470, 28,000, 321,000, 2,190,000 to that for toluene was measured six times. The resulting relative standard deviation (RSD (%)) was less than 0.2% for the volume of mesopores. Therefore, the comparison of porosity for the capillary columns can be discussed with confidence. Using ISEC, PSD of a monolithic silica capillary column was determined from the estimation reported previously by Al-Bokari and co-workers [31]. Pore diameter of monolithic silica was calculated according to the classical method by Hàlasz and Martin as represented using Eq. (1) [27,31].

$$D_{pore}[\text{Å}] = 0.62(M_w)^{0.59} \quad (1)$$

The measurements in RPLC was carried out for a monolithic silica columns using alkylbenzens in methanol/water = 80/20 or acetonitrile/water = 80/20 at $T = 30\,^{\circ}C$. In addition, using leucine-enkephalin, angiotensin II, and insulin, column efficiency was evaluated in acetonitrile/water/TFA = 28/72/0.1 or acetonitrile/water/TFA = 33/67/0.1 at $T = 30\,^{\circ}C$ after the modification of the capillary columns with TMSI. MU701 UV-VIS detector with 2 nl of UV capillary cell (GL Sciences, Tokyo, Japan) was utilized in the all measurements in RPLC.

3. Results and discussion

3.1. Characterization of monolithic silica rods

In order to study the influence of hydrothermal treatment on the mesoporosity in monolithic silica, monolithic silica rods were prepared using different hydrothermal conditions (see Fig. 1). The hydrothermal treatment procedures only differed in the duration of the treatment at $T = 80\,^{\circ}C$ and the presence/absence of an additional treatment at $T = 120\,^{\circ}C$. Note that in all cases the treatment at $T = 80\,^{\circ}C$ is mandatory to obtain a well-defined monolithic structure and mechanically stable monoliths.

Fig. 2(a) and (b) shows the isotherm curves and PSD of TMOS and TMOS/MTMS hybrid silica rods using nitrogen physisorption. The NDLFT method for silica was applied on the adsorption branch, because it was demonstrated that it is more suitable, especially to evaluate microporosity, compared to the Barett–Joyner–Halenda (BJH) method [34].

These analyses reveal several interesting insights into the dependence of mesoporosity on the hydrothermal treatment applied (Fig. 2(a) and (b)). The treatment of a TMOS rod at $T = 80\,^{\circ}C$ for 25 h provided only slight differences in the isotherm curve compared to that for 15 h.

However, the additional hydrothermal treatment at $T = 120\,^{\circ}C$ for 3 or 4 h significantly changes the mesoporosity compared to that at $T = 80\,^{\circ}C$. This trend was observed for both, TMOS and hybrid rods.

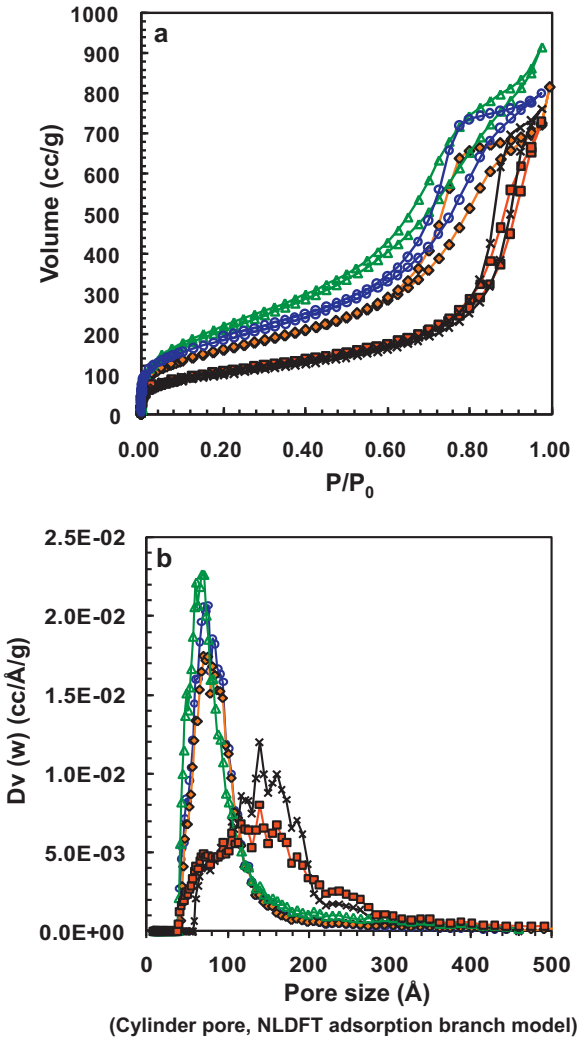

Fig. 2. Pore characterization of monolithic silica rods by nitrogen physisorption. (a) Isotherm curves. (b) Pore size distribution obtained from NLDFT method. Silica rod: TMOS rod treated at 80 °C for 15 h (●), TMOS rod treated at 80 °C for 25 h (◆), TMOS rod treated at 80 °C for 15 h + 120 °C for 3 h (✕), hybrid rod treated at 80 °C for 15 h (▲), hybrid rod treated at 80 °C for 15 h + 120 °C for 4 h (■).

The additional treatment of TMOS or hybrid rods at $T = 120\,^{\circ}C$ provided larger pores and a wider PSD than the hydrothermal treatment at $T = 80\,^{\circ}C$ only, as shown in Fig. 2(b). Comparing the pH values, measured at T = ca. 23 °C, of solutions of urea (0.09 g/ml) in 0.01 N acetic acid exposed to hydrothermal treatment at $T = 80\,^{\circ}C$ for 15 h with the pH value of a solution treated additionally at $T = 120\,^{\circ}C$ for 3 h, the former solution provided pH = 9.8 and the latter pH = 10.2. This increase in the pH value is a result of the transformation from urea to ammonium carbonate by heat. This interpretation is supported by the observation that a solution heated from room temperature to $T = 80\,^{\circ}C$ during 10 h possessed a pH value of 8.1. These results show that the solutions of urea

Table 2
Average pore size, BET surface area and pore volume of monolithic silica rods.

Monolithic silica	Average pore size (Å)	Surface area (m^2/g)	Pore volume (cc/g)
TMOS silica rod-80-15h	74	668	1.2
TMOS silica rod-80-25h	78	582	1.1
TMOS silica rod-120-3h	130	352	1.1
Hybrid silica rod-80-15h	66	789	1.3
Hybrid silica rod-120-4h	118	380	1.1

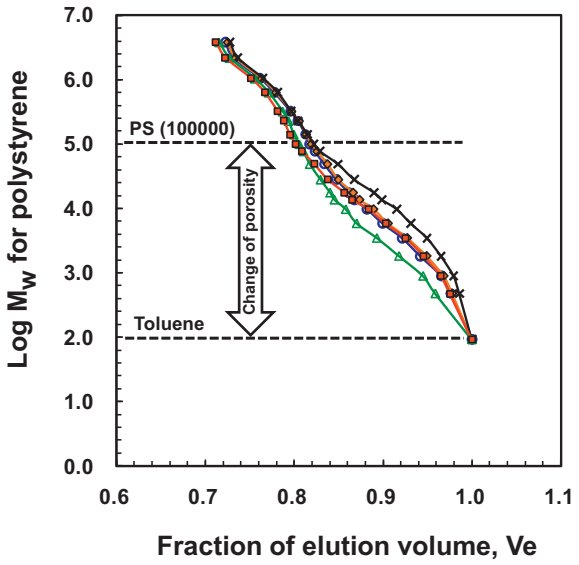

Fig. 3. Selective permeation curves of polystyrene standards in THF for bare monolithic silica capillary columns. Column: MS(100)-T80-15h ( ), MS(100)-T80-25h ( ), MS(100)-T120-3h ( ), MS(100)-Hy80-15h ( ), MS(100)-Hy120-4h ( ). Temperature: 30 °C. Fraction of elution volume: $V_e = V_{PS}/V_{toluene}$.

can provide alkaline conditions needed for generating mesopores in silica according to the process shown in Fig. 1. The PSD becomes wider and the average mesopore size larger with higher temperature and a larger pH value due to Ostwald ripening. This result agrees well with that obtained from an ammonia solution used to obtain mesopores [19,20].

Our study also allowed the comparison of TMOS silica rods and hybrid silica rods regarding the influence of the additional hydrothermal treatment at $T = 120$ °C. The influence of methyl groups on the formation of mesopores was recently described for MTMS monolithic silica [35]. Our result suggests that it is more difficult to obtain hybrid monolithic silica rods featuring well-defined mesopores above ca. 12 nm as expected. The additional treatment at $T = 120$ °C results in an increased mesopore size, but the PSD is substantially wider and less defined compared to TMOS based rods treated identically (see Fig. 2b).

For the all materials under study, no microporosity (pores below 20 Å according to IUPAC) was observable, in particular not for three silica rods carried out with the treatment at $T = 80$ °C for 15 h, which can be expected to construct more smaller pores than the rods with additional treatment at $T = 120$ °C. The differences in the mesopore size at similar pore volume are in line with the surface areas determined from the BET approach (see Table 2): the materials possessing a larger average mesopore size featured an accordingly smaller surface area, and vice versa.

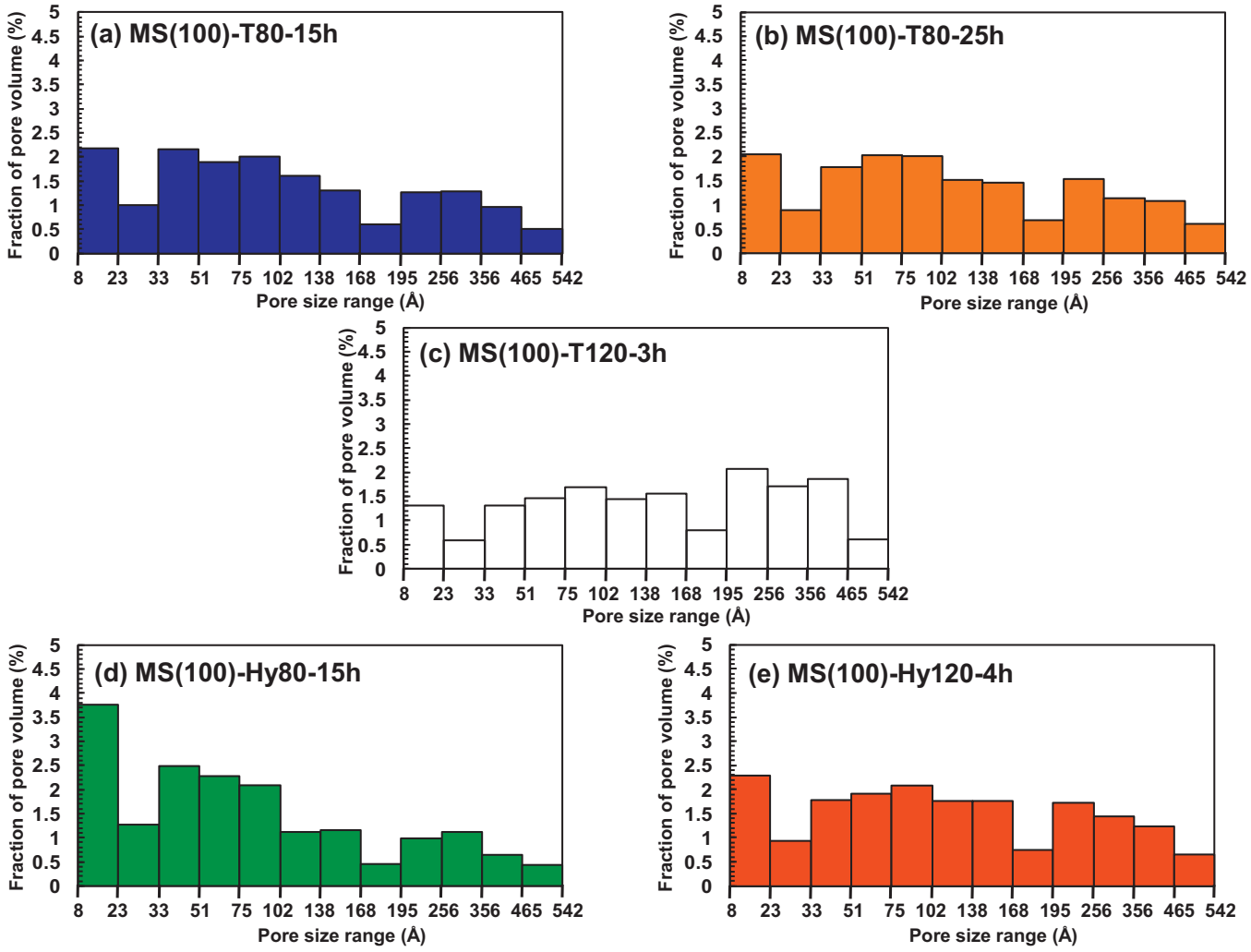

Fig. 4. Pore size distribution of bare monolithic silica columns obtained from ISEC. Column: (a) MS(100)-T80-15h, (b) MS(100)-T80-25h, (c) MS(100)-T120-3h, (d) MS(100)-Hy80-15h, (e) MS(100)-Hy120-4h. Fraction of pore volume and pore size was calculated according to Ref. [31].

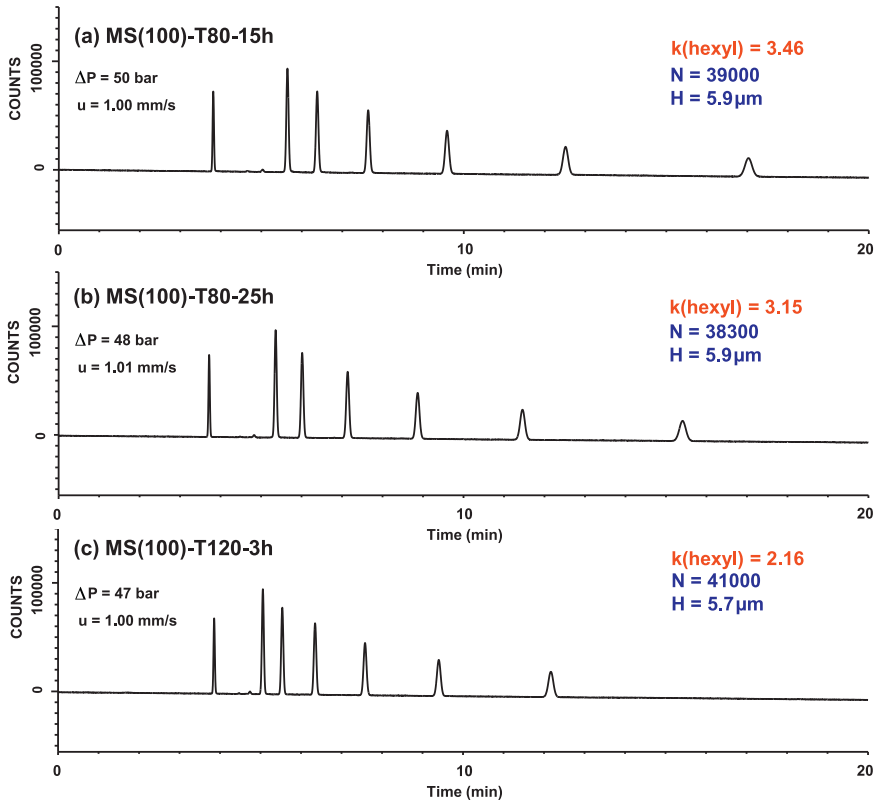

Fig. 5. Chromatograms obtained for alkylbenzenes with ODS-modified monolithic silica columns. Column: (a) MS(100)-T80-15h (column length 23.0 cm), (b) MS(100)-T80-25h (22.5 cm), (c) MS(100)-T120-3h (23.2 cm). Mobile phase: methanol/water = 80/20. Solute: thiourea, alkylbenzenes ($C_6H_5(CH_2)_nH$, n = 1–6). Temperature: 30 °C. Detection: 210 nm. Pressure drop and linear velocity are shown. Retention factor, number of theoretical plates, and plate height for hexylbenzene are also indicated.

3.2. ISEC for monolithic silica capillary columns

As shown in Fig. 3, the relationship between elution volume and the molecular weight of a polystyrene standard (PSS) for the bare monolithic silica capillary columns was evaluated for the materials under study. The fraction of elution volume is estimated as the ratio of the volume of each polystyrene standard to that of toluene which provides the total permeation volume of a column. The elution curve, using of PSS of varying molecular weight, correlates to corresponding column porosity, because a small molecule can penetrate into pores, but a large molecule cannot, according to the principle of size exclusion chromatography (SEC). For example, a column with small pores can provide a significantly large change in the fraction with a low molecular weight range, but not in a large molecular weight range, and vice versa [36]. The elution curves shown in Fig. 3 prove that the hydrothermal treatments influence the column porosity corresponding to change of elution volume for PSSs and toluene with molecular weight spanning 92–100,000. According to Eq. (1), the estimation of pore size of silica using those PSSs and toluene corresponds to 8 Å to 542 Å. The additional hydrothermal treatment at T = 120 °C significantly affects the column porosity both for TMOS and hybrid capillary columns, but a treatment solely at T = 80 °C did not, comparing MS(100)-T80-15h with MS(100)-T80-25h. Consequently, these elution curves agree well with nitrogen physisorption for silica rods with similar hydrothermal treatment.

In Fig. 4, the PSD of the monolithic silica capillary columns is shown using toluene and PSSs with molecular weight from 474 to 100,000 in ISEC. The extension (mimimal and maximum pore size) and increment in the pore size (x-axis) is determined by the polystyrene standards available. Several relevant insights were obtained from these ISEC measurements regarding the hydrothermal treatment applied to the monolithic capillaries.

First, it is observed that the PSD of the monolithic silica capillary columns depends strongly on the temperature of hydrothermal treatment, as already demonstrated in a previous study [33]. Applying T = 120 °C as hydrothermal treatment leads to an enhanced fraction of larger mesopores contributing to the pore volume, being in agreement with the physisorption analysis performed on monolithic silica rods.

Second, the difference in the silica precursor of a preparation feed between TMOS and hybrid columns influences the PSD. Especially, MS(100)-Hy80-15h possess the highest degree of small pores in comparison with other columns. Subsequently, it can be confirmed that the change in PSD of the monolithic silica capillary columns from ISEC corresponds to that for silica rods determined by nitrogen physisorption.

Fig. 5 shows the chromatograms for alkylbenzenes in methanol/water = 80/20 at T = 30 °C using ODS-modified TMOS monolithic silica capillary columns. For the retention factors (k) with hexylbenzene, we obtained k = 3.46 for MS(100)-T80-15h, k = 3.15 for MS(100)-T80-25h, and k = 2.16 for MS(100)-T120-3h. In addition, k = 5.31 was provided by MS(100)-Hy80-15h and k = 3.16 by MS(100)-Hy120-4h (not shown in Fig. 5). As expected, the retention factors for the columns treated at T = 120 °C were comparable with those obtained from the columns with the same treatment in the previous report [7]. It shows that surface modification with ODS-DEA for a monolithic silica column was carried out properly. The difference in hydrothermal treatment of the capillary columns corresponded to differences in the retention factors for hexylbenzene between the series of TMOS and hybrid monolithic silica, as expected from surface area for silica rods. The capillaries featuring a relatively small retention factor were those possessing a larger average mesopore size and a consequently smaller BET surface area, as determined from physisorption on the rods and also as determined from ISEC performed on the capillaries themselves. On

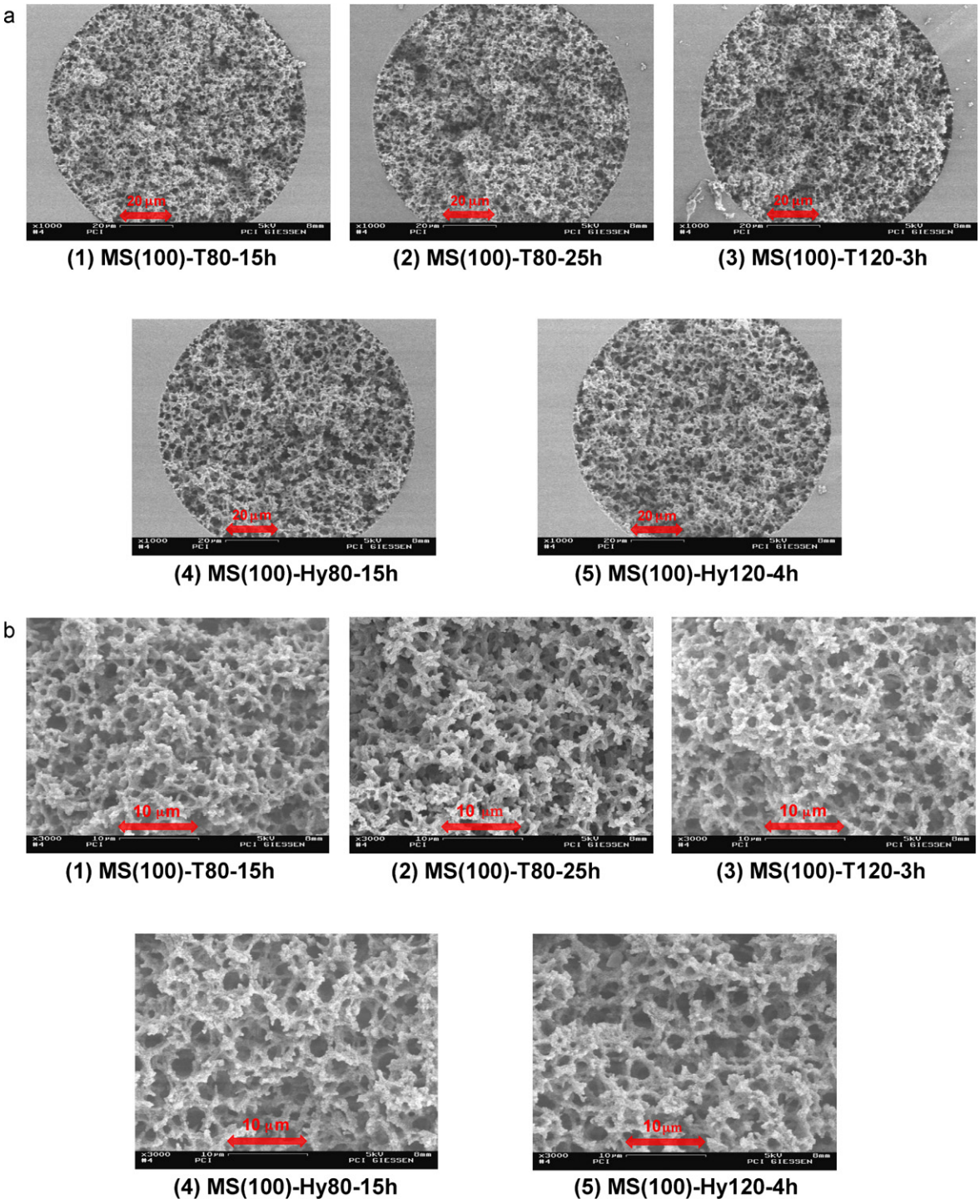

Fig. 6. Scanning electron micrographs of monolithic silica columns prepared from TMOS and MTMS in an i.d. 100 μm fused-silica capillary. (a) Scale bars correspond to 20 μm (×1000). (b) Scale bars correspond to 10 μm (×3000). Column: (1) MS(100)-T80-15h, (2) MS(100)-T80-25h, (3) MS(100)-T120-3h, (4) MS(100)-Hy80-15h, (5) MS(100)-Hy120-4h.

the other hand, comparing MS(100)-T80-15h and MS(100)-Hy80-15h or MS(100)-T120-3h and MS(100)-Hy120-4h, the difference in the retention factor is larger as assumed from comparing the surface area for the corresponding silica rods. This result indicates an important effect of the methyl group incorporated by MTMS, due to hydrophobic retention ability in RPLC, as shown in our previous report [7].

These differences in the analyses might also originate from the estimation of pore sizes of silica applying Eq. (1), which is derived from the basic assumption that the pore size is 2.5 times larger than the rotational coil of polystyrene molecules in THF or methylene chloride [27]. For a detailed interpretation, it is essential to estimate a computationally simulated PSD with the result obtained from mercury porosimetry or nitrogen physisorption [30,32,37,38]. Considering the accessibility into pores, nitrogen physisorption is more reliable than ISEC because of the small molecular size of nitrogen. However, ISEC is helpful to semi quantitatively analyze mesoporosity of capillary columns and the change in porosity. Our study

Table 3
Column properties of monolithic silica capillary columns.

Column	Total porosity (ε_T)[a]	External porosity (ε_e)[b]	Permeability (K) (10^{-14} m^2)[c]	Plate height (μm)[d]	$D_{pore(ISEC)}$ (Å)[e]
MS(100)-T80-15h	0.918	0.797	4.4	4.0	68
MS(100)-T80-25h	0.928	0.804	4.5	4.1	77
MS(100)-T120-3h	0.922	0.797	4.7	4.0	108
MS(100)-Hy80-15h	0.916	0.780	7.0	4.4	61
MS(100)-Hyl20-4h	0.925	0.782	6.8	4.4	100

[a] Total porosity was obtained with toluene by six times measurements in SEC (RSD ≤ 0.2%).
[b] External porosity was estimated according to Ref. [39] and [40].
[c] According to Eq. (2), permeability (K) was calculated in methanol/water = 80/20 at T = 30 °C assuming the porosity of monolithic silica is 92%.
[d] Plate height number was measured for thiourea in methanol/water = 80/20 at T = 30 °C.
[e] Pore size was estimated from ISEC according to Ref. [40] and [41].

proved that the trends in porosity, as a function of hydrothermal treatment, corresponded well to that for a silica rod with similar treatment.

3.3. Column property

As shown Fig. 6(a) and (b), SEM photographs for monolithic silica capillary columns were taken in order to qualitatively study the influence of the synthesis parameters on the macroporosity. It was possible to prepare a monolithic silica capillary column as connecting the structures with inner wall in a capillary, as confirmed in Fig. 6(a). It is seen that TMOS capillary columns possessed smaller skeletons and through pores compared to those for hybrid capillary columns (see Fig. 6(b)). The TMOS columns feature a domain size of 2–3 μm (sum of through pore size and skeleton size), and the hybrid columns 3–4 μm. In addition, the macropore structure of monolithic silica does not significantly depend on the difference in hydrothermal treatment because similar preparation feed solutions were utilized for TMOS columns or hybrid columns. This finding is consistent with the results for a monolithic silica rod reported by Tanaka and co-workers [2].

In Table 3, total porosity ε_T, external porosity ε_e, pore size from ISEC, permeability, and the plate height for thiourea in methanol/water = 80/20 at T = 30 °C for the bare monolithic silica capillary columns are summarized. The total porosity ε_T was calculated by averaging six measurements using toluene in THF, and the external porosity ε_e was estimated from the plot of the elution volume for PSSs in THF against $M_w^{1/3}$ using SEC (not shown) [39,40]. In addition, using that plot and Eq. (1) it was recently described by Tallarek and co-workers that the pore size estimated with the smallest polystyrene standards, the chains of which are completely size-excluded from mesopores in THF, enables a comparison with the nominal pore size for particles in a particulate column provided by manufacturers [40,41]. We applied such a method for the

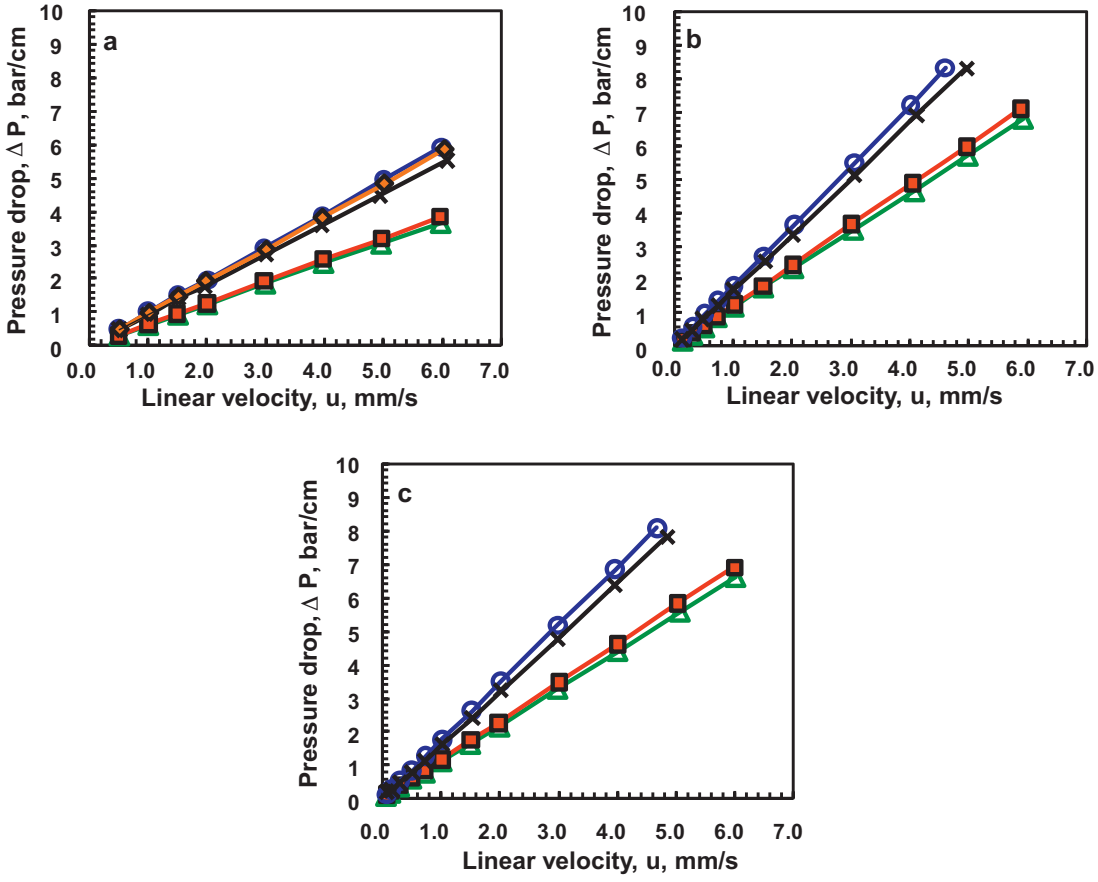

Fig. 7. Plots of column back pressure against linear velocity of mobile phase. Mobile phase: (a) acetonitrile/water = 80/20, (b) acetonitrile/water/TFA = 28/72/0.1, (c) acetonitrile/water/TFA = 33/67/0.1. Temperature: 30 °C. Symbols are same as shown in Fig. 3 for the columns.

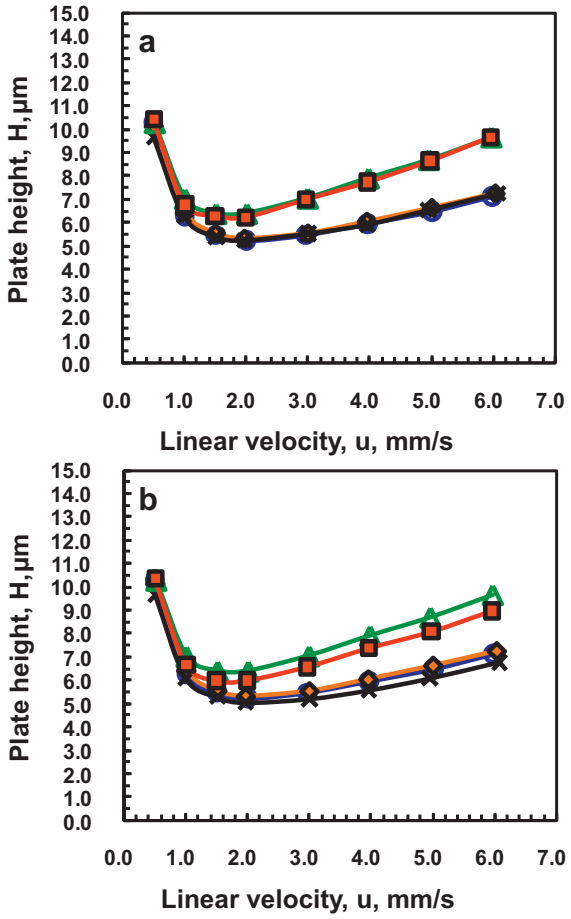

Fig. 8. Van Deemter plots obtained for ODS-modified monolithic silica columns with alkylbenzenes. (a) Plots are shown using pentylbenzene and hexylbenzene. (b) Plots are shown using pentylbenzene. Mobile phase: acetonitrile/water = 80/20. Temperature: 30 °C. Detection: 210 nm. Retention factor (k) for pentylbenzene: MS(100)-T80-15h (k = 1.23), MS(100)-T80-25h (k =1.14), MS(100)-T120-3h (k = 0.82), MS(100)-Hy80-15h (k = 1.65), and MS(100)-Hy120-4h (k = 1.10). Retention factor for hexylbenzene: MS(100)-T120-3h (k = 1.16), MS(100)-Hy120-4h (k = 1.55). The symbols are the same as in Fig. 7 for the columns.

estimation, to compare pore sizes for monolithic silica capillary columns. The permeability (K) of a capillary column was obtained from Darcy's law by using Eq. (2) [7,42–44].

$$K = \frac{\varepsilon_T u_0 \eta L}{\Delta P} \quad (2)$$

where u_0 is linear velocity and η viscosity of a mobile phase, L column length, and ΔP column pressure drop, respectively.

First, it has been already reported that ε_T and ε_e for a monolithic silica column are dominated by the initial preparation conditions, especially by the concentration of silica precursor in the feed [5]. In our study, the same preparation conditions were applied to the preparation for the capillary columns with same silica support except for hydrothermal treatment, resulting in the observed similarities of ε_T, ε_e and internal porosity ε_i for micro- and mesopores ($\varepsilon_i = \varepsilon_T - \varepsilon_e$), but influencing mesopore size and PSD, as observed in Fig. 4. Interestingly, the pore size of monolithic silica capillary columns obtained from ISEC nearly corresponded to the average pore size of the silica rods extracted from nitrogen physisorption (see Table 2).

Second, it was already shown that the domain size for a monolithic silica capillary column can be controlled by adjusting the amount of PEG in a preparation feed [5,7]. Permeability reflects the domain size of monolithic silica, as found in previous studies [7,13]. It can be assumed that the difference in permeability between TMOS and hybrid columns corresponds to that in domain size obtained from SEM observation in Fig. 6(b).

Furthermore, column efficiency depends strongly on the domain size of a monolithic silica capillary column, as shown in previous reports [5,7]. The column efficiency for thiourea (no retentive solute) in methanol/water = 80/20 at T = 30 °C is quite similar between the columns with same silica support because of the similar domain sizes, but different between TMOS and hybrid columns due to the differences in domain sizes, as shown in Table 3.

Therefore, it should be emphasized that column properties for the capillary columns prepared by applying the same initial conditions shown in Table 1 are quite similar except for the difference in the average mesopore size and PSD derived from different hydrothermal treatments, as confirmed in Fig. 4 and Table 3. Note that this enables us to attribute the difference in column efficiency to that in the pore size and PSD.

Fig. 7 shows the relationship between column pressure drop for ODS-modified monolithic silica capillary columns and linear velocity using thiourea and three kinds of mobile phase, acetonitrile/water = 80/20, acetonitrile/water/TFA = 28/72/0.1, and acetonitrile/water/TFA = 33/67/0.1 at T = 30 °C. The hybrid columns provided quite similar values for column back pressure. Even for TMOS columns, the values could be obtained within difference in column back pressure of 8% at same linear velocities, as expected from the permeability in Table 3. Therefore, it was possible to confirm that the domain size is quite similar between the columns with same silica support, but different between TMOS and hybrid monolithic silica by using column back pressure due to permeability.

3.4. Column efficiency for C18 monolithic silica capillary columns

Fig. 8(a) and (b) shows the plots of plate height (H) for pentylbenzene and hexylbenzene against linear velocity u in acetonitrile/water = 80/20 at T = 30 °C for the ODS-modified columns. Using pentylbenzene and hexylbenzene, it was possible to provide similar retention factors between the capillary columns with same silica support (see the information in Fig. 8). This allows for a simple comparison of column efficiency, for a similar retention factor, between the capillary columns assuming that diffusivity and a molecular size are similar, because the structural difference between pentylbenzene and hexylbenzene is only a methylene group (CH_2).

As shown in Fig. 8(a), it is observed that the plots are obviously similar between the capillary columns with same silica support, and

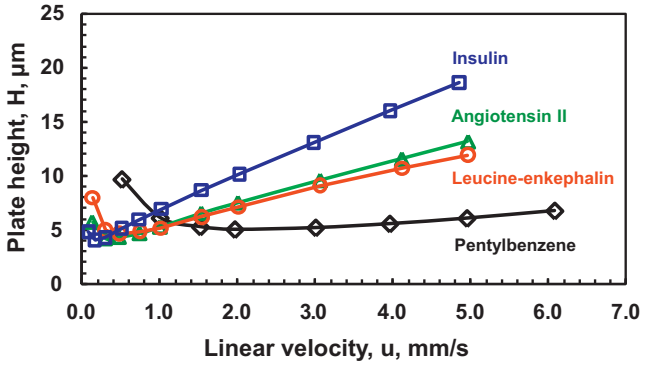

Fig. 9. Van Deemter plots obtained for MS(100)-T120-3h-e with four kinds of solutes. Symbol: pentylbenzene in acetonitrile/water = 80/20 (◆), leucine-enkephalin (●) and angiotensin II (▲) in acetonitrile/water/TFA = 28/72/0.1, insulin (■) in acetonitrile/water/TFA = 33/67/0.1. Temperature: 30 °C. Detection: 210 nm for pentylbenzene, 220 nm for the peptides. The last abbreviation "e" for a column name shows endcapping with TMSI.

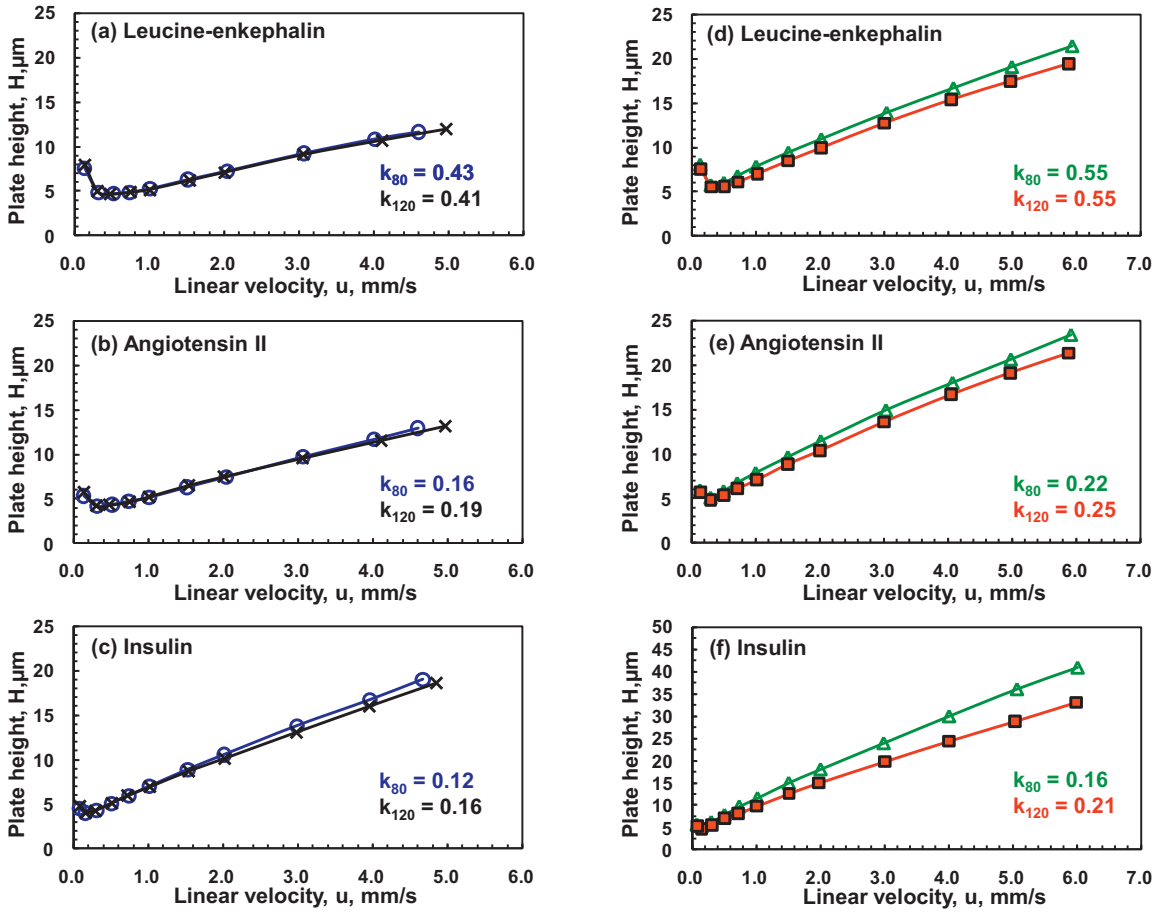

Fig. 10. Van Deemter plots obtained for ODS-modified monolithic silica columns with three kinds of peptides. (a)–(c) Plots are shown using TMOS columns. (d)–(f) Plots are shown using hybrid columns. Column: MS(100)-T80-15h-e (○), MS(100)-T120-3h-e (×), MS(100)-Hy80-15h-e (▲), MS(100)-Hy120-4h-e (■). Solute and mobile phase: (a) and (d) leucine-enkephalin in acetonitrile/water/TFA = 28/72/0.1, (b) and (e) angiotensin II in acetonitrile/water/TFA = 28/72/0.1, (c) and (f) insulin in acetonitrile/water/TFA = 33/67/0.1. Temperature: 30 °C. Detection: 220 nm. Retention factors k_{80} are represented for columns treated at 80 °C, and k_{120} for other columns at 120 °C.

there is no effect of pores on the column efficiency for the solutes. It suggests that the influence of small mesopores on the column efficiency with small molecules like alkylbenzenes is negligible for a monolithic silica column, as reported previously by Guiochon and co-worker [24].

Fig. 8(b) shows the plots only for pentylbenzene in acetonitrile/water = 80/20 at T = 30 °C for the ODS-modified columns. A plate height of H = 5.3 μm for pentylbenzene was obtained for MS(100)-T80-15h (k = 1.23) and MS(100)-T80-25h (k = 1.14), and H = 5.2 μm for MS(100)-T120-3h (k = 0.82) at u = 2.0 mm/s. The slight difference in column efficiency is also observed for hexylbenzene in methanol/water = 80/20 at T = 30 °C between the capillary columns, as shown in Fig. 5. This difference in column efficiency is probably due to the different values for retention factors. It has been already observed by Tanaka and co-workers that the column efficiency for a monolithic silica capillary column tends to become slightly lower with increase in retention factor using split injection/flow system [45].

However, comparing the column efficiency even at u = 6.0 mm/s, a value of H = 7.1 μm was obtained for MS(100)-T80-15h, and H = 6.8 μm for MS(100)-T120-3h. Also, the column efficiency was similar for MS(100)-Hy80-15h (H = 9.6 μm, k = 1.65) and MS(100)-Hy120-4h (H = 9.0 μm, k = 1.10). There is no significant difference in column efficiency with increase in u between the series of TMOS or hybrid columns. Consequently, this result suggests that hydrothermal treatment at T = 120 °C, for both TMOS and hybrid columns, has no significant influence on column efficiency, but retention factors, regarding the behavior of relatively small molecules. It can be assumed that the capillary column exposed to a treatment at T = 80 °C results in higher resolution and larger sample loading capacity on separation of small molecules because of the larger retention ability derived from higher surface area without significant loss of column efficiency, compared to the columns with the additional treatment at T = 120 °C.

In addition, this measurement allowed determining the difference in column efficiency between TMOS columns and hybrid columns. Such difference is related to the domain size due to mass transfer of solute, and the domain size of a hybrid column is significantly larger than that of a TMOS column, as examined in Section 3.3. It has been already shown that column efficiency tends to be higher with decrease in domain size for a monolithic silica capillary column [5,7].

Hence, a series of further experiments were performed to address the question, if the changes in porosity generated by hydrothermal treatment in both, TMOS and hybrid columns, affect HPLC performance for larger molecules. As probe molecules we used leucine-enkephalin (M_w = 555), angiotensin II (M_w = 1046), and insulin (M_w = 5770).

As shown in Fig. 9, the relationship of plate height against linear velocity for pentylbenzene in acetonitrile/water = 80/20, leucine-enkephalin and angiotensin II in acetonitrile/water/TFA = 28/72/0.1, and insulin in ace-

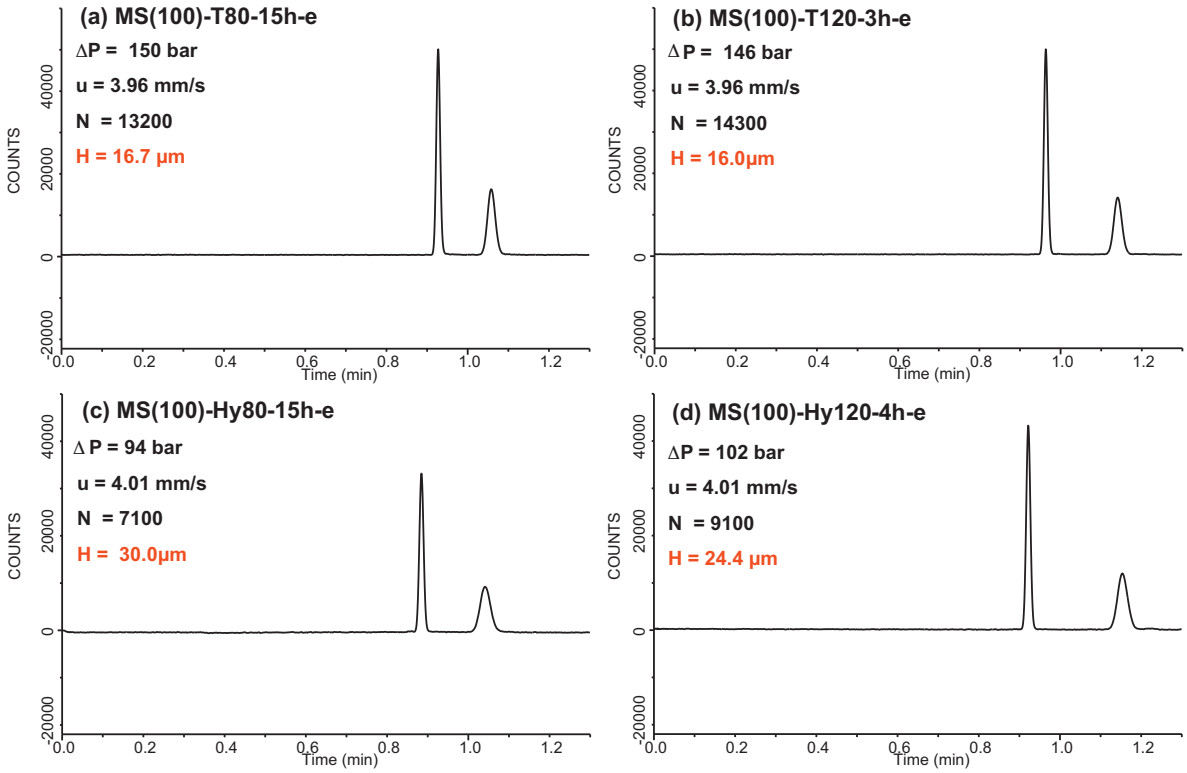

Fig. 11. Chromatograms obtained for insulin with ODS-modified monolithic silica columns. Column: (a) MS(100)-T80-15h-e (column length 22.1 cm), (b) MS(100)-T120-3h-e (22.9 cm), (c) MS(100)-Hy80-15h-e (21.3 cm). (d) MS(100)-Hy120-4h-e (22.2 cm). Mobile phase: acetonitrile/water/TFA = 33/67/0.1. Solute: thiourea, insulin. Temperature: 30 °C. Detection: 220 nm. Pressure drop, linear velocity, number of theoretical plates and plate height for insulin are indicated.

tonitrile/water/TFA = 33/67/0.1 at $T = 30\,^{\circ}C$ was obtained for MS(100)-T120-3h-e. The abbreviation "e" for a column name indicates the endcapping with TMSI. It can be observed that column efficiency is lower with increasing molecular weight for a solute at high linear velocity because of slower mass transfer inside pores, especially comparing the column efficiency for the case of insulin to that with the other solutes. In general, it has been known that diffusivity of solute decreases with increase in molecular weight. For an estimation of molecular diffusivity of the peptides, Eq. (3) from Young and co-workers was used [39,46].

$$D_m = 8.341 \times 10^{-8} \frac{T}{\eta M^{1/3}} \quad (3)$$

where D_m is the diffusion coefficient of solute in mobile phase and η the viscosity of a mobile phase, and M molecular weight of solute, and T temperature, respectively. According to Eq. (3) with $T = 303$ K and a viscosity of mobile phase $\eta = 0.86$ cP, a diffusion coefficient $D_m = 3.6 \times 10^{-6}$ cm^2/s was obtained for leucine-enkephalin, 2.9×10^{-6} cm^2/s for angiotensin II, and 1.6×10^{-6} cm^2/s for insulin. In addition, the diffusion coefficient of pentylbenzene in acetonitrile/water = 80/20 at $T = 30\,^{\circ}C$ is 1.7×10^{-5} cm^2/s from the experimental data reported by Li and Carr [47]. With the estimation of diffusion coefficient of solute, it can be concluded that an increase in molecular weight (molecular size) leads to a significantly lower column efficiency.

In addition, interestingly the plate height curve is convex upward for the peptides in comparison with the curve for pentylbenzene. This tendency was also observed for the other capillary columns despite the differences in pore size or PSD. It supports that the phenomenon is not dependent on intraskeleton mass transfer due to pores in monolithic silica, but may be related to Eddy diffusion, as represented with the coupling theory by Giddings [44,48].

To confirm the effect of pore size on column efficiency for the peptides, a comparison of MS(100)-T80-15h-e with MS(100)-T120-3h-e or MS(100)-Hy80-15h-e with MS(100)-T120-4h-e can be useful, as mentioned in Section 3.3.

Fig. 10 shows Van Deemter plots obtained for the monolithic silica capillary columns with leucine-enkephalin, angiotensin II, and insulin at similar retention factors. The column efficiency is quite similar for leucine-enkephalin, angiotensin II, and slightly different even for insulin at linear velocities between 0.08 and 5 mm/s, using MS(100)-T80-15h-e and MS(100)-T120-3h-e. In contrast, there is significant difference in column efficiency between MS(100)-Hy80-15h-e and MS(100)-Hy120-4h-e, especially for insulin with increase in linear velocity from 0.08 to 6 mm/s. The difference in column efficiency for the hybrid columns tends to be larger with gradual increase in molecular weight of solute.

In Fig. 11, the chromatograms for insulin at $u = 4.0$ mm/s in acetonitrile/water/TFA = 33/67/0.1 at $T = 30\,^{\circ}C$ are shown using ODS-modified monolithic silica capillary columns. Comparing the difference in plate height between MS(100)-T80-15h-e and MS(100)-T120-3h-e to that between MS(100)-Hy80-15h-e and MS(100)-Hy120-4h-e, it is seen that hydrothermal treatment at $T = 120\,^{\circ}C$ for a hybrid column can result in higher column efficiency.

It can be confirmed by ISEC that there are more small pores inside bare MS(100)-Hy80-15h compared to bare MS(100)-T80-15h (see Fig. 4). Corresponding to that result, the hybrid silica rod treated at $T = 80\,^{\circ}C$ for 15 h possesses a larger quantity of small pores compared to TMOS rods exposed to $T = 80\,^{\circ}C$ for 15 h (see Fig. 2). For example, for pores below 60 Å, the hybrid rod possesses a 1.7 times larger pore volume (0.32 cc/g) than the TMOS rod treated at $T = 80\,^{\circ}C$ for 15 h (0.19 cc/g). The pore volume was 0.06 cc/g for the hybrid silica rod with the additional treatment at $T = 120\,^{\circ}C$ for 4 h and 0.01 cc/g for the TMOS rod with the

treatment at $T = 120\,^{\circ}C$ for 3 h. Therefore, more small pores in bare MS(100)-Hy80-15h contribute to the lower column efficiency for the peptides compared to those in bare MS(100)-Hy120-4h. It can be suggested that it is necessary to carry out hydrothermal treatment for a hybrid column with higher temperature or longer time in the preparation process, compared to that for a TMOS column, to provide higher column efficiency with increase in molecular weight (molecular size). In contrast, for a TMOS column, hydrothermal treatment at $T = 120\,^{\circ}C$ does not influence column efficiency even for the peptides. This result implies that the preparation method of the monolithic silica columns exerts a drastic impact on structural homogeneity (through pores and silica skeletons), allowing a significant improvement of column efficiency [5,7,24,43,49–51].

4. Conclusion

In our study, using nitrogen physisorption and ISEC, a reasonable correlation was observed between monolithic silica rods and monolithic silica capillary columns regarding the change of pore size caused by variation of hydrothermal treatment or silica precursor. ISEC performed on monolithic silica capillaries and nitrogen physisorption on corresponding monolithic rods allowed a systematic comparison, enabling the determination of mesopore sizes even in capillaries with sub-millimeter diameter. In addition, the retention factors for hexylbenzene with ODS-modified capillary columns in methanol/water = 80/20 at $T = 30\,^{\circ}C$ also support the results from nitrogen physisorption.

The column efficiency for pentylbenzene and hexylbenzene was not significantly different between ODS-modified columns with a treatment at $T = 80\,^{\circ}C$ and columns treated at $T = 120\,^{\circ}C$. For small molecules like alkylbenzenes, it is suggested that there is no significant effect of pores on column efficiency for the monolithic silica columns, and the treatment at $T = 120\,^{\circ}C$ is not necessary. This supports the interpretation the monolithic silica capillary columns with the treatment at $T = 80\,^{\circ}C$ can be expected to provide higher resolution and larger sample loading capacity on separation of small molecules because of the higher surface area, resulting in larger retention ability without significant loss of column efficiency, compared to the columns with the additional treatment at $T = 120\,^{\circ}C$ [5,7]. Using leucine-enkephalin, angiotensin II, and insulin, a lower column efficiency was observed for the hybrid column treated at $T = 80\,^{\circ}C$ with increasing molecular weight of peptides, compared to that for the column treated at $T = 120\,^{\circ}C$. For TMOS columns with and without the treatment at $T = 120\,^{\circ}C$, there was no significant difference in column efficiency for leucine-enkephalin, angiotensin II, and even for insulin. It is concluded that the higher content of small mesopores in the hybrid column treated at $T = 80\,^{\circ}C$ contributed to the lower column efficiency, as predicted by nitrogen physisorption and ISEC. Therefore, we suggest that hydrothermal treatment of a hybrid column with higher temperature or longer time in preparation process is essential, compared to that for a TMOS column, to provide higher column efficiency with increase in molecular size of solute.

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