Focusing Review

Simple LC using New Macroporous Polymers

Ken Hosoya

Graduate School of Life and Environmental Sciences, Kyoto Prefectural University

1–5 Shimogamo–hangi–cho, Sakyo–ku, Kyoto 606–8522, Japan

Abstract

New macroporous polymers have been prepared based on two different "platforms" to simplify rather complicated pretreatment processes for LC analyses. For example, uniformly sized, molecularly imprinted polymer particles modified with sulfonic acids on outer surface easily removed humic acids in environmental water sample to improve detectability of really low level of the target molecule. Nicely controlled, 3D skeletal co–continuous polymers (monolithic polymer) have been prepared using visco–elastic phase separation mechanism as well as the utilization of epoxy monomers and a diamine. In addition, a hydrophilic monolithic media based on ethylene oxide units simplified affinity chromatography through avoiding non–specifically bound proteins even if those were utilized in protein lysate. All the macroporous polymers have contributed to simple and accurate LC analyses.

Keywords : Macroporous polymer, HPLC, Selectivity, Monolithic media

Introduction

Modern HPLC can realize really high performance separation for highly complicated bio–related samples, for example, using multi–dimensional, sophisticated HPLC system [1,2] coupled with MS/MS detection. Environmental samples including various misplaced materials can be also selectively determined even if those are in ppt level concentration. Those analyses are now highly automated, therefore, the data obtained seems to be reliable. This is because the data observed was appropriately processed and calculated based on the "sample" introduced into HPLC system and separated. In this context, we have to become aware of importance of the quality of sample injected. In MS detection, especially selective ion mode, we tend to pay no attention to sample quality.

If we look at overall process or strategy of LC analysis, sample preparation processes are not negligible to obtain suitable and accurate sample for LC system utilized. One of the important roles of sample preparation processes is separation and/or isolation of the target compound from matrix of impurities as much as possible and selective concentration of target molecule is of course essential in the case of low concentration of the target molecules, therefore various types of pretreatment devices including particulate type, membrane type, as well as the other formats are now commercially available, while the detailed information will be published somewhere else [3]. In recent years, the importance of analysis for relatively hydrophobic molecules, such as dioxins and/or poly–aromatic hydrocarbons (PAH) has been continuing, however, highly hydrophilic molecules related to pharmaceuticals and personal care products (PPCPs) become more important [4]. Because of highly hydrophilic properties of those compounds, activated sludge treatment is not effective therefore, it is now serious problem that possible high concentration of those compounds was determined in river water just after sewage plant. A role of pretreatment device as well as its process has been more important for LC analyses even if the HPLC system has been highly automated. Because commercially available solid phase extraction (SPE) media are not effective for some of PPCPs.

Corresponding author: Ken Hosoya
Tel: +81–75–703–5444
Fax: +81–75–703–5444
E–mail address: hosoya@kpu.ac.jp

In this brief contribution, I will focus on preparation and application of new macroporous, polymer–based materials to simplify the LC separation and/or analysis through unique pretreatment concept effectuated by some useful traps. Two different platforms were employed for the purpose, where particulate type and co–continuous type polymer–based materials will be introduced including experimental details. There two platforms have been actively studied in the world, however, the focus in this contribution will be the macroporous polymers having multi–dimensional separation mechanism including retention and exclusion on one platform. Using these polymers, LC strategy and process must be simplified. In addition, it will be discussed that cursory pretreatments have a significantly increased risk of inaccurate analytical results.

Particulate type, polymer –based packing material

First of all, particulate type, macroporous polymer is briefly introduced. Usually particulate type polymer "beads" are prepared using suspension polymerization method [5], namely pearl polymerization. This method is really easy to use, but one of the draw–backs of the method is to afford rather broad particle size distribution. Size classification is essential to improve particle size distribution, but this procedure is highly complicated. Therefore, some of the special polymerization methods have been introduced to obtain uniformly sized, macroporous polymer particles through easier processes. I have employed one of the seed polymerization methods, namely, multi–step polymerization method [6,7]. Uniformly sized polymer particles, packing materials realized high column efficiency with much lower column pressure drop [8], however, essential draw–back of the polymer–based packing materials has not been improved by only size uniformity of polymer particles. Another draw–back of traditional polymer–based packing materials was much lower column efficiency toward aromatic compounds such as naphthalene and anthracene. I have had an improvement in the draw–back using a hydrophilic cross–linking agent with co–monomer [9]. Fig. 1 reveals greatly improved column efficiency of polymer–based HPLC column packed with uniformly sized, polymer packing materials. This column afforded 13,300 plates/150 mm length for naphthalene with larger k values compared with that of typical silica–based C_{18} column. Through this work, compatibility of the packed column in 100% water mobile phase has been also proved because of hydrophilic properties of the polymer–based packing materials. This is really one of the contributions of

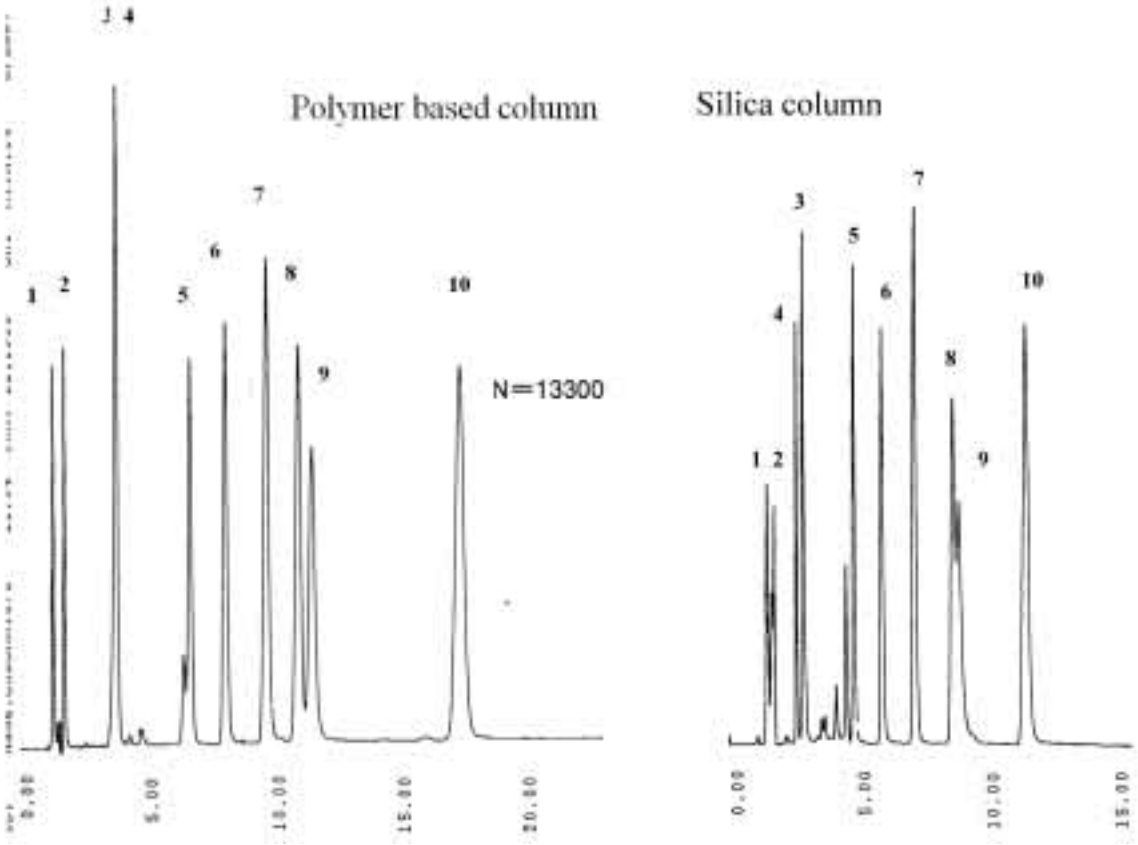


Fig. 1 Column performance of polymer–based column and C_{18} column.
Column size: 2 mm ID × 150 mm, Mobile phase: 50% (v/v) aqueous acetonitrile, Flow rate: 190 μL/min., Temperature: 40°C, Detection: UV 254 nm, Solutes: 1. Uracil, 2. Caffeine, 3. 2–Ethylpyridine, 4. Phenol, 5. Butyl benzoate, 6. Benzene, 7. N, N–diethylaniline, 8. Toluene, 9. Phenylacetyl acetone, 10. Naphthalene, C18 column: Shiseido, CAPCELL PAK C18 UG120.

uniformly sized polymer packing material to LC.

Another contribution of the uniformly sized polymer–based packing materials is that detailed comparison of column characteristics and efficiency among various columns can be determined on their appearances of chromatograms. This is simply because parameter of particle size and its distribution can be ignored. Fig. 2 offered a glimpse into tiny differences in retention properties for alkyl benzenes from benzene to pentylbenzene on the same stationary phases derived from the monomer, ethylene dimethacrylate (EDMA), except the porogenic solvent utilized [10]. The found differences in retention properties were due to so–called porogen imprinting effect [11,12], which was another contribution of polymer –based packing materials for molecular recognition in LC process. The left chromatogram (a) was obtained on the column packed with EDMA polymer prepared using benzene as porogenic solvent, where retention time of benzene was slightly larger compared with that of on the other two chromatograms (b) and (c), while the center (b) is on that using toluene as porogenic solvent, where toluene resulted in relatively larger retention time to narrower peak distance between toluene and ethylbenzene. The right one is chromatogram on EDMA by ethyl benzene as the porogenic solvent. These facts mean that polymer–based packing materials can memorize the environment of polymerization conditions such as the shape of porogenic solvent in these cases. In fact, the porogen imprinting effects realized isomer separation between xylenes as shown in Fig. 3 [11]. The separation (a) was done using the EDMA packing material prepared using *ortho* xylene as the porogenic solvent, while separation (b) was realized the packing material prepared using *para* xylene as the porogenic solvent. It is really interesting because EDMA packing material prepared using toluene as porogenic solvent afforded only one peak for three isomers of xylenes. Only porogen utilized for preparation of uniformly sized polymer particles can contribute the separation of isomers in LC

Contribution of molecularly imprinted packing materials

If we utilize some analytical target molecule as temple molecule in the preparation of polymers, the above mentioned “imprinting” effect is called as molecular imprinting effect [13]. Molecularly imprinted packing material [14–18] has greatly contributed to target selective concentration toward pharmaceutical samples or environmental pollutants existing with really low concentration in its matrix. In molecular imprinting, real target molecule is usually utilized as the “template” molecule directly, however, I have utilized pseudo template molecule having similar structure or a part of structure of the target molecules [19–28]. When *tert*–butyl phenol was utilized the pseudo template for bisphenol A, Fig. 4 proved selective concentration of bisphenol A in environmental water including one of the natural organic matters, humic acid on the imprinted polymer cartridge [29]. The concentrated bisphenol A can be easily detected using general UV detector. However, in the case of high concentration ratio up to 1,000 times, tiny impurities such as humic acid retained on the treatment device become possible problem for detectability of bisphenol A in HPLC analysis [30–34]. Therefore I introduced new surface modification [35–38] to molecularly imprinted polymer packing material using some functional monomers to exclude humic acid [39–42].

As shown in Fig. 5, on non–surface modified column, impurities were detected at the peak front. The hydrophilic modification using

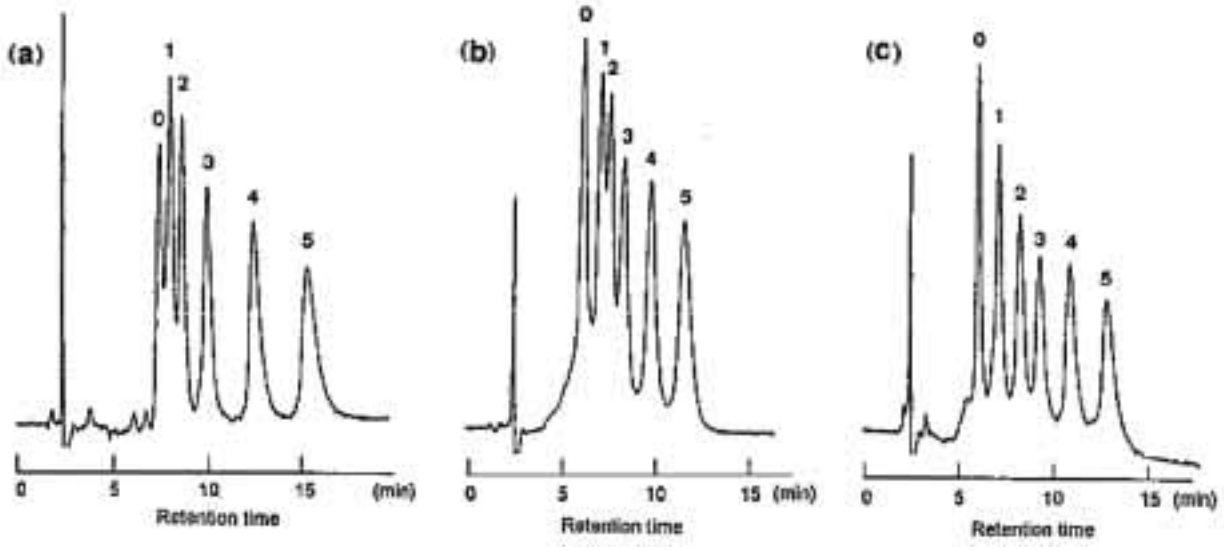


Fig. 2 Chromatograms of alkylbenzenes on EDMA stationary phases prepared with (a) benzene, (b) toluene, and (c) ethylbenzene as porogen. Mobile phase, 80% aqueous methanol. Flow rate, 0.7 mL/min. Detection, UV 254 nm. Solutes: 0, benzene; 1, toluene; 2, ethylbenzene; 3, propylbenzene; 4, butylbenzene; and 5, pentylbenzene.

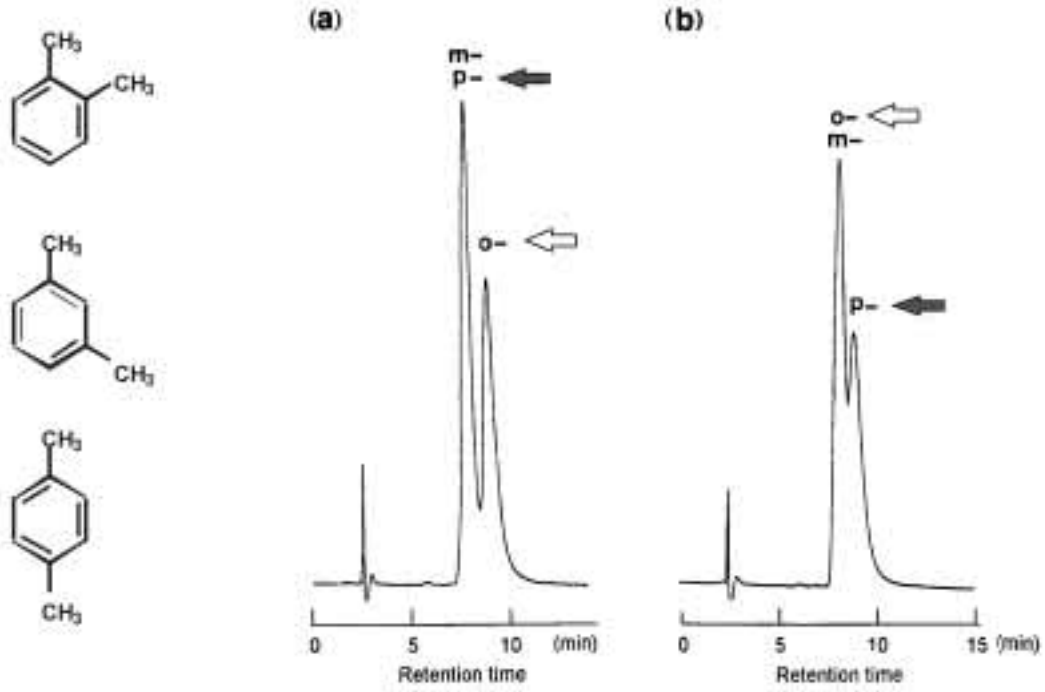


Fig. 3 Chromatograms of xylene isomers on EDMA stationary phases prepared with (a) *o*−xylene and (b) *p*−xylene as porogen. Mobile phase, 80% aqueous methanol. Flow rate, 0.7 mL/min. Detection, UV 254 nm.

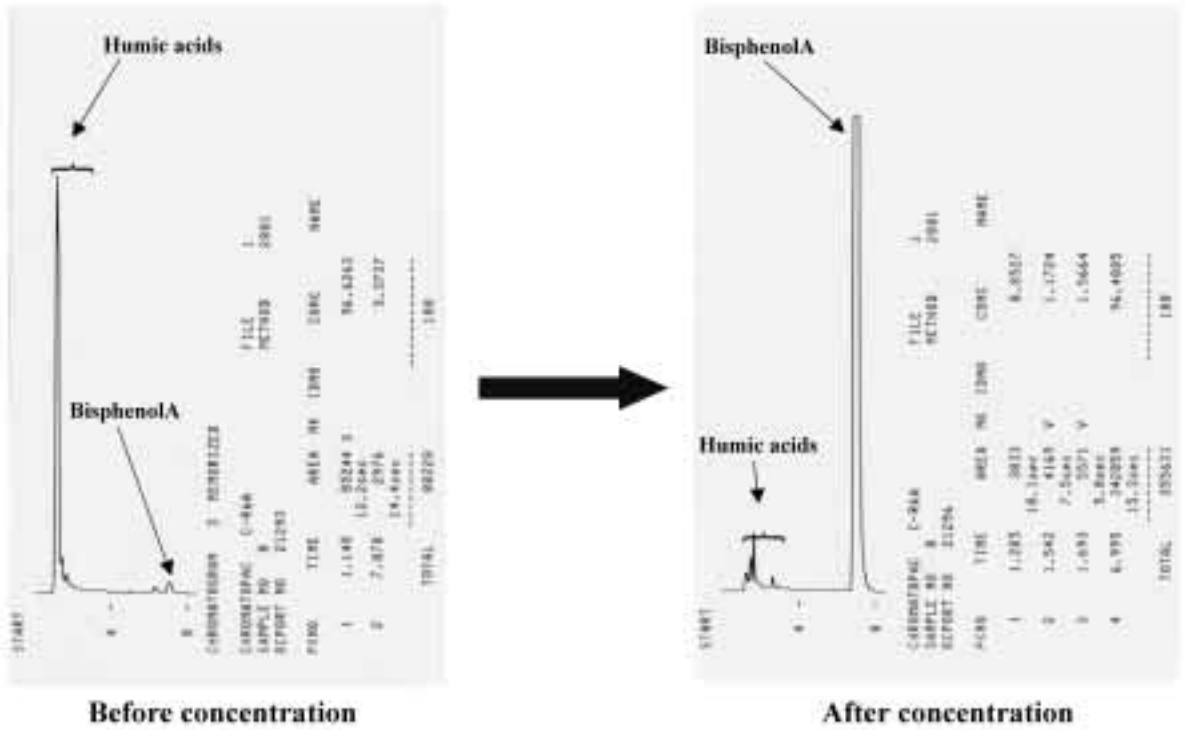


Fig. 4 Chromatograms of humic acid and bisphenol A before and after the treatment with prepared polymer packed cartridge.
Condition of HPLC evaluations: Mobile phase: 50% methanol aq. Flow rate: 1.0 mL/min Detection: UV 254 nm., Column: C_{18} column (Merck) 100 mm × 4.6 mm (I.D.) Temperature: 30°C.

GDMA (glycerol di–methacrylate)/GMMA (glycerol mono–methacrylate) much reduced the impurities, but not perfect level. Packing materials modified with a functional monomer, namely MASK (methacrylic acid 3–sulfopropyl potassium salt) electrostatically excluded impurities to realize nearly perfect exclusion of the impurities. The exclusion of impurities greatly contributed efficiency of ionization process of MS detection as shown in Fig. 6. The MASK modified column clearly afforded much greater SIM (selected ion monitoring) peak compared with that on non–modified column. This phenomenon is probably due to concentrated impurities as shown on lower chromatogram of Fig. 6 by UV detection. Unexpectedly, MS detection with SIM mode is affected by concentrated impurities, which is possible problem for environmental analysis.

New automated pre–concentration HPLC system [43] with two multi–channel valves as illustrated in Fig. 7 was introduced further contribution for accurate analysis of environmental samples requiring pre–concentration [44]. This two valve system resulted in much longer running time for analytical column due to avoiding direct charge of environmental water sample. Using surface modified, molecularly imprinted, pre–concentration column with the HPLC system, practically detectable peak of the target molecule, bisphenol A was obtained as proved in Fig. 8 using simple UV detection [40]. This combination of the pre–concentration column and the two valves HPLC system highly contributed to simple and accurate LC analyses.

Co–continuous type separation media

Monolithic columns have been extensively investigated for HPLC applications as an alternative to a packed column with silica particles conventionally used. The merits of the monolith have been cited such as simpler preparation *in situ* (confined in mold), the alleviation of the time–consuming packing process, and higher permeability enhancing the diffusion of solute molecules to the porous stationary phase, and the capability of manufacturing a long column with higher efficiency (larger plate number) as well.

The monolithic column is commonly classified into two categories, (organic) rigid polymer and silica monolith. Firstly, the polymer monoliths have been widely investigated for HPLC application since the early 1990s. The polymer monolith preparation has been carried out mainly by the free radical polymerization of vinyl or methacrylate monomers in a rather poor porogenic solvent selected among alcohols, aromatic hydrocarbons, ethers or their mixture as claimed in the patent by Svec and Frechet [45]. The recent status of the polymer monoliths has been reviewed by Svec [46].

However, the preparation of polymer monoliths is usually carried out with the said poor porogenic solvents as major component more than 50 vol% (isometric composition) for creating a macroporous structure for liquid flow. Under such solvent rich condition, the van der Waals attraction of the growing polymer chains overcomes their mutual steric hindrance and dominates the interaction between the polymer chain and the solvent [47]. This enhances the segregation of polymer (phenomenally, free energy of mixing in-

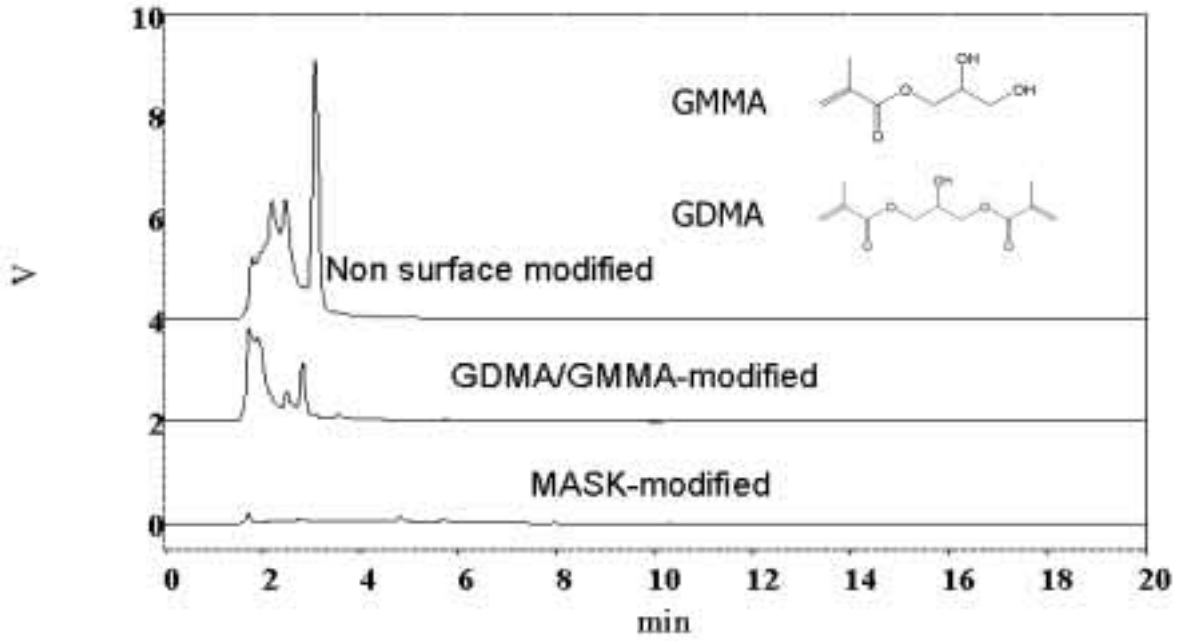


Fig. 5 Comparative chromatograms of lake water obtained MASK–(MIP–TM), GDMA/GMMA– (MIP–TG), non– (MIP–TN) modified MIPs used for the pretreatment column in the column switching HPLC system.
HPLC conditions: mobile phase, 20 mM sodium phosphate buffer (pH 7.0)–acetonitrile (70: 30 (v/v)); flow rate, 0.8 mL/min for analysis and 2.5 mL/min for pre– treatment; column, Shim–pack VP–ODS (150 mm × 4.6 mm); detection, UV 220 nm; temperature, 40°C concentration volume, 50 mL.

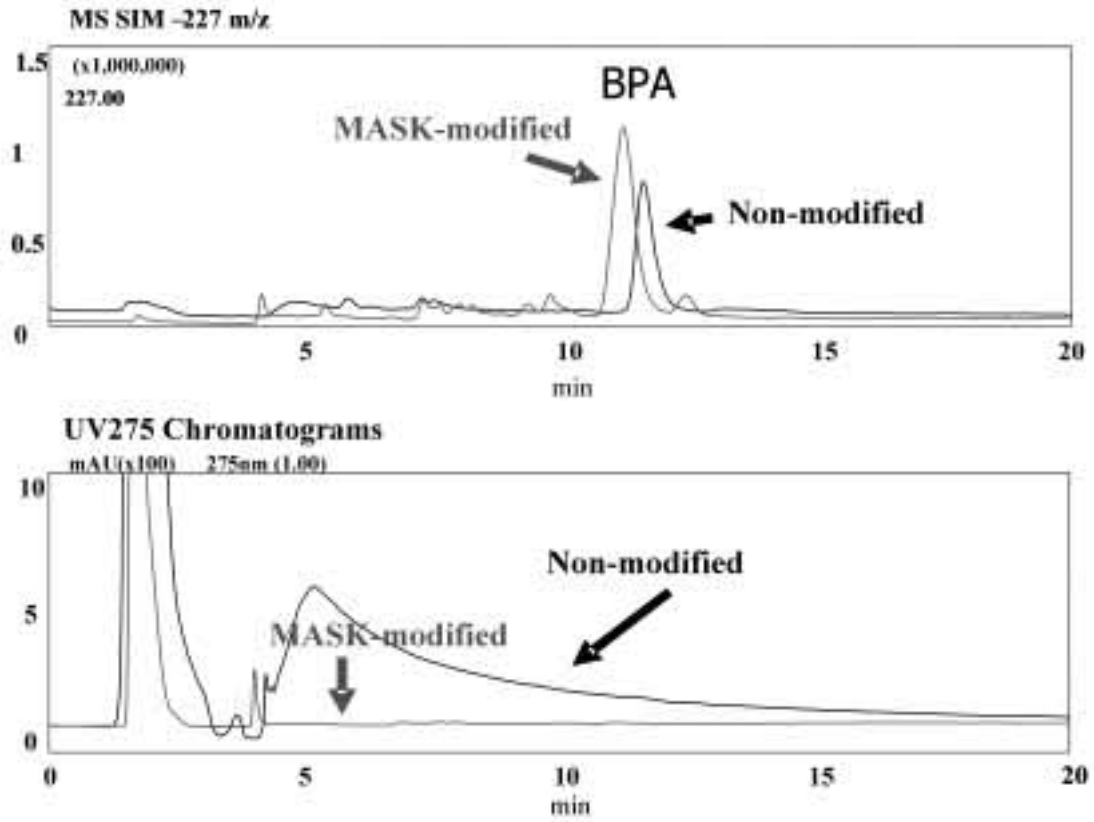


Fig. 6 Increased sensitivity by the removal of interference in MS detection.
LC/MS conditions were same as Table 1 except UV detection and pretreatment column.
UV detection was performed at 275 nm and for confirming the effect of MAS modification, non–surface modified MIPs were employed.

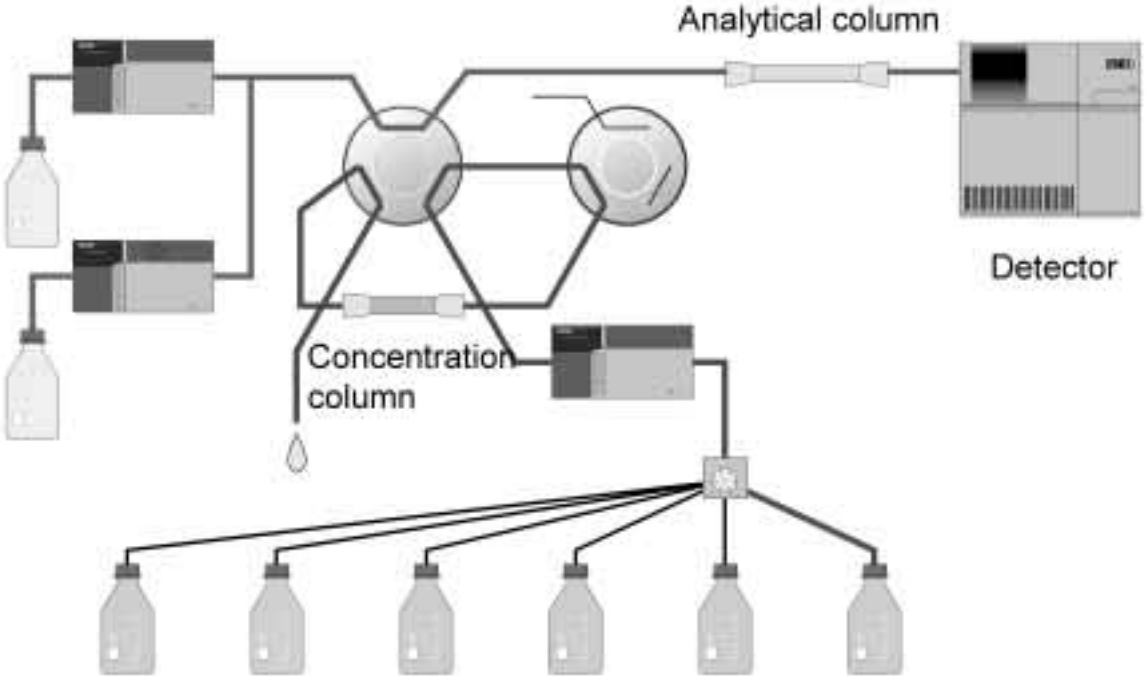


Fig. 7 Automated two valves HPLC system with pre–concentration column.

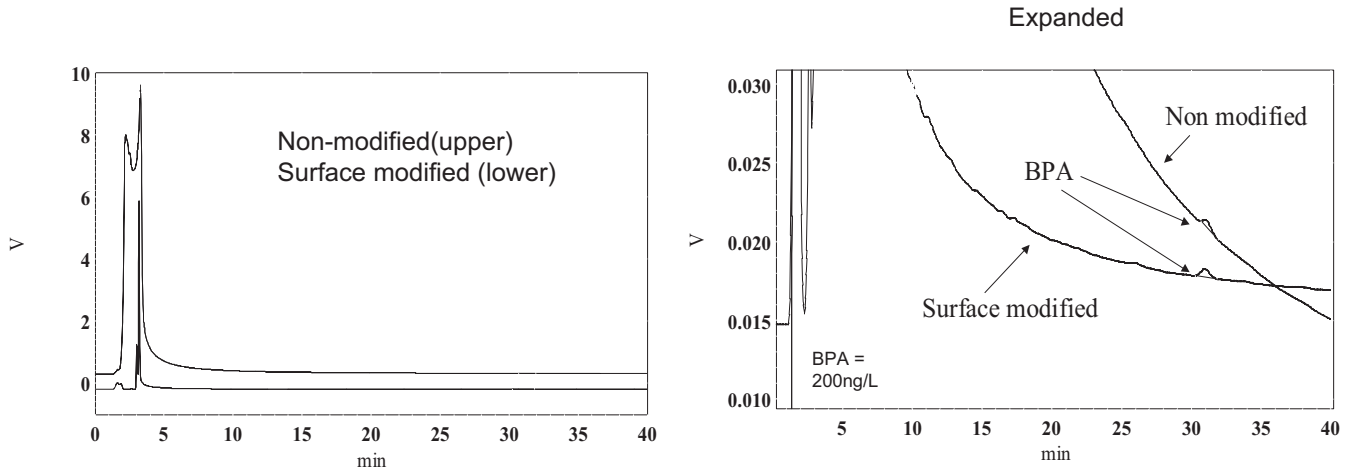


Fig. 8 Comparative chromatograms of Suwannee River NOM obtained with or without surface modification of MIP used for the pretreatment column in the column switching HPLC system (left). Right figure is close up, expanded one.
HPLC conditions: mobile phase, 20 mM sodium phosphate buffer (pH 7.0)–acetonitrile (70: 30 (v/v)); flow rate, 0.8 mL/min for analysis and 2.5 mL/min for pretreatment; column, Shim–pack VP–ODS (150 mmL × 4.6 mm i.d.); detection, UV 275 nm; temperature, 40°C; concentration volume, 50 mL.

creases) [48] and the phase separation rapidly proceeds *via* spinodal decomposition. Therefore, the polymer monolith thus prepared becomes a brittle agglomerated structure of globules with small mechanical integrity. Such macroporous monoliths usually reveal a coarse macroporous structure with the maze–like flow channels and insufficient population of the mesopores at the surface for small solute retention (according to IUPAC definition, mesopore is defined in size as less than 2 nm, mesopore is defined as 2–50 nm, and macropore as larger than 50 nm, [49] respectively).

My attention focuses on freezing such time–evolved bi–continuous polymer structure induced via visco–elastic phase separation by thermal or photo–initiated polymerization before the coarsening of the structure so to optimize the monolith morphology as a HPLC column. In terms of this, the porogenic solvent is very important. Recently, we reported the poly–GDMA monoliths with bi–continuous structures by thermally initiated free radical polymerization using azo initiator [50–54]. We chose as a porogen the ultra–high molecular weight mono–disperse polystyrene solution in chlorobenzene. Our experiment indicated that the said polystyrene solution presumably induced the visco–elastic phase separation affording the bi–continuous structures of poly–GDMA monoliths. I preliminarily reported μ–HPLC measurement of poly–GDMA monolith column. As shown in Fig. 9, much improved, 3D skeletal structures of GDMA polymer were obtained using thermal radial polymerization. The bi–continuous (co–continuous) polymer has reasonable bimodal pore size distribution as shown in Fig. 10 due to combination of low molecular weight solvent and ultra–high molecular weight polymer. This method contributed the great improvement in A term as well as C term of van Demeter equation as summarized in Table 1, which was obtained in capillary format of GDMA monolithic column. There results were the first experimental prove of visco–elastic phase separation mechanism. But unfortunately, the monolithic columns prepared using the above mentioned preparation method did not improve their column efficiency compared with those on silica–based monolithic columns.

Another improvement in co–continuous polymer was done by the use of epoxy resin type monolithic media [55]. Two monomers as depicted in Fig. 11 were simply polymerized in polyethylene glycol as porogenic solvent to afford nicely controlled co–continuous polymers, where the morphology can be controlled by the change of molecular weight of polyethylene glycol as well as polymerization temperature as shown in Fig. 12. The detailed study revealed that the formation of polymer morphology was seriously affected by the change of polymerization temperature as shown in Fig. 13 based on expected two–step polymerization mechanism [56]. This is unimaginable from nicely controlled 3D skeletal structure, The monolithic polymer did not directly contribute the improvement in high column efficiency. However a kind of joke revealed that reverse “J” letter shape of the rod type monolithic polymer purified dirty water including sludge spontaneously involving anti–bacterial effect [57]. The driving power of liquid flow was capillary action and rather large sand or sludge was excluded by micron–size through pores by filtration. The adsorption towards impurities were achieved by rather hydrophobic properties of the polymer, while anti–bacterial action was probably due to combination of rigid hydrophobic part and rather hydrophilic amino– alco-

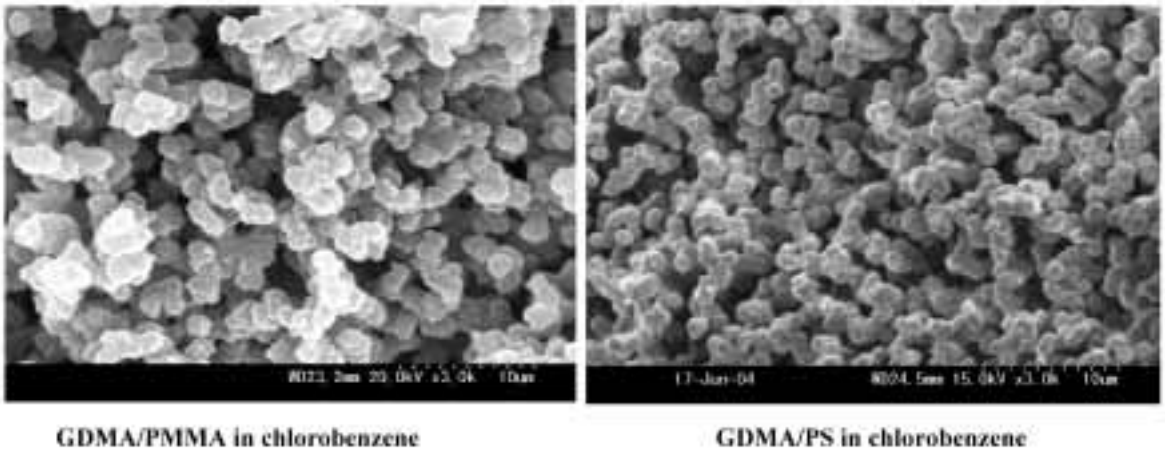


Fig. 9 Comparison of GDMA gel morphology between GDMA gels prepared with different polymer porogen (Ps and PMMA solution in chlororbenzene) magnification = 3,000 right: PMMA Mw = 2,000,000, 3% w/v, GDMA/porogen = 35/65, v/v, polymerized at 60°C for 24 h left: Ps Mw = 3,840,000, 3% w/v, GDMA/porogen = 35/65, v/v, polymerized at 60°C for 24 h.

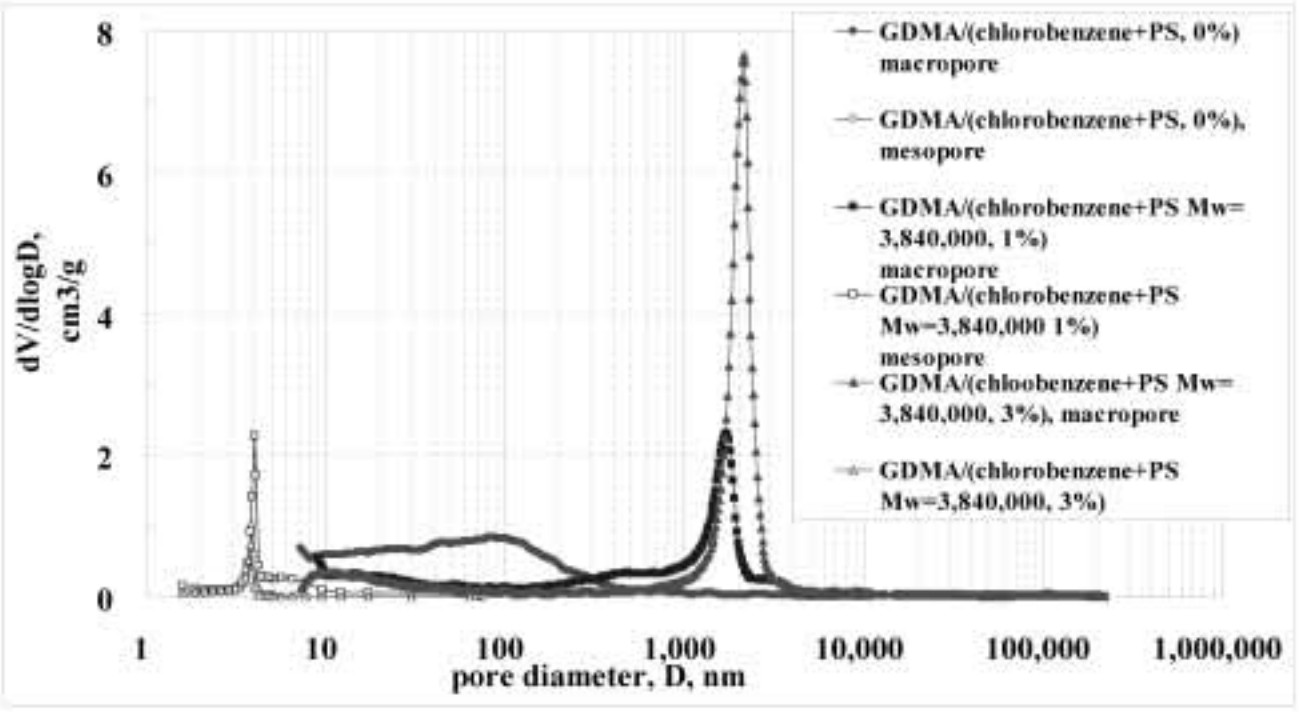


Fig. 10 Mesopore and macropore data by BET and Hg intrusion combined, respectively; PS solution in chlorobenzene, Ps Mw = 3,840,000, Ps % = 0, 1, 3 , w/v; polymerized at 60°C for 24 h with AIBN 10 mg/ml, GDMA/porogen = 35/65, v/v.

hol functional groups through disturbance of the cell membrane of bacteria.

The great improvement in column efficiency was effectuated by the use of three functional epoxy monomer namely TEPIC with the diamine BACM utilized in the previous section [58]. These two monomers afforded nicely controlled co–continuous structure also by the polymerization in polyethylene glycol as the porogen again as shown in Fig. 14. The characteristics of TEPIC–based co–continuous structure are sub–micron size skeleton and relatively large through pores, where size exclusion chromatography proved existence of mesopores as well as macro–pores. A capillary column having 21.5 cm long realized up to 46,000 theoretical plates for alkyl benzenes in aqueous acetonitrile mobile phase. Due to relatively hydrophilic characteristics, TEPIC based stationary phase can be utilized as a HILIC stationary phase in higher concentration of acetonitrile in water. Therefore, TEPIC based column can be utilized for the separation of highly hydrophilic bio–related com-

Table 1. A and C term of van Deemter equation of poly–GDMA filled capillary.

Solute	Thiourea (non–retentive)		Acetophenone (retentive)	
column samples	A term μm	C term ms	A term μm	C term ms
1. GDMA/PS (Mw = 50,000, 5%) solution in chlorobenzene = 35/65, v/v 340 mm × 200 μm i.d. 50% aqueous methanol	34	223	327	377
2. GDMA/PS (Mw = 3,840,000, 3%) solution in chlorobenzene = 35/65, v/v 330 mm × 200 μm i.d. 50% aqueous methanol	20	71	92	99
3. GDMA/PS (Mw = 3,840,000, 3%) solution in chlorobenzene = 33/67, v/v 320 mm × 200 μm i.d. 60% aqueous methanol	11	41	26	63
4. GDMA/PS (Mw = 3,840,000, 3%) solution in chlorobenzene = 33/67, v/v 320 mm × 200 μm i.d. 80% aqueous methanol	11	35	10	62

Conditions: Detection UV at 214 (entry 1) and 245 nm (entry 2,3) by off–column adaptor with a window defined 10 cm from the outlet from the capillary; Solute, 0.1 mg/ml in methanol except thiourea (0.01 mg/ml).

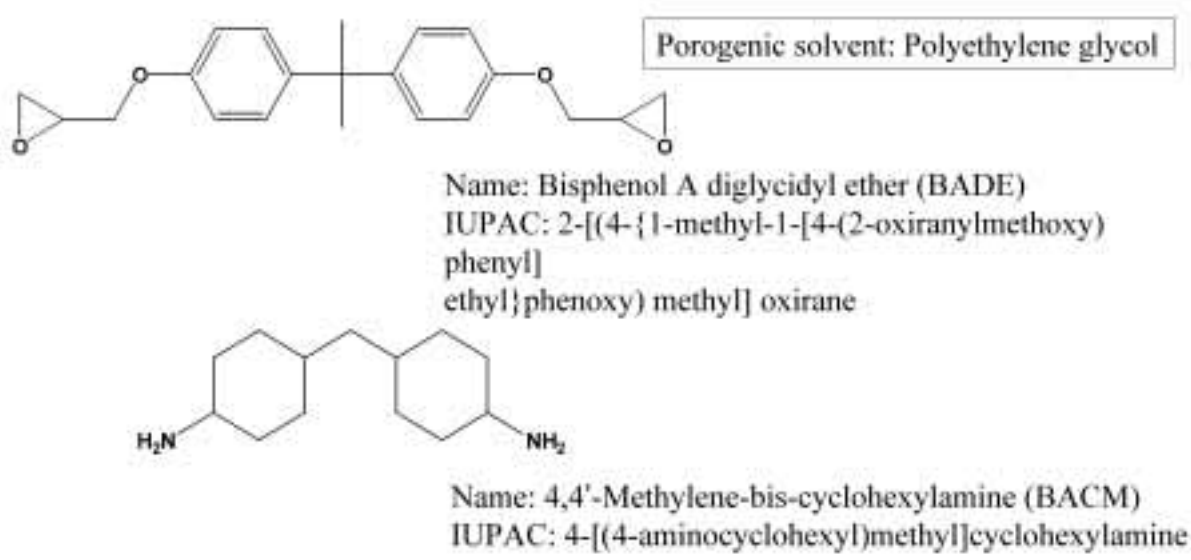
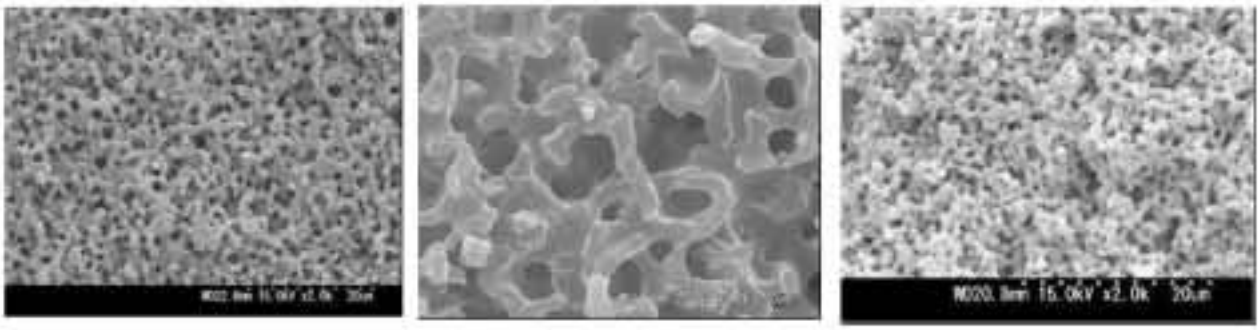


Fig. 11 Monomers utilized for epoxy resin based monolithic polymers.

pounds. It was striking that relatively long capillary column filled with TEPIC based co–continuous polymer could be wired on name card size, plastic plates as shown in Fig. 15 [59]. The "wired" column having 95 cm length afforded up to 150,000 plates for alkylbenzenes with low column pressure drop. This wired type column is simply easy to use in capillary HPLC.

Spongy type bi–continuous columns for fast concentration

Morphology of co–continuous, monolithic polymers is controllable by the change of polymerization condition including the change of monomers, but has clear limitation of upper pore size. For example, sub–millimeter size pores can't be usually obtained by usual phase separation mechanism. A thermoplastic co–polymer was focused to realize to create sub–millimeter size through pores. Poly (ethylene–co–vinyl acetate) was utilized for this purpose, after kneading process with so–called "pore template" at higher temperature, the mixture was excluded to create polymer–rod. The pore template was washed with water to result in co–continuous



Condition	PEG Mw	Porogen (g)	Temperature (°C)
1	200	7.2	120
2	150	6	160
3	300	7	80

Fig. 12 Change in morphology of epoxy resin based monolithic polymers based on the feed ration listed in table under the SEM pictures.

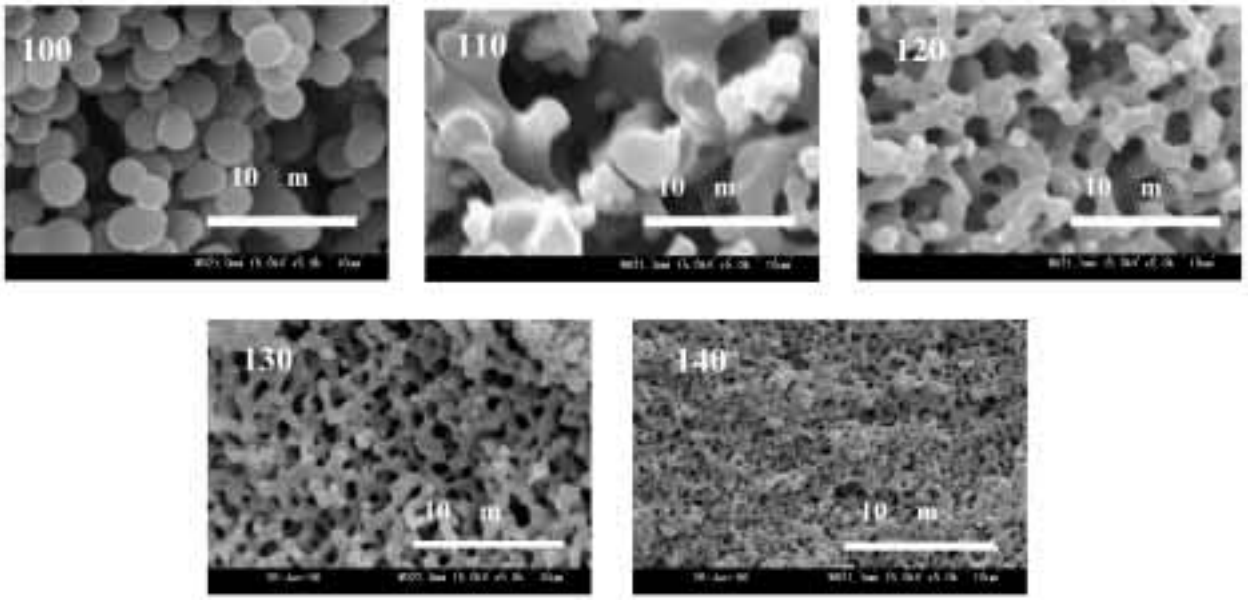


Fig. 13 Change in morphology by the change of polymerization temperature.

spongy like polymer. Normal size column having 4.6 mm I.D can be easily fabricated by insertion of the spongy type polymer rod as shown in Fig. 16 [60–64]. Interestingly, this spongy type column showed preferable retention toward poly–aromatic hydrocarbons, while relatively poor retention properties for hydrophilic solutes such as alcohols, phenol, and carboxylic acids. In addition, to this retention characteristics and sub–millimeter size through pores, relatively higher flow rate for concentration was employed with previously introduced automated pre–concentration HPLC system, where as shown in Fig. 17 benzo[a]pyrene in a pyroligneous acid was detected using general UV detection in sub–ppb level concentration in it. Exclusion of relatively hydrophilic solutes as mentioned before nicely contributed effective concentration of aromatic target molecule. In addition, the spongy type pre–concentration column was also useful for total analysis of PAHs in river water as shown in Fig. 18. The analysis of PAHs has been still very important for environmental evaluation.

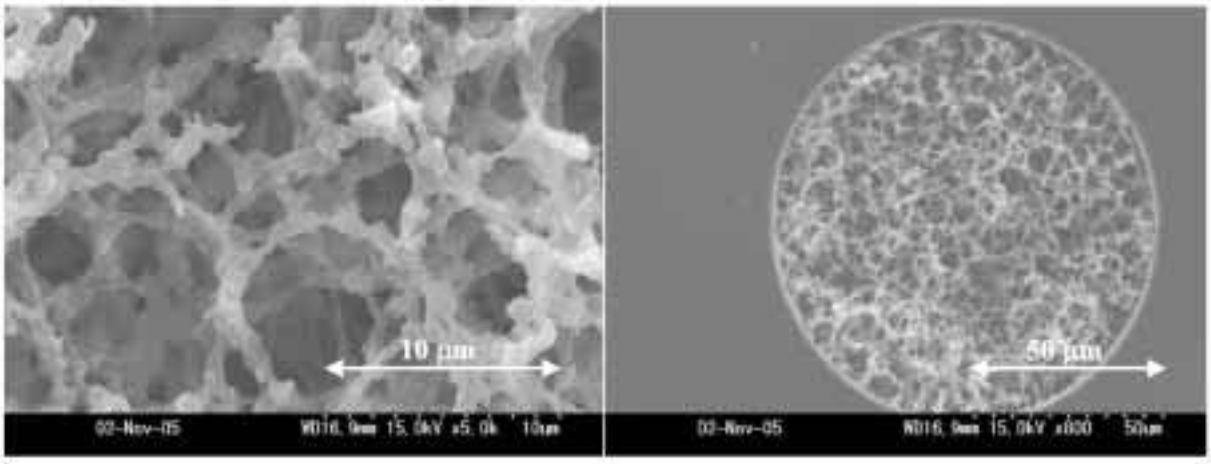


Fig. 14 SEM pictures of TEPIC–based capillary columns.

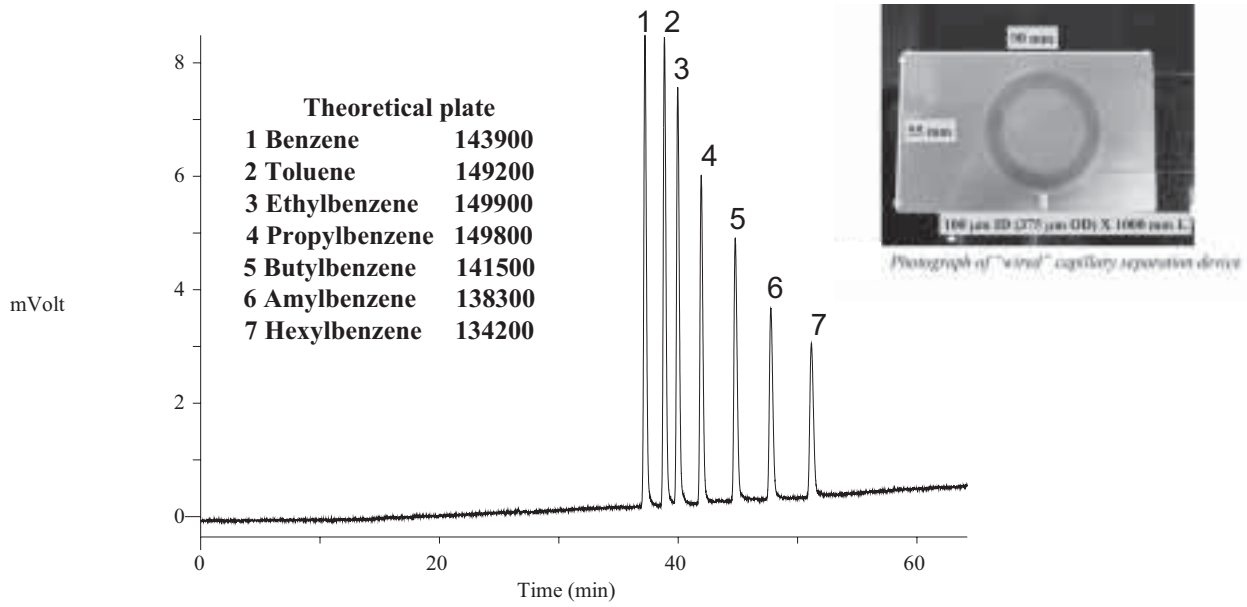


Fig. 15 Separation of alkylbenzenes using wired capillary column.
Conditions: Mobile phase: CH_3CN/20 mM Sodium Phosphate buffer pH 7.0 = 60/40 (v/v), Column: TEPIC – BACM Sheet column, Effective length: 95 cm, ΔP = 6.5 MPa, u = 1.53 mm/s, Detection: 210 nm, Temperature: 20°C, Off column: 9 cm × 50 mm i.d., Sample: alkylbenzenes (from benzene to hexyl benzene).

Contribution of monolithic polymer to affinity chromatography

Research on search of the target proteins using ligand immobilized affinity resin is a classic yet new method, which is getting much attention for drug discovery, because the resin immobilizing bioactive compounds as ligand is able to capture its target proteins directly from a protein lysate prepared using possibly affected organs and/or cells.

Traditionally the solid supports immobilizing ligand were packed into columns to capture the target proteins in fluid (affinity chromatography), but this column method tends to require rather large volume of materials including the gels as well as protein lysate, while handling of particulate affinity resins might be simplified. This presumably results in loss of valuable items (ligand as well as protein lysate). Therefore, it can be hardly utilized for very rare ligand such as naturally occurring compounds or toxic compounds. In addition, proteins are possibly denatured through this column method due to relatively large volume in the column.

To avoid the disadvantages of column method, tiny amount of affinity resins might be directly added into protein lysate to capture the target proteins. In comparison to the column method, experimental procedures using dispersed affinity resins should be complicated, because collection of affinity resin is essential. Therefore,

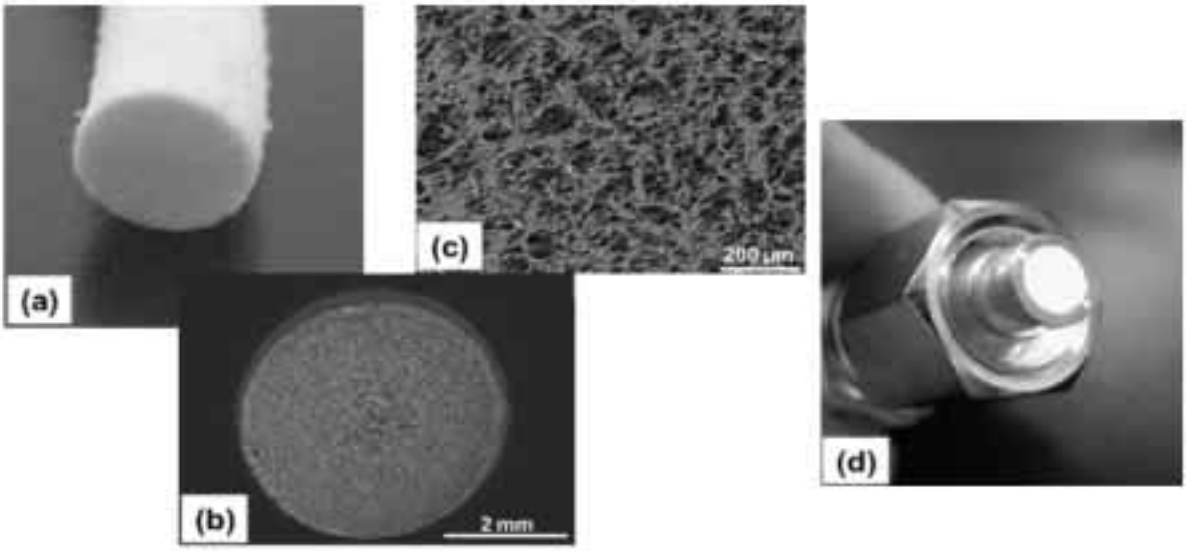


Fig. 16 Appearances of spongy type polymers and packed column. (a) Physical appearance, (b and c) SEM image, and (d) photo of column end of the spongy monolithic column.

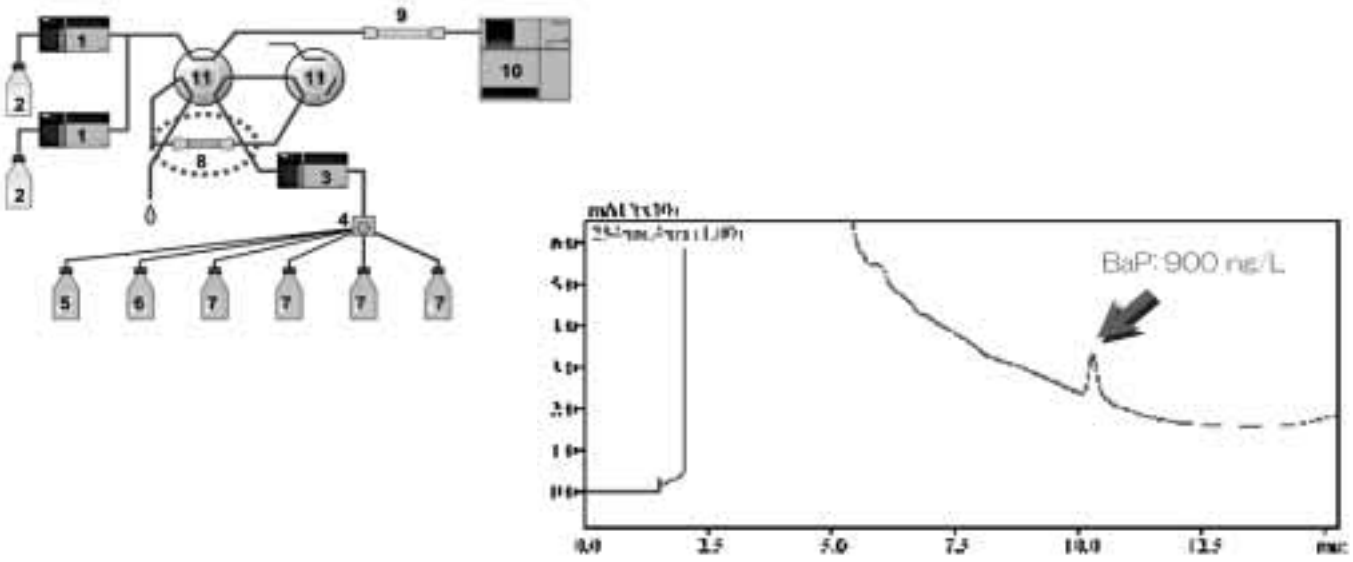


Fig. 17 Determination of benzo[a]pyrene using two valve HPLC system.
LC condition: Flow rate: 1.0 mL/min, Mobile phase: 80% MeCN aq., Column: ODS (150×4.6 mm i.d.), Pretreatment column: Spongy monolith (EVA D 50×4.6 mm i.d.), Temperature: 40°C, Detection: PDA (254 nm), Concentrate flow rate: 2.5 mL/min, Concentrate time: 12 min.

some devisal should be required for preparation of the affinity resins. In addition to the devisal, quantitative immobilization of ligand to the affinity gel, simplified experimental procedures including washing and elution steps, and effective binding of target proteins without non–specifically bound proteins will be required.

Monolithic type, hydrophilic affinity resins were prepared using ethylene oxide base methacrylate monomers including functional monomer depicted in Fig. 19 [65–69]. By the change of ratio of the functional monomer that is utilized for ligand immobilization, variety of ligand concentration was available. As summarized in Table 2, maximum ligand concentration was 125 μmol/ml, which is much greater than that of a typical commercially available affinity beads. Because ethylene oxide based, relatively hydrophilic monomers were utilized, non–specifically bound proteins were greatly reduced compared with that on commercial methacrylate type affinity beads. The monolithic type affinity reins prepared were called as Moli–gel, which is abbreviation of monolithic affinity gel.

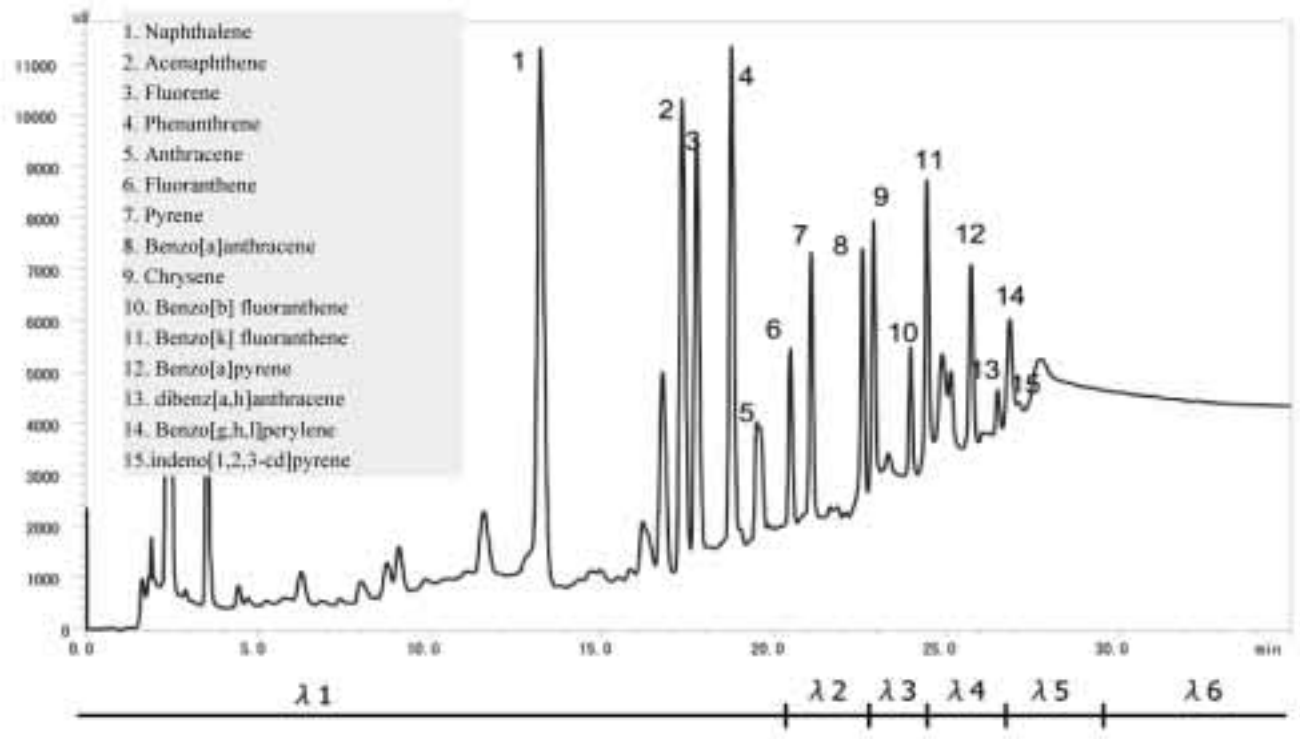


Fig. 18 Separation of PAHs.
Chromatographic conditions: mobile phase: water/acetonitrile gradient, Flow rate: 1.5 mL/min for analysis, 1 mL/min for concentration, sample: 10 mL of Kamo river water spiked with PAH samples.Analytical column: Restek Pinacle II PAH (250 mmL. × 4.6 mmI.D.)
Concentration column: SPONGE (50 mmL. × 4.6 mmI.D.)
Column temperature: 40°C, Detection: RF–20A
Sample concentration: 2,3,4,7,8,12,13,14,15; 10 ng/L, 5,6,9,10,11; 20 ng/L, 1,13; 100 ng/L.

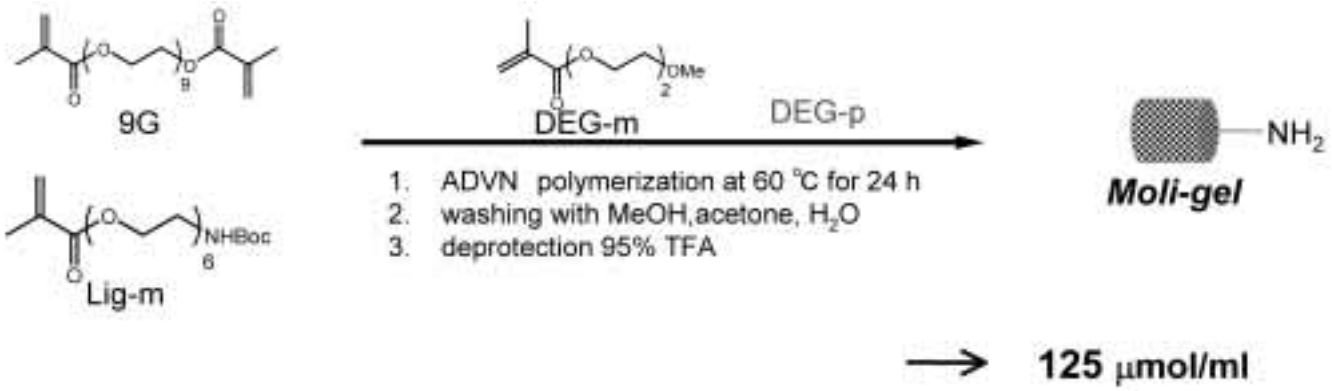


Fig. 19 Preparation of monolithic type affinity resin, Moli–gel.

Interesting experimental facts were observed using Ketoprofen, Ibuprofen, and Aspirin as ligands using the monolithic type affinity resins as well as commercial Toyopearl as base resins. As summarized Western blot data in Fig. 20 [70–72], completely opposite results in capture of one of the common target protein of the ligands, COX–1 were observed. These unexpected results in capturing were affected by the density of ligand as shown in Fig. 21. On Moli–gel, higher ligand density captured COX–1, while lower density captured the target protein on commercial Toyopearl. The detailed reasons have not been elucidated yet but, these investigations strongly suggested that micro–environment on solid support seriously affected the results of affinity chromatography to result in misunderstanding of the essential. Hydrophilic affinity gel, Moli–gel was applied to elucidate possible target proteins of one of algae toxins, Microcystin LR. The results were reported in our recent paper [73].

Table 2. Feed ratio of monolithic type affinity gels.

Lig–m (μl) (ratio)	DEG–m (μl)	9G (μl)	DEG–p (ml)	ADVN (mg)
3.5 (0.1)				
17.4 (0.5)				
25.9 (0.75)				
34.5 (1)	15.8	390.8	750	10
68.9 (2)				
120.8 (3.5)				
172.3 (5)				

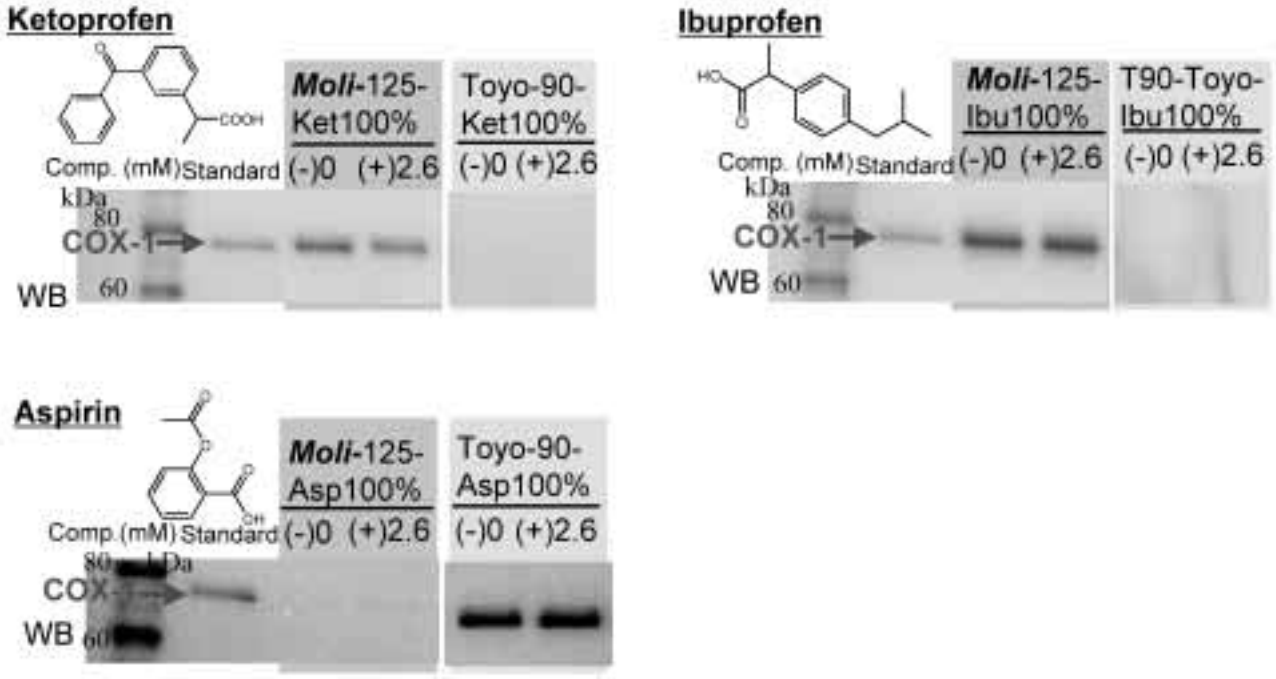


Fig. 20 Capture of COX–1 using Moli–gel and Toyopearl.
Buffer: 0.25 M sucrose, 0.3 mM DDC, 25 mM Tris–HCl pH 7.5, 0.5% Tween 20, Time for capture: 4°C, 4 h, Protein solution: Buffer spiked with COX–1 (1.4 pmol/ml), Detection: SDS–sample buffer (25°C, 1000 rpm, 10分), WB: Anti–COX–1 Mouse-mono, Anti–mouse–IgG-HRP.

Summary

Through this contribution, "simple LC" was set up as the theme. However, the point of this contribution is to reveal importance of preparation of pre–treatment procedure of samples. Modern HPLC system has been greatly improved and highly automated rapidly, however, we have to still pay attention very carefully, whether the sample applied to analysis or detection is really feasible enough or not. HPLC system probably reflects us some fact based on the sample injected, but we have to consider the fact obtained is really what reflects inside science information of target samples. In other word, sample preparation and/or pre–treatment have to be really suitable and accurate method for modern LC analyses. After this summary, detailed experimental information will be listed. I sincerely thank all the students and stuff for their really great help and work.

Experimental

Packing material utilized in Fig. 1

Solvents: Acetonitrile and tetrahydrofuran (THF) were of the highest grade for HPLC and used as received. Water utilized for mobile phase was ultra–pure water produced in the laboratory using a Yamato, AUTO STILL Model wg–22 followed by a Branstead, E–PURE. Cyclohexanol as porogen was also purified using the standard distillation technique.

Materials: Glycerol dimethacrylate (GDMA) was a gift from Kyoeisya Chemicals Inc., (Osaka, Japan) and another alkyl methacrylate monomers were all purchased from Wako Pure Chemical

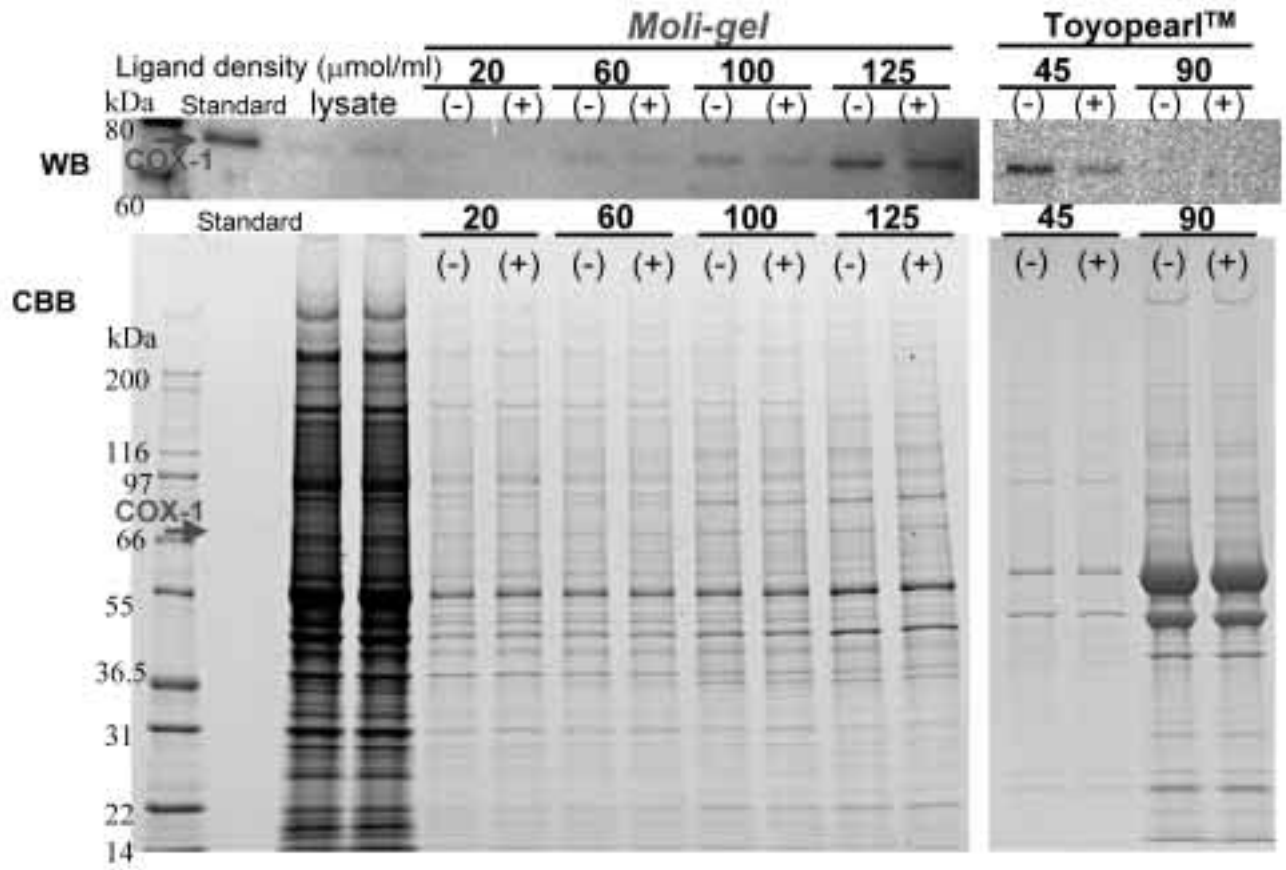


Fig. 21 Capture of COX–1 with Moli–gel and Toyopearl having different ligand density.
Buffer: 0.25M sucrose, 0.3 mM DDC, 25 mM Tris–HCl pH 7.5, 0.5% Tween 20, Capture test: 4°C, 4 h, rat brain lysate spiked with COX–1 (1.4 pmol/ml), resolution: SDS–sample buffer (4°C, 1000 rpm, 10 min), WB: Anti–COX–1 Mouse-mono, Anti–mouse–IgG–HRP, CBB stained.

Ltd., (Osaka, Japan). All the monomers utilized for preparation of stationary phases [methyl methacrylate (MMA), butyl methacrylate (BMA), and 2–ethylhexyl methacrylate, (2–EHMA)] were purified using standard distillation techniques under reduced pressure to remove polymerization inhibitors.

Styrene monomer for the preparation of the seed particle utilized for following multi–step swelling and polymerization method, was washed using 5% sodium hydroxide solution followed by saturated sodium chloride solution and dried over calcium chloride followed by the distillation in vacuum. Benzoyl peroxide (BPO) as a radical initiator was purified using re–precipitation technique from the chloroform solution into methanol to remove aqueous stabilizer. Potassium peroxysulfide as a water–soluble initiator was also purified using re–crystallization technique.

Preparation of the seed particle: To completely de–oxygenized water (300 ml) through helium bubbling and boiling, 0.39 g of sodium chloride and 6 ml of the purified styrene were added. The suspended system was heated up to 75°C under argon atmosphere. Then, aqueous solution of 0.27 g of potassium peroxysulfide in 50 ml of the purified and de–oxygenized water was added to initiate the soap free polymerization. After the initiation, 7 ml of styrene was added every one hour and finally 2 ml of styrene was added after 7 hours from the initiation. Total amount of styrene utilized was 50 ml. After the completion of 24 hours' polymerization, the emulsion system was purified using a centrifugation technique at 5,000 rpm for 30 minutes. The yield was up to 61% and the seed styrene particle was re–dispersed into pure water (5.52×10^{-2} g/ml) to be utilized as a seed dispersion.

Preparation of stationary phases through multi–step swelling and polymerization method: To the suspension of seed particle (1.44 ml), was added micro–emulsion of the activating solvent prepared from dibutyl phthalate (0.405 ml), sodium dodecylsulfate (0.028 g) and the water 10 ml by sonifier. The swelling was completed in 4 hours at room temperature.

The micro–emulsion was prepared by sonification from the porogen, cyclohexanol (5 ml), BPO (0.10 g) and water (25 ml) containing polyvinyl alcohol (dp = 500, 0.48 g). This micro–emulsion was added into the swollen system prepared above and the

swelling was continued for 5 hours. The further swelling was carried out using the micro–emulsion of 5 ml of monomers (GDMA + monomer) and 25 ml of water containing polyvinyl alcohol (dp = 500, 0.48 g) and completed in 5 hours at room temperature.

The polymerization was carried out at 70°C for 24 hours. After the polymerization, the prepared particles were washed using water, methanol, THF, and acetone and dried for calculation of the chemical yields. The prepared stationary phases were packed into semi–micro size, stainless steel columns (2 mm ID × 150 mm) by slurry method using mixtures of water, acetonitrile, and 2–propanol, and the composition of slurry solvent is varied depending on the properties of polymer packing materials.

Semi–micro HPLC system: **NANOSPACE S–1** (Shiseido Co., Tokyo, Japan) was used for evaluation of stationary phases equipped by UV detector..

Packing materials utilized in Fig. 2 & 3

Materials: Ethylene dimethacrylate (EDMA) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and purified by a distillation technique to remove radical inhibitors before use. All other materials for the preparation of polymer stationary phases were used without further purification. *o*–Xylene and *p*–xylene as porogens, and 2,2'–azobis–(2,4–dimethylvaleronitrile) as a radical initiator, were of the highest grade available from Wako Pure Chemical Industries, Ltd. (Kyoto, Japan). Other materials were of the highest grade available from Nacalai Tesque (Kyoto, Japan).

Preparation of Uniformly Sized Polymer Stationary Phases.

Uniformly sized polystyrene seed particles were prepared by an emulsifier–free emulsion polymerization method. The diameter of the polystyrene seed particles was ca. 1 μm. Uniformly sized, macroporous, cross–linked polymer stationary phases were prepared by a multistep swelling and polymerization method as follows. The polystyrene seed particles (7.0×10^{-2} g/mL, 7.8×10^{-1} mL) were admixed with a microemulsion prepared from dibutyl phthalate (2.5×10^{-1} mL), sodium dodecyl sulfate (3.5×10^{-2} g), 2,2'–azobis (2,4–dimethylvaleronitrile) (5.0×10^{-2} g), and distilled water (40 mL) by sonication. This suspension was stirred at 125 rpm until oil droplets of the added emulsion were completely absorbed on the seed particles at room temperature. A suspension of cross–linking agent (5.0 mL), porogen (5.0 mL), poly (vinyl alcohol) (DP) 500, saponification degree – 96 mol%) (7.2×10^{-1} g), and distilled water (45 mL) was added to the swollen polystyrene seed particles. This suspension was stirred at 125 rpm for 2 h at room temperature. The polymerization was carried out at 50°C for 24 h under argon atmosphere. The resulting polymer particles were washed with methanol and tetrahydrofuran by a repeated sedimentation– redispersion process and the yield was quantitative. The final particle size was 5.5 μm in diameter, and CV values of the prepared particles were about 5%.

Chromatography: The polymeric stationary phases were packed into stainless steel column (150 mm × 4.6 mm i.d.) by slurry method using a mixture of methanol, 2–propanol, and glycerol as slurry medium. HPLC was performed using a Shimadzu–LC 4 A pump equipped with a Rheodyne 7125 valve loop injector, a Shimadzu SPD–2A UV detector, and a Shimadzu C–R4A integrator.

Packing material utilized in Fig. 4

Materials: Monomers, Ethylene glycol dimethacrylate (EDMA) as the cross–linking agent, and 4–vinylpyridine as the functional monomer, both from Wako Chemicals (Osaka, Japan) were effectively purified by vacuum distillation techniques to remove polymerization inhibitor. Template molecules, p–*t*–butylphenol and bisphenol A were purchased from Nacalai Tesque (Kyoto Japan) and used as received. A polymerization radical initiator, 2,2'–azobis–(2,4–dimethyl– valeronitrile) (ADVN) was purchased from Wako Chemicals (Kyoto Japan) and purified using a standard purification method. A solvent realizing porous structure (porogenic solvent), toluene from Nacalai Tesque was the highest grade and used as received.

Preparation of the molecular imprinting polymer: To prepare polymer–based separation devices, we utilized two–step swelling and polymerization method, which afforded uniformly sized polymer particles, utilizing polystyrene seed particles as shape template. The polystyrene seed particles were prepared through an emulsifier free emulsion polymerization, which has been reported [9].

The two–step swelling and polymerization method easily afforded uniformly sized polymer particles with following feed ratio; EDMA: 10.0 ml, 4–vinylpyridine as functional monomer: 0.992 ml, toluene: 10.0 ml, p–*t*–butylphenol: 0.173 g, ADVN: 0.7 g. (EDMA/4–vinylpyridine/*t*–butylphenol = 46/4/1, in mole ratio). The polymerization was carried out at 50°C for 24 h.

The prepared polymer particles were dispersed into methanol and the supernatant was discarded after sedimentation of the polymer particles. This procedure was repeated three times in methanol and twice in tetrahydrofuran (THF), and then the polymer particles were filtered with a membrane filter and dried at room temperature to determine the chemical yields. The chemical yields were almost quantitative. The polymer particles had 10.4 μm in diameter with excellent size uniformity.

Concentration of bisphenol A: We prepared water solution of bisphenol A including excess of humic acids as contaminant. The

water solution contained bisphenol A (2 ppm), while humic acids was saturated in the water solution.

First, the prepared polymer particles (0.5 g) were packed into a glass cartridge having a syringe shape followed by water flow as a pre–treatment of the polymer adsorbent layer.

Second, the prepared water solution was pumped into the glass cartridge packed with the polymer adsorbents continuously (about 300 ml) followed by pure water (10 ml). Third, methanol was pumped into the glass cartridge to recover the adsorbed bisphenol A. Finally, the concentration of bisphenol A recovered from the glass cartridge was determined by HPLC with C_{18} column.

Packing materials utilized in Fig. 5, 6, and 8

Materials:

Monomers, ethylene glycol dimethacrylate (EDMA) as a cross–linking agent, and 4–vinylpyridine (4–VP) as the functional monomer, both from Wako Pure Chemicals (Osaka, Japan) were effectively purified by vacuum distillation techniques to remove polymerization inhibitor. Glycerol dimethacrylate (GDMA) and glycerol monomethacrylate (GMMA) were purchased from Kyoeisya Chemical (Osaka, Japan) and used without further purification. The template molecule, p–*tert*–butyphenol (TBP) was purchased from Nacalai Tesque (Kyoto, Japan) and 4,4'–methylenebisphenol (MBP) and butyl methacrylate (BMA) were purchased from Wako Pure Chemicals. A polymerization initiator, 2,2'–azobis–(2,4–dimethyvaleronitrile) (ADVN) and benzoil peroxide were purchased from Wako Pure Chemicals.

A solvent realizing porous structure (porogenic solvent), toluene from Nacalai Tesque was of the highest grade. All chemicals for preparing HPLC mobile phase, sodium dihydrogen phosphate, disodium hydrogenphosphate and acetonitrile were purchased from Wako Pure Chemicals. Water for preparing BPA standard solution was obtained from Milli–Q water purification system of Millipore (Bedford, MA, U.S.A).

Preparation of the molecularly imprinted polymer:

Uniformly sized polystyrene seed particles were prepared by a typical emulsifier–free emulsion polymerization method and purified by a centrifugation method. The size of seed particles was around 1 μm in diameter with excellent size mono–dispersity.

Preparation of uniformly sized macro–porous polymer particle by a multi–step swelling and polymerization method was carried out as follows. In the first step, 0.162 ml of aqueous dispersion of the purified polystyrene seed particles (2.23×10^{-1} ml/ml) was admixed with micro–emulsion of dibutyl phthalate (activating solvent), 0.04 g of sodium dodecylsulfate, and 10 ml of distilled water by sonication.

This first step swelling was carried out at room temperature while the solution was stirred at 125 rpm. Completion of the first step swelling was determined by the vanishing point of oil droplets in added micro–emulsion using an optical microscope.

A dispersion of 3 ml of toluene (porogenic solvent), 0.34 mL of 4–VP, 0.06 g of TBP or 0.04 g of MBP, 0.15 g of ADVN and 0.06 g of sodium dodecylsulfate into 35 ml of water containing 0.45 g of poly (vinyl alcohol) (degree of polymerization, DP = 2000; saponification value = 86.5–89 mol%) as dispersion stabilizer was added to the dispersion of swollen seed particles. This second step swelling was carried out at room temperature with stirring at 125 rpm.

After the second step swelling was completed, the other dispersion of 3 ml of EDMA, 0.06 g of sodium dodecylsulfate into 35 ml of water containing 0.45 g of poly (vinyl alcohol) was added to the dispersion of the swollen particles. This swelling step was carried out for 6 h at room temperature while the solution was stirred at 125 rpm. For the polymerization of swollen particles, the aqueous dispersion was stirred at 50°C for 24 h under argon atmosphere. The polymer particles obtained were washed with water, methanol, and tetrahydrofuran to remove the porogenic solvent, template molecules other impurities. The feed ratio was as follows, EDMA–4–VP–template, 40: 8: 1 in mole ratio.

Some of obtained MIPs were surface modified as described in following sections.

Surface modification methods:

The non–modified polymer particles prepared using the multi–step swelling and polymerization method (0.8 g) (base polymer particles) were dispersed in 50 ml of acetone and the hydrophilic monomers of a mixture of GMMA and GDMA, same as described previous section and ADVN were added (5% in weight ration of monomers) and polymerized at refluxing temperature of acetone. The polymerization was continued for 24 h and the obtained particles were washed with acetone and water in order.

The base polymer particles were dispersed in 50 ml of methanol and the ionic monomer of methacrylic acid 3–sulfopropyl potassium salt (MASK) and benzoyl peroxide (BPO) were added (5% in weight ratio to monomers) and polymerized at refluxing temperature of methanol. The polymerization was continued for 24 h and the obtained particles were washed with methanol and 1N HCl and water in order.

Column packing method:

The prepared particles were packed into a stainless steel column (30 mm × 4.6 mm i.d.) by slurry techniques to evaluate their characters. We mainly utilized mixture of water, isopropanol, and methanol as packing medium.

Chromatographic measurement

HPLC measurement was carried out with the LC–VP HPLC sys-

tem from Shimadzu (Kyoto, Japan) consisted of a LC−10 Avp solvent delivery pump, CTO−10 Avp column oven, FCV−12 AH two −position flow changeover valve, FCV−13 AL six−port flow selection valve, SIL−10 Avp automatic injector, Rheodyne 7725 manual injector (Cotati, CA) with 100 μL loop, SCL−10A system controller and a CLASS−VP work station software. A Coulochem II, electrochemical detector (ECD) was purchased from ESA (Chelmsford, MA, USA).

Small hydrocarbons including BPA were analyzed by HPLC to compare the retention times on respective MIPs.

HPLC conditions for small hydrocarbons were as follows, Mobile phase: water−acetonitrile = 55/45 (v/v), Flow rate: 0.3 mL/min, Detection: UV 220 nm, Temp.: 40 deg., Column: packed with prepared MIPs (150 mm × 4.6 mmi.d.).

Chromatographic applications:

Surface modified MIPs were applied as pretreatment columns to actual determination of BPA with the column switching HPLC. To accomplish suppression of BPA contamination and determine the BPA concentration in water samples, a special technique was required. A column switching HPLC with a pump injection system was one of the solutions. The pump delivered 50 mL of BPA standard solutions or environmental water samples and the BPA was concentrated on the pretreatment column. Then mobile phase was delivered a via six−port switching valve and then the concentrated BPA was directed to the analytical column and detected by the detector after the separation on the analytical column.

HPLC conditions employed for column switching HPLC were as follows; mobile phase: 20 mM (sodium) phosphate buffer (pH = 7)/acetonitrile = 65/35 (v/v), rinsing solvent: 20% (v/v) of acetonitrile aqueous solution, flow rate for analysis: 0.8 mL/min, flow rate for pretreatment: 2.5 mL/min, concentrated volume: 50 mL, analytical column: Shim−pack VP−ODS (150 mm L × 4.6 mm I.D.), temperature: 40 ℃, electrochemical detection: at + 0.35/ + 0.55 V (analytical cell, CH1/CH2, 1 μAF.S.) and + 0.6 V (guard cell), UV detection: at 220 or 275 nm. For the reference, LCMS analysis was compare to HPLC column switching analysis.

Polymers prepared in Fig. 9

Materials:

Crosslinking agents

GDMA (glycerol dimethacrylate,

$CH_2 = C(CH_3)OCOCH_2CH(OH)CH_2OCO(CH_3)C = CH_2$, Mw = 227, was provided by Kyoeisha Chemical, GP−101P, and used as it was.

Initiator:

The following initiators for free radical polymerization were used as received.

1) 2,2'−azobisisobutyronitrile, AIBN, Mw = 164.21, 98%, Purchased from Nacalai Tesque, Kyoto, Japan.

2) 2,2'−azobis(2,4−dimethyl)valeronitrile, ADVN, Mw = 248.37, 95%, Purchased from Wako Pure Chemical, Osaka, Japan.

Porogenic solvent:

1) Toluene

Purchased from Nacalai Tesque and used after distillation from calcium hydride at bp (110.6˚C).

2) Polymer porogenic solution

Polymer porogenic solution was prepared by dissolving polystyrene powder in chlorobenzene.

i) Chlorobenzene, 99% was purchase d from Nacalai Tesque and was distilled at 35˚C, 20 mmHg before use.

ii) Polystyrene, standard monodisperse samples were used. Mw= 50,000, 600,000, 3,840,000, Mw/Mn = 1.04 (Tohso Co., Japan), added to chlorobenzene at 1% − 5% (w/v) and left at room temperature for 24 h without any mechanical stirring for complete dissolution

Preparation of polymerization solution:

An initiator, 10 mg was weighted in 20 ml beaker with a plastic spoon. Next, 2 ml of the porogenic solvent was added and stirred. GDMA was filtered through 0.2 μm PTFE filter (DISMIC−25JP; ADVANTEC TOYO, Japan) mounted to a syringe (5 ml; B. Braun Melsungen AG), then added to the porogenic solvent and stirred gently. Then, the mixture was poured into a test tube (10 mm inner diameter and 10 cm long). Then, the solution was bubbled with argon gas through for 10 min. All operations described above were carried out at room temperature.

Capillary preparation:

Commercial fused silica capillary coated with polyimide of 250 or 200 μm ID was sampled with about 1.8 m long. Then, the capillary wound in a coil was filled with 1 N sodium hydroxide and immersed in a bath at 60˚C for 1 h, and washed several times repeatedly with water and acetone flown through manually by a syringe. The capillary inner wall was not specially treated for increasing interfacial adhesion. GDMA/toluene (35/65, v/v) solution was filled in the capillary wound in a coil with a syringe pump (Harvard Apparatus Model 11). The pumping was continued until the solution of about 100 times of the capillary volume (0.57-0.88 ml) was pumped through. Then, the filled capillary coil was sealed with a packaging film (PARA Film, American National Can) at the both ends and immersed in an oil bath kept at 60˚C for polymerization. The coil was picked up after 24 h and connected to LC pump (Shimadzu LC 6 A) for washing with tetrahydrofuran (THF) and then methanol, for 24 h, respectively. The capillary of test length was

cut out with a ceramic cutter.

Measurements:

Poly–GDMA prepared in a test tube

Test tubes frozen in liquid nitrogen were broken manually to extract gel samples. The gels were immersed in THF for 24 h and dried in a draft for another 24 h. Then those were dried at 60°C for another 24 h and then in vacuum for 60 min.

Observation with SEM:

SEM apparatus was a Hitachi S–3000N. The observation of polymers was made after gold was vapor deposited on them. The magnification was 400 to 5,000.

Pore measurement:

BET (Brunauer, Emmett, Teller's equation) apparatus, GEMINI II (Micrometritics, USA) was used for mesopore measurement. Hg intrusion apparatus (Micrometrics, USA) was partly used for macro pore measurement.

Polymer preparation described in Fig. 11 & 12:

4–[(4–aminocyclohexyl) methyl] cyclohexylamine (BACM) 0.52 g (2.47 mmol) was dissolved in poly–ethylene glycol (PEG), then 2.33 g (6.84 mmol) of 2–[(4–{1–methyl–1–[4–(2–oxiranylmethoxy) phenyl] ethyl} phenoxy) methyl]oxirane (BADE) was added into this solution with stirring. This polymerization mixture was poured into a glass test tube having 12 mm inner diameter. The polymerization took place in oil bath at the prescribed temperature for 3 hours. The structures of monomers utilized and the amount of monomers as well as porogen were shown in Fig. 11. The resulting polymers were removed from the test tube to be washed with water followed by acetone, then dried at 60°C for 24 hours. We have prepared three types of polymers with different molecular weight PEG and polymerization temperature.

Polymer monolith in Fig. 14:

Monomers: 4–[(4–aminocyclohexyl) methyl] cyclohexylamine (BACM) was purchased from Tokyo Kasei Co., (Tokyo, Japan) and utilized as received. *Tris*–(2,3–epoxypropyl)–isocyanurate (TEPIC) (racemic and chiral) was kindly donated by Nissan Chemical Co., (Tokyo, Japan) and utilized without further purification.

Other materials: polyethylene glycol #200 (PEG 200) and #300 (PEG 300) were purchased from Nacalai Tesque (Kyoto, Japan) and utilized as porogenic solvents. 3–Aminopropyltriethoxysilane was purchased from Nacalai Tesque and used as surface modifying agent of capillary inner wall, while 100 μm i.d. × 375 μm o.d. fused silica capillary was purchased from POLYMICRO TECHNOLOGIES, USA)..

Solvents: ultra–pure water was obtained through Milli–Q GPA system, while methanol, acetonitrile, and tetrahydrofuran (THF) were purified by suitable distillation techniques.

Chromatographic solutes; all the alkylbenzenes as well as alkyl phenyl ketones were commercially available and used as received. Polystyrene standard samples were purchased from Showa Denko, Co., (Tokyo Japan) as size exclusion makers.

Equipment; A constant temperature oven DNE 400 (Yamato Co.) was utilized as polymerization reactor. High performance liquid chromatograph was consisted of a LC–20 AT chromatographic pump (Shimadzu), DGU–20 A as an on–line degasifier, and CE–2075 UV detector (Jasco) equipped with RHEODYNE 7725 injector (RHEODYNE). Scanning electron micrograph was obtained using a Hitachi S–510. Pore size distribution was performed on a Micromeritics PORESIZEZR 9320.

Surface modification of inner wall of the capillary; the 100 μm i.d. capillary was washed with 1 N NaOH aqueous solution and kept at 70 °C for 30 min, followed by washing with pure water. The capillary was washed with 1N HCl and kept at 70 °C for 30 min. followed by washing with pure water and acetone. After complete removal of acetone by air, tetrahydrofuran and 3–aminoproplyltriethoxysilane (1:1 v/v) were flowed through the capillary and kept at 80 °C for 24 hours. After the reaction, the modified capillary was washed with ethanol repeatedly.

Preparation of capillary column: TEPIC (1.60 g) and BACM (0.37 g) were completely dissolved in PEG #200 (7.00 g). This solution was injected into the modified capillary by a syringe. Polymerization reaction was carried out at 80 °C for 12 hrs. The resulting capillary was washed out by water and methanol and dried *in vacuo* for 5 hrs.

Measurement: Monolithic bulk material was prepared in test tube as well as preparation of capillary column. Pore size distribution measured by mercury intrusion method. The surface area measured by BET.

Preparation of Spongy monolith in Fig. 16:

The spongy–monolith was prepared as follows: polyolefin chips (consist of polyethylene and polyvinyl acetate: EVA resin) and pore templates (water–soluble compounds: multiple alcohol) were melted at 130 °C and stirred to combine. The combined material was extruded in columnar style at 130 °C. Then, the columnar material was cooled in water immediately and the stick shaped material was obtained. After cooling, the stick material was washed with water using ultrasonication to remove the water–soluble compounds. Here, the water–soluble compounds worked as pore templates. The porosity of obtained spongy–monolith was 74% and its diameter of cross section was globally 4.7 mm.

For packing of spongy–monolith to stainless column, we utilized the empty column having 4.6 mm–i.d. The diameter of spongy–monolith (4.7 mm) was larger than that of inter diameter of column (4.6 mm). Therefore, we carried out the packing with taking advantage of elastic characterization of spongy–monolith. The procedures of packing are shown as follows: One end of spongy–monolith was compressed with thermal shrinkage tube at 120 ºC. After cooling, the shrinkage tube was removed and the diameter of the end of spongy–monolith was become smaller than 4.6 mm. Then, the spongy–monolith was macerated into water and the shrunk end of spongy–monolith was set into empty column. Finally, the excessive part of spongy–monolith was cut down and the column end module was connected. Here, the shrunk part of spongy–monolith was completely cut down and only normal part (4.7 mm) could be packed into column.

Preparation method of Moli–gel:

Reagents:

Solvents and reagents were utilized without further purification unless it was particularly mentioned. Monomers and porogen were structurally illustrated in our previous report.

Nacalai Tesque, INC (Kyoto, Japan), Wako Pure Chemical Industries, Ltd (Osaka, Japan), Tokyo Chemical Industry Co., LTD. (Tokyo, Japan), Bio–Rad Laboratories (Tokyo, Japan) were abbreviated to be shown simply as Nacalai, Wako, TCI, and Bio–Rad in the following experimental description.

Trifluoro acetic acid (TFA) and ninhydrin were purchased from Nacalai. 2,2'–Oxydiethanol (DEG–p), 2,2'–azobis (2,4–dimethylvaleronitrile) (ADVN), oxalic acid, 0.1 N NaOH: 1 mol/l sodium hydroxide solution, 1 mol/l hydrochloric acid, 1.0 w/v% phenolphthalein ethanol (90) solution, octylamine, dodecylamine, 3–phenyl–1–propylamine, ethanol (EtOH), pyridine, acetonitrile, and (*S*)–2–(4–(((2,4–diaminopteridin–6–yl)methyl)(methyl)amino) benzamido) pentane–dioic acid (Methotrexate: MTX) were purchased from Wako. 2–(2–Methoxyethoxy) ethyl methacrylate (DEG–m) and 2–(3–benzoyl phenyl)–propionic acid (Ketoprofen) were purchased from TCI. 4–Carboxybenzenesulfonamide (Sulfonamide) was purchased from SIGMA–ALDRICH, (Tokyo, Japan). 2–(2–Methoxyethoxy) ethyl methacrylate (DEG–m) was purchased from TCI. *N*–Methyl–2–pyrrolidinone dehydrated (dry–NMP) was purchased from Kanto Chemical Co., INC., (Tokyo, Japan). 1–Ethyl–3–(3–dimethylaminopropyl)carbodiimide (water–soluble carbodiimide: WSCD) and 1–hydroxybenzotriazole (HOBt) were purchased from PEPTIDE INS., (Osaka, Japan). *N*–*t*–Butoxy–17–amino–3,6,9,12,15–pentaoxaheptadecane–1–nyl methacrylate (Lig–m) was kindly donated by Reverse Proteomics Research Institute (Tokyo, Japan). Polyethylene glycol #400 dimethacrylate 9G NK ESTER (9G) was donated from Shin–Nakamura Chemical CO., LTD. (Wakayama, Japan). Toyopearl; AF–Amino–650 M (Toyopearl) was purchased from Tosoh Bioscience (Pennsylvania, USA). The certified surface density of amino groups is as high as 92 μmol/ml. Affigel 102 (Affigel) was purchased from Bio–Rad. The certified surface density of amino groups is as high as 17 μmol/ml. 76% w/w phenol/EtOH, (phenol/EtOH) was purchased from Applied Biosystems (Tokyo, Japan).

Devices:

UV–mini 1240 UV–VIS spectrophotometer (SHIMADZU, Kyoto, Japan), E–1010 Ion Spattering Apparatus (HITACHI), and Miniscope TM–1000 (HITACHI) were used.

Preparation of Moli–gel (monolithic type affinity gel) and calculation of theoretical amino group density:

A standard Moli–gel was prepared. In this case, Lig–m 186.4 mg (172.3 μl), DEG–m 16.6 mg (15.8 μl), 9G 435.7 mg (390.8 μl), DEG–p 844.7 mg (750 μl), and ADVN 10 mg were admixed in a vial at once and polymerized at 60 ºC for 24 hr. The resulting polymer was washed and comminuted, and then de–BOC reaction was carried out. Moli–gel with free amino group was then obtained.

To calculate the theoretical amino group density about above prepared Moli–gel, 20 μl of Moli–gel (water dispersion) was washed by 20% aqueous ethanol and dried. (3.92 mg/20 μl). The amino group density of Moli–gel was measured by titration and ninhydrin method using above mentioned processes. In the case of ninhydrin method, resulting colored supernatant was diluted 8 or 16 times by 20% aqueous ethanol.

References:

[1] Tanaka, N.; Kimura, H.; Tokuda, D.; Hosoya, K.; Ikegami, T.; Ishizuka, N.; Minakuchi, H.; Nakanishi, K.; Shintani, Y.; Furuno, M,; Cabrera, K. *Anal. Chem.* **2004**, *76*, 1273–1281.

[2] Ikegami, T.; Hara, T.; Kimura, H.; Kobayashi, H.; Hosoya, K.; Cabrera, K.; Tanaka, N. *J. Chromatgr. A* **2006**, *1106*, 112–117.

[3] Pawliszyn, J. Handbook of Solid Phase Microextraction; Elsevier, London, **2012**.

[4] Brausch, J. M.; Connors, K. A.; Brooks, B. W.; Rand, G. M. Reviews of Environmental Contamination and Toxicology, **2012**, *218*, 1–99.

[5] Hosoya, K.; Maruya, S.; Kimata, K.; Kinoshita, H.; Araki, T.; Tanaka, N. *J. Chromatogr*. **1992**, *625*, 121–129.

[6] Hosoya, K.; Kishii, Y.; Tanaka, N.; Kimata, K.; Maruya, S.; Araki, T.; Fréchet, J. M. J. *Chem. Lett.* **1992**, 1145–1148.

[7] Hosoya, K.; Fréchet, J. M. J. *J. Polym. Sci., Part A: Polym. Chem.* **1993**, *31*, 2129–2141.

[8] Hosoya, K.; Fréchet, J. M. J. *J. Liq. Chromatogr*. **1993**, *16*, 353–365.

[9] Hosoya, K.; Teramachi, M.; Tanaka, N.; Kobayashi, A.;

Kanda, T,; Ohtsu, Y. *Anal. Chem.* **2001**, *73*, 5852–5857.

[10] Yoshizako, K.; Hosoya, K.; Iwakosh, Y.; Kimata, K.; Tanaka, N. *Anal. Chem.* **1998**, *70*, 386–389.

[11] Hosoya, K.; Yoshizako, K.; Sasaki, H.; Kimata, K.; Tanaka, N. *J. Chromatogr. A* **1998**, *828*, 91–94.

[12] Tominaga, Y.; Kubo, T.; Kobayashi, A.; Yasuda, K.; Kato, K.; Hosoya, K. *Chemosphere* **2012**, *89*, 378–382.

[13] Hosoya, K.; Yoshizako, K.; Shirasu, Y.; Kimata, K.; Araki, T.; Tanaka, N.; Haginaka, J. *J. Chromatogr. A* **1996**, *728*, 139–147.

[14] Haginaka, J.; Takehira, H.; Hosoya, K.; Tanaka, N. *Chem. Lett.* **1997**, 555–556.

[15] Hosoya, K.; Shirasu, Y.; Kimata, K.; N. Tanaka, N. *Anal. Chem.* **1998**, *70*, 943–945.

[16] Haginaka, J.; Takehisa, H.; Hosoya, K.; Tanaka, N. *J. Chromatogr. A* **1998**, *816*, 113–121.

[17] Haginaka, J.; Takehira, H.; Hosoya, K,; Tanaka, N. *J. Chromatogr. A* **1999**, *849*, 331–339.

[18] Sanbe, H.; Hosoya, K.; Haginaka, J. *Anal. Sci.* **2003**, *19*, 715–719.

[19] Kubo, T.; Hosoya, K.; Watabe, Y.; Ikegami, T,; Tanaka, N.; Sano, T.; Kaya, K. *J. Chromatogr. A* **2004**, *987*, 389–394.

[20] Kubo, T.; Tanaka, N.; Hosoya, K. *Analytical and Bioanalytical Chemistry*, Paper in Forefront, **2004**, *378*, 84–88.

[21] Hosoya, K.; Watabe, Y.; Ikegami, T.; Tanaka, N.; Kubo, T.; Sano, T.; Kaya, K. *Synthetic receptors section of Biosensors and Bioelectronics.* **2004**, *20–6*, 1185–1189.

[22] Kubo, T.; Hosoya, K.; Watabe, Y.; Ikegami, T.; Tanaka, N.; Sano, T.; Kaya, K. *J. Chromatogr. A* **2004**, *1029*, 37–41.

[23] Kubo, T.; Hosoya, K.; Watabe, Y.;Tanaka, N.;Takagi, H.; Sano, T.;Kaya, K. *J. Chromatogr. B.* **2004**, *806*, 229–235.

[24] Nomachi, M.; Kubo, T.; Hosoya, K.; Kaya, K. *Anal. Bioanal. Chem.* **2006**, *384*, 1291–1296.

[25] Kubo, T.; Nomachi, M.; Nemoto, K.; Sano, T.; Hosoya, K.; Tanaka, N.; Kaya, K. *Anal. Chemica Acta* **2006**, *577*, 1–7.

[26] Nemoto, K.; Kubo, T.; Nomachi, M.; Sano, T.; Matsumoto, T.; Hosoya, K.; Hattori, T.; Kaya, K. *J. Am. Chem. Soc.* **2007**, *129*, 13626–13632.

[27] Kubo, T.; Tominaga, Y.; Watanabe, F.; Kaya, K.; Hosoya, K. *Anal. Sci.* **2008**, *24*, 1633–1636.

[28] Kubo, T.; Kaya, K.; Hosoya, K.; Tominaga, Y. *Macromolecules* **2009**, *42*, 2911–2915.

[29] Kubo, T.; Hosoya, K.; Watabe, Y.; Ikegami, T.; Tanaka, N.; Sano, T.; Kaya, K. *J. Chromatogr. A* **2003**, *987*, 389–394.

[30] Watabe, T.; Kondo, T.; Imai, H.; Morita, M.; Tanaka, N.; Kubo, T.; Hosoya, K. *Anal. Chem.* **2004**, *76*, 105–109.

[31] Watabe, Y.; Kondo, T.; Imai, H.; Morita, M.; Tanaka, N.; Haginaka, J.; Hosoya, K. *Anal. Sci.* **2004**, *20*, 133–137.

[32] Watabe, Y.; Kondo, T.; Morita, M.; Tanaka, N.; Haginaka, J.; Hosoya, K. *J. Chromatogr. A* **2004**, *1032,* 45–49.

[33] Sambe, H.; Hoshina, K.; Hosoya, K.; Haginaka, J. *Analyst* **2005**, *130*, 38–40.

[34] Kubo, T.; Hosoya, K.; Sano, T.; Tanaka, N.; Kaya, K. *Anal. Chim. Acta* **2005**, *549*, 45–50.

[35] Hosoya, K.; Sawada, E.; Kimata, K.; Araki, T.; Tanaka, N.; Fréchet, J. M. J. *Macromolecules* **1994**, *27*, 3973–3976.

[36] Yoshizako, K.; Hosoya, K.; Kimata, K.; Araki, T.; Tanaka, N. *J. Polym. Sci. Part A: Polym. Chem.* **1997**, *35*, 2747–2757.

[37] Hosoya, K.; Yoshizako, K.; Kubo, T.; Ikegami, T.; Tanaka, N.; Haginaka, J. *Anal. Sci.* **2002**, *18*, 55–58.

[38] Hosoya, K.; Watabe, Y.; Kubo, T.; Hoshino, N.; Tanaka, N.; Sano, T.; Kaya, K. *J. Chromatogr. A* **2004**, *1030*, 237–246.

[39] Watabe, Y.; Hosoya, K.; Tanaka, N.; Kubo, T.; Kondo, T.; Morita, M. *Chem. Lett.* **2004**, *33–7*, 806–807.

[40] Watabe, Y.; Hosoya, K.; Tanaka, N.; Kubo, T.; Kondo, T.; Morita, M. *J. Chromatogr. A* **2005**, *1073*, 363–370.

[41] Watabe, Y.; Hosoya, K.; Tanaka, N.; Kubo, T.; Kondo, T.; Morita, M. *J. Polym. Sci., Part A, Polymer Chemistry* **2005**, *43*, 2048–2060.

[42] Watabe, Y.; Hosoya, K.; Tanaka, N.; Kondo, T.; Morita, M.; Kubo, T. *Anal. Bioanal. Chem.* **2005**, *381*, 1193–1198.

[43] Watabe, Y.; Fujita, T.; Kubo, T.; Kaya, K.; Nishikawa, T.; Hosoya, K. *J. Chromatogr. A* **2006**, *1120*, 252–259.

[44] Ogura, T.; Watabe, Y.; Fujita, T.; Kubo, T.; Hosoya, K.; Kaya, K. BUNSEKI KAGAKU, **2009**, *58*, 295–299.

[45] Frechet, J. M. J.; Svec, F. (Cornell Research Foundation Inc.). U. S. Patent 5, 344, 310, Aug. 2, **1994**.

[46] Svec, F. *J. Sep. Sci.* **2004**, *27*, 747–766.

[47] de Gennes, PG. In Scaling Concept of Polymer Physics, Cornell Univ. Press, Ithaca, New York, **1979**; Chapter 4, pp. 113–115.

[48] Flory, P. J. In Polymer Chemistry, Cornell Univ. Press, Ithaca, New York, **1953**; Chapter 17, pp. 509.

[49] Macintyre, FS.; Sherrington, DC. Macromolecules **2004**, *37*, 7628–7638.

[50] Aoki, H.; Kubo, T.; Watabe, Y.; Tanaka, N.; Norisuye, T.; Hosoya, K.; Shimbo, K. *Chem. Lett.* **2004**, *33*, 1134–1135.

[51] Aoki, H.; Hosoya, K.; Norisuye, T.; Tanaka, N.; Tokuda, D.; Ishizuka, N.; Nakanishi, K. *J. Polym. Sci, Part A, Polymer Chemistry* **2006**, *44*, 949–958.

[52] Aoki, H.; Kubo, T.; Ikegami, T.; Tanaka, N.; Hosoya, K.; Tokuda, D.; Ishizuka, N. *J. Chromatogr. A* **2006**, *1119*, 66–79.

[53] Aoki, H.; Tanaka, N.; Kubo, T.; Hosoya, K. *J. Polym. Sci., Part A, Polymer Chemistry* **2008**, *46*, 4651–4673.

[54] Aoki, H.;Tanaka, N.; Kubo, T.; Hosoya, K. *J. Sep. Sci.* **2009**, *32*, 341–358.

[55] Tsujioka, N.; Hira, N.; Aoki, S.; Tanaka, N.; Hosoya, K. *Macromolecules* **2005**, *38*, 9901–9903.

[56] Tsujioka, N.; Ishizuka, N.; Tanaka, N.; Kubo, T.; Hosoya, K. *J. Polym. Sci., Part A, Polymer Chemistry* **2008**, *46*, 3272–3281.

[57] Kubo, T.; Tominaga, Y.; Yasuda, K.; Fujii, S.; Watanabe, F.; Mori, T.; Kakudo, Y Hosoya, K. *Anal. Methods* **2010**, *2*, 570–574.

[58] Hosoya, K.; Hira, N.; Yamamoto, K.; Nishimura, M.; Tanaka, N. *Anal. Chem.* **2006**, *78*, 5729–5735.

[59] Hosoya, K.; Sakamoto, M.; Akai, K.; Mori, T.; Kubo, T.; Kaya, K.; Okada, K.; Tsujioka, N.; Tanaka, N. *Anal. Sci.* **2008**, *24*, 149–154.

[60] Kubo, T.; Watanabe, F.; Kaya, K.; Hosoya, K. *Chem. Lett.* **2008**, *37*, 950–951.

[61] Watanabe, F.; Kubo, T.; Kaya, K.; Hosoya, K. *J. Chromatogr. A* **2009**, *1216*, 7402–7408.

[62] Tanigawa, T.; Kato, K.; Watabe, Y.; Kubo, T.; Hosoya, K. *J. Sep. Sci.* **2011**, *34*, 2193–2198.

[63] Tanigawa, T.; Watabe, Y.; Kubo, T.; Hosoya, K. *J. Separation Sci.* **2011**, *34*, 2840–2846.

[64] Tanigawa, T.; Kubo, T.; Hosoya, K. *Chem. Lett.* **2012**, *41*, pp. 1265–1266.

[65] Takahashi, T.; Shiyama, T.; Hosoya, K.; Tanaka, A. *Bioorganic & Medicinal Chem. Lett.* **2005**, *16*, 447–450.

[66] Takahashi, T.; Shiyama, T.; Mori, T.; Hosoya, K.; Tanaka, A. *Anal. Bioanal. Chem.* **2006**, *385*, 122–127.

[67] Mori, T.; Takahashi, T.; Shiyama, T.; Tanaka, A.; Hira, N.; Tanaka, N.; Hosoya, K. *Bioorganic and Medicinal Chemistry* **2006**, *14*, 5549–5554.

[68] Mori, T.; Tanaka, A.; Kubo, T.; Kaya, K.; Sakamoto, M.; Hosoya, K. *Bioorg. Med. Chem.* **2008**, *16*, 1983–1991.

[69] Mori, T.; Kubo, T.; Kaya, K.; Hosoya, K. *Colloid and Polym. Sci.* **2009**, *287(5)*, 513–523.

[70] Mori, T.; Kubo, T.; Kaya, K.; Hosoya, K. *Bioorg. Med. Chem.* **2009**, *17*, 1587–1599.

[71] Iwaoka, E.; Mori, T.; Shimizu, T.; Hosoya, K.; Tanaka, A. *Bioorg. Med. Chem, tters* **2009**, *19*, 1469–1472.

[72] Mori, T.; Kubo, T.; Hosoya, K. *Colloids Surf. B* **2011**, *84*, 181–186.

[73] Mori, T.; Kubo, T.; Kaya, K.; Hosoya, K. *Anal. Bioanal. Chem.* **2012**, *402,* 1137–1147.