A Simple Synthetic Route for the Preparation of a Reversed-Phase Stationary Phase Based on Monosized-Porous Hydrogel Beads and Its Chromatographic Use for Separation of Small Molecules

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Summary. New polyacrylate-based monosized-porous polymer beads were proposed as a stationary phase for the separation of polar compounds by microbore reversed-phase chromatography. For this purpose, monosized-porous poly(glycerol dimethacrylate-co-glycerol-1,3-diglycerolate diacrylate), poly(GDMA-co-GDGDA), beads with hydroxyl functionality were synthesized by a modified seeded polymerization. The selected octadecylating agent, stearoyl chloride (SC), was covalently attached onto the hydrophilic beads via a direct, single stage reaction with a simple synthetic route. SC attached-poly(GDMA-co-GDGDA) beads were slurry-packed into the microbore columns and used as separation medium microbore reversed-phase chromatography. The stationary phase was used for separation of alkylbenzenes and polar analytes by micro reversed-phase chromatography, using mobile phases with low acetonitrile content. Theoretical plate number (TPN) values up to 12,000 plates m^{-1} and 10,000 plates m^{-1} for alkylbenzenes and polar analytes, respectively, were achieved. The results also showed that poly(GDMA-co-GDGDA) hydrogel beads are a promising starting material for a number of chromatographic applications like reversed-phase (RP) chromatography, hydrophilic interaction chromatography (HILIC), ion-exchange chromatography (IEC), and affinity chromatography with a single-stage surface modification.

Key Words: reversed-phase chromatography, stearoyl chloride, hydrogel beads, microbore columns, polar analytes

Introduction

Octadecyl functionalized silica-based particles have been commonly used as separation medium in reversed-phase chromatography. Although silica-based stationary phases have been used so much in reversed-phase liquid chromatography applications, it has some disadvantages. While dissolution of silica takes place when the pH is above 7.5, cleavage of siloxane linkages

occurs when the pH is lower than 2 [1]. The insufficient mechanical and chemical stability is known as the major disadvantage of silica-based separation media. For this reason, polymer-based separation media with hydrophilic character are a significant alternative for the reversed-phase chromatography. A polymer silica hybrid particle-based stationary phase was developed by using an electrostatically driven self-assembly process for reversed-phase and ion-exchange separation mode [2]. A phospholipid-modified reversed-phase amide column was obtained by the dynamic coating method for the determination of lipophilic properties in high-performance liquid chromatography (HPLC) [3]. Porous polymer particle-based stationary phases were prepared by click chemistry and used for separation of peptides and proteins by reversed-phase chromatography [4]. Poly(dihydroxypropyl methacrylate-co-ethylene dimethacrylate), poly-(DHPM-co-EDM), particles obtained by the acidic hydrolysis of poly(GMA-co-EDMA) particles were successfully used as column-packing material in the reversed-phase separation of alkylbenzenes [5]. Monodisperse poly(styrene-co-divinylbenzene) particles with relatively small pore size were used for separation of alkyl benzenes [6]. Similar particles were also used as stationary phase for the reversed-phase separation of proteins with high-resolution and high-column efficiency [7]. Bis[4-(2-hydroxy-3-methacryloyloxypropoxy) phenyl] sulfide and divinylbenzene porous microspheres were synthesized as highly crosslinked copolymer stationary phase for the reversed-phase HPLC [8].

In reversed-phase chromatography, monolith-based stationary phases have also been developed in recent years [9–12]. Narrow-bore monolithic silica columns [13] and methacrylate-based organic polymer monolithic columns [14] were introduced to the literature as stationary phases for HPLC in early 1990's. Organo-silica-based hybrid monolithic columns with different functional groups such as methyl [15] octyl [16] and chloropropyl [17] have also drawn attention in liquid chromatography. Monolithic columns have some advantages like easy preparation, high efficiency, good permeability, and little solvent consumption compared with conventional particle based columns. Silica monoliths, which have relatively high surface area, are used for separation of small molecules [18] while organic polymer monoliths are used for separation of large molecules such as protein [19], nucleic acids [20], and peptides [21]. Different studies were conducted with organic polymer monoliths. The effects of synthesis conditions on the chromatographic performance of polyacrylate-based capillary monoliths with different functional groups were studied [22]. Hydrophobicity of monoliths was compared by working methacrylate ester monomers with

different alkyl chains, and it was concluded that C18-methacrylate mono-liths exhibited the most promising performance in chromatographic separation [23]. All monolithic columns have long-term mechanical stability problems, even though silica and organo-silica monoliths have solved it partially. Since polymeric stationary phases can be used in the pH range of 1–13, they have been still developed as an alternative for monolithic and silica-based stationary phases.

In the present study, a highly hydrophilic polyacrylate-based structure, newly synthesized monodisperse macroporous beads obtained by the copolymerization of glycerol dimethacrylate (GDMA) and glycerol-1,3-diglycerolate diacrylate (GDGDA) were used as starting material for the synthesis of a stationary medium for microbore reversed-phase chromatography. The beads were octadecylated by reacting with stearoyl chloride using their hydroxyl functionality. Octadecylated-poly(GDMA-co-GDGDA) beads were slurry-packed into microbore columns 1.5 and 2.0 mm i.d. The separation of small molecules was investigated in microbore reversed-phase chromatography mode.

Experimental

Materials

The monomers, glycidyl methacrylate (GMA), glycerol dimethacrylate (GDMA, 85%), and glycerol-1,3-diglycerolate diacrylate (GDGDA) were supplied by Aldrich Chemical, USA, and used without further purification. Cyclohexanol (Cyc-OH), dibutyl phthalate (DBP), benzoyl peroxide (BPO), ethanol (Et-OH, HPLC grade), tetrahydrofuran (THF, HPLC grade), and isoamylalcohol (IAm-OH) were supplied by Aldrich Chemical, USA. Sodium dodecyl sulfate (SDS), polyvinyl alcohol (PVA, 87% hydrolyzed), and polyvinylpyrrolidone K-30 (PVP K-30) were obtained from Sigma Chemical Co., USA. 2,2′-Azobisizobutyronitrile (AIBN) was obtained from Merck A.G., Darmstadt, Germany. Functionalization agent stearoyl chloride (SC) was supplied by Aldrich Chemical, USA, and used without further purification. Acetonitrile (ACN) (99.9%, Sigma-Aldrich) and methanol (99.8% Riedel de Haen, Germany) were used as supplied. The alkylbenzenes and polar analytes were supplied by Aldrich and used without purification in the chromatographic runs.

Preparation of Seed Latex

The poly(glycidyl metacrylate) seed latex was obtained by dispersion polymerization of GMA as 2.1 µm microspheres [24]. Typically, GMA (3 mL) and AIBN (0.24 g) were added into the organic phase obtained in sealed pyrex glass reactor by dissolving PVP K-30 (0.45 g) in Et-OH (30 mL). In order to ensure complete dissolution of AIBN, the medium was ultrasonicated at room temperature for 5 min (Bransonic, UK). The reactor was heated to 70 °C in a temperature-controlled shaking water-bath (Gallenkamp, USA), and the medium was purged with nitrogen for 3 min. Polymerization was carried out at 70 °C for 24 h with a shaking rate of 120 cpm. By using distilled deionized water, the resulting seed latex was cleaned by a successive centrifugation-decanting procedure. The seed particles were then suspended in distilled deionized water. Monodisperse poly(glycidyl methacrylate) seed latex (2.1 µm in size) was obtained by dispersion polymerization.

Synthesis of Monodisperse Poly(GDMA-co-GDGDA) Particles

Multistage swelling and polymerization technique was used for the synthesis of poly(GDMA-co-GDGDA) [25]. In the first stage, following the preparation of aqueous solution (60 mL) containing SDS (0.15 g) and PVA (0.8 g), the organic phase containing DBP (1.8 mL) and Cyc-OH (3.1 mL) was emulsified in the aqueous medium by sonication for 20 min. Then, the seed latex, 2.1 µm in size (0.2 g), was added into the resulting emulsion, and the medium was magnetically stirred in room temperature for 24 h. In the next stage, the monomer phase containing GDMA (2.6 mL), GDGDA (2.6 mL), isoamyl alcohol (IAA) (0.25 mL), and initiator BPO (0.12 g) was emulsified in the aqueous medium (60 mL), containing SLS (0.15 g). The resulting emulsion was added into the previous emulsion, and the medium was stirred magnetically for 24 h. Thus, the seed latex was swollen by the organic phase containing monomers, porogen, and initiator. In the last stage, the polymerization was conducted at 80 °C at 120 cpm shaking rate for 24 h. The monodisperse porous poly(GDMA-co-GDGDA) particles, 5.5 µm in size, were obtained as polymerization product. The resulting monodisperse particles were then washed in water, ethanol, and tetrahydrofuran (THF) in succession by means of the centrifugation-decanting procedure. Finally, the resulting particles were then suspended in distilled deionized water.

Functionalization of Monodisperse Poly(GDMA-co-GDGDA) Particles

The particles (0.5 g) suspended in water were washed in ethanol and methylene chloride before being treated with stearoyl chloride. Stearoyl chloride (0.5 g), methylene chloride (9 mL), and pyridine (1 mL) were mixed in a bottle, and the resulting solution was sonicated for 1 min. When the homogenous emulsion was obtained, poly(GDMA-co-GDGDA) particles (0.5 g) were added into it. Then, particles were treated with stearoyl chloride solution at room temperature for 24 h. After functionalization, the resulting solution was washed with ethanol and distilled deionized water. Then, poly(GDMA-co-GDGDA) particles carrying octadecyl groups were obtained.

Characterization of Monodisperse Poly(GDMA-co-GDGDA) Particles

The average particle size (Da) and the coefficient of variation (CV) for size distribution of poly(GDMA-co-GDGDA) particles were determined by scanning electron microscopy (SEM) (Zeiss, Evo-50, Germany). The surface morphology of particles was also examined by scanning electron microscopy with higher magnification. The porous properties, specific surface area (SSA), and pore volume (Vp) were determined using a Brunauer–Emmet–Teller apparatus (Quantachrome, Nova, 2200E, UK).

Chromatographic Study

Poly(GDMA-co-GDGDA) particles were dispersed in water for the packing process. The dispersion containing approximately 20% *w/w* of particles was used. The particles were slurry-packed into 300 mm × 2.0 mm i.d. and 300 mm × 1.5 mm i.d. stainless steel microbore HPLC columns, under 25 MPa pressure [26]. Reversed-phase chromatographic studies were carried out by using a micro-liquid chromatography system (Dionex, Ultimate 3000, USA) with a ternary gradient pump and a diode array detector (DAD). The chromatographic separations of alkyl benzenes (toluene, benzene, ethylbenzene, propylbenzene, butylbenzene, pentylbenzene) and the polar analytes (caffeine, uracil, aniline, anisole, and toluene as a reference analyte) were conducted under isocratic conditions, using mobile phases containing ACN

and water at room temperature. For both analyte groups, first, the ACN–water volume ratio of the mobile phase was optimized, and then, the effect of the mobile phase flow rate on the chromatographic separation was investigated. In the chromatographic run, the analyte mixture was injected automatically via a closed loop with a volume of 1 μL. The isocratic mode separations were performed by a DAD operated at 254 nm for the alkylbenzenes and polar analytes. Retention factor, peak resolution, and theoretical plate number were evaluated as chromatographic parameters [27].

Results and Discussion

Characterization of Poly(GDMA-co-GDGDA) Particles

Highly hydrophilic polyacrylate-based monodisperse macroporous beads synthesized by the copolymerization of glycerol dimethacrylate (GDMA) and glycerol-1,3-diglycerolate diacrylate (GDGDA) were selected as starting material for the synthesis of chromatographic stationary phase for microbore reversed-phase chromatography. Both monomers, GDMA and GDGDA, also acting as cross-linking agents contain hydroxyl groups that can be easily tailored. The octadecylated stationary phase was easily obtained using the reaction between stearoyl chloride and hydroxyl groups of the poly(GDMA-co-GDGDA) beads. The reaction taking place between
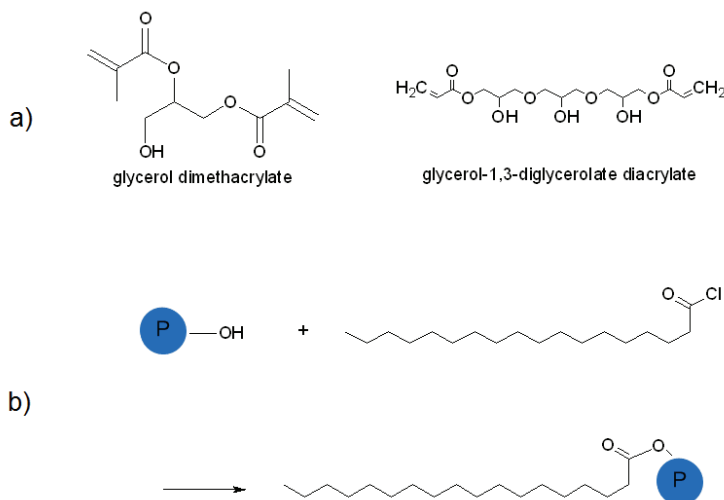

Fig. 1. The chemical structure of (a) monomer and crosslinking agent (b) reaction route for the attachment of stearoyl chloride onto the poly(GDMA-co-GDGDA) particles

poly(GDMA-co-GDGDA) particles and stearoyl chloride is given in *Fig. 1.* Scanning electron microscope (SEM) photographs of poly(GDMA-co-GDGDA) beads giving an idea about the size distribution and surface morphology are given in *Fig. 2.* As seen here, nearly monodisperse porous particles were obtained by the seeded copolymerization of GDMA and GDGDA. The average particle size and the coefficient of variation were determined as 5.5 µm and 5.5%, respectively, using the SEM photo in *Fig. 2A* and *2B.* On the other hand, the surface of poly(GDMA-co-GDGDA) particles had a sponge-like pore structure containing homogeneously distributed small macropores. The porous properties of poly(GDMA-co-GDGDA) beads were determined by inverse size-exclusion chromatography (ISEC) [25]. By using the pore-size distribution curve of poly(GDMA-co-GDGDA) beads, the results showed that the pore size lies in the range of 2–120 nm for when seed latex monomer ratio was 0.038 g mL^{-1} [25]. The variation of column back-pressure with the flow rate change is given in *Fig. 3.* Here, ACN–water solution containing 20% *v/v* ACN was used as mobile phase. The backpressure linearly increased with the increasing flow rate for the microbore columns. The upper limit allowed in micro-HPLC system studied was 30 MPa. The pressure values obtained in the studied range of the flow rate showed that poly(GDMA-co-GDGDA) beads synthesized were suitable for microbore reversed chromatography.
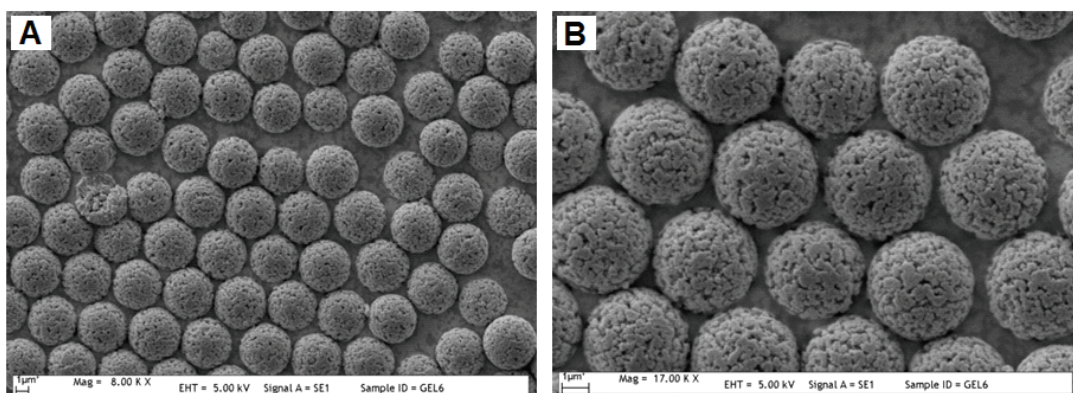

Fig. 2. The SEM images of stearoyl chloride attached-poly(GDMA-co-GDGDA) particles. Magnification: (A) 8000×, (B) 17,000×
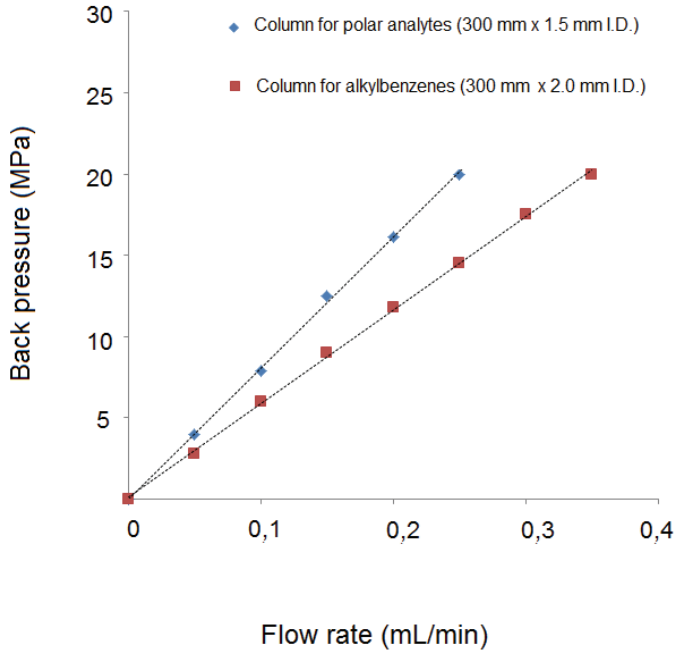
Fig. 3. The variation of back-pressure with flow rate in the column packed with poly(GDMA-co-GDGDA) particles. Column: 300 × 2.0 mm i.d., mobile phase: ACN–water mixture containing 20% *v/v* ACN

Chromatography

Reversed phase is a successful mode for a wide range of hydrophilic, hydrophobic, and ionizable compounds; on the other hand, poor retention occurs with unsuccessful peaks for separation of polar molecules [28, 29]. Polar compounds are generally separated with normal-phase liquid chromatography or hydrophilic interaction chromatography (HILIC) mode. By the synthesized poly(GDMA-co-GDGDA) stationary phase, separation of polar and nonpolar compound in the same HPLC columns were aimed for separating of small molecules. Two analyte groups were selected as test compounds to be able to test chromatographic performance of the columns. For nonpolar test compounds, alkyl benzenes (toluene, benzene, ethylbenzene, propylbenzene, butylbenzene, pentylbenzene) were used. Alyklbenzenes which do not ionize in mobile phase are easily available compounds and typical homolog series for evaluating of the chromatographic performance of the stationary phases in reversed-phase liquid chromatography [30]. Caffeine, uracil, anilin, anisole with decreasing polarity, and toluene as the ref-

erence analyte were chosen as second analyte group as relatively polar test compounds.

   Microbore columns offer minimal loss of performance compared to the conventional HPLC columns. The requirement for less solvent and the reduction of total analysis time make microbore columns more attractive. Thus, the reversed-phase chromatography in our case was performed using microbore columns 1.5 and 2.0 mm i.d. For the isocratic separations of alkylbenzenes, the liquid chromatograms obtained with different mobile phase ACN concentrations using the column (300 × 2.0 mm i.d.) packed with poly(GDMA-co-GDGDA) particles are given in *Fig. 4*. The resolution values calculated from the chromatograms obtained with different ACN concentrations are given in *Table I*. The highest resolution values were obtained in the case where the lowest mobile phase ACN concentration was used. As the ACN concentration increased, the resolutions decreased and the total analysis time was shortened. By considering the resolution values in *Table I* and the total analysis times obtained, 35% *v/v* ACN concentration was selected as the appropriate value for testing the effect of mobile phase flow rate on the separation behavior of the microbore column packed with the poly(GDMA-co-GDGDA) beads.
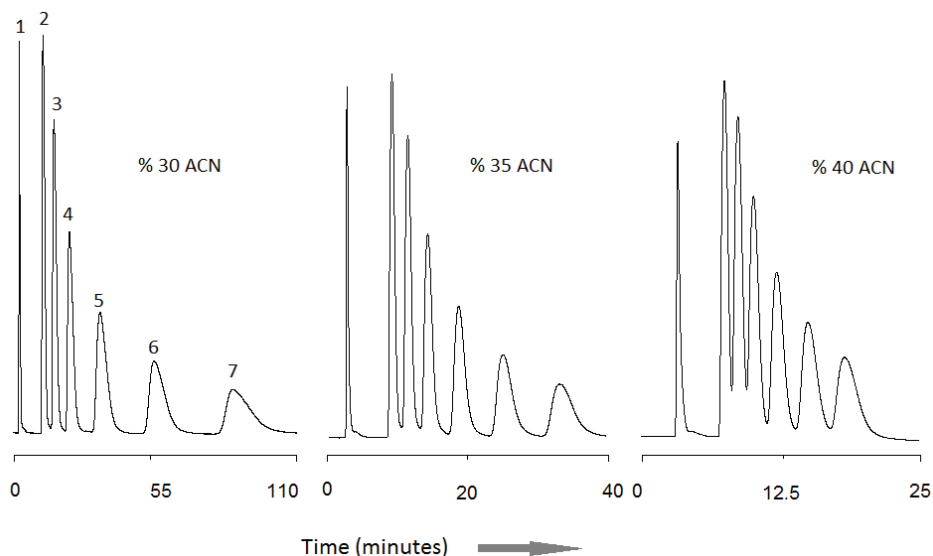

Fig. 4. The liquid chromatograms obtained with different ACN concentrations in the mobile phase using the column packed with stearoyl chloride attached-poly(GDMA-co-GDGDA) particles. Column: 300 × 2 mm i.d., flow rate: 0.2 mL min^{-1}, DAD at 254 nm, order of elution: 1. thiourea, 2. toluene, 3. benzene, 4. ethylbenzene, 5. propylbenzene, 6. butylbenzene, 7. pentylbenzene

Table I. Effect of ACN concentration on peak resolution in the separation of alkylbenzenes

Poly(GDMA-co-GDGDA)	Peak resolutions					
ACN % (*v/v*)	R (2/1)	R (3/2)	R (4/3)	R (5/4)	R (6/5)	R (7/6)
30	7.13	2.14	2.23	2.30	2.31	2.29
35	8.00	1.65	1.77	1.89	2.00	1.78
40	5.68	0.97	1.20	1.22	1.19	1.13

Column: 300×2 mm i.d., flow rate: 0.2 mL min^{-1}, DAD at 254 nm, order of elution: 1. thiourea, 2. toluene, 3. benzene, 4. ethylbenzene, 5. propylbenzene, 6. butylbenzene, 7. pentylbenzene.

The variation of the logarithm of retention factor with the number of alkyl carbon atoms of alkylbenzenes is given in *Fig. 5*, using the chromatograms obtained with different ACN concentrations. The retention factor increased linearly with increasing number of alkyl-carbon atom. The slope of the graph is an important parameter for the evaluation of polarity and separation behavior of the column [6]. The slopes between 0.10 and 0.19 were obtained with different ACN concentrations, and the slope increased with increasing ACN concentration. The results are in accordance with the
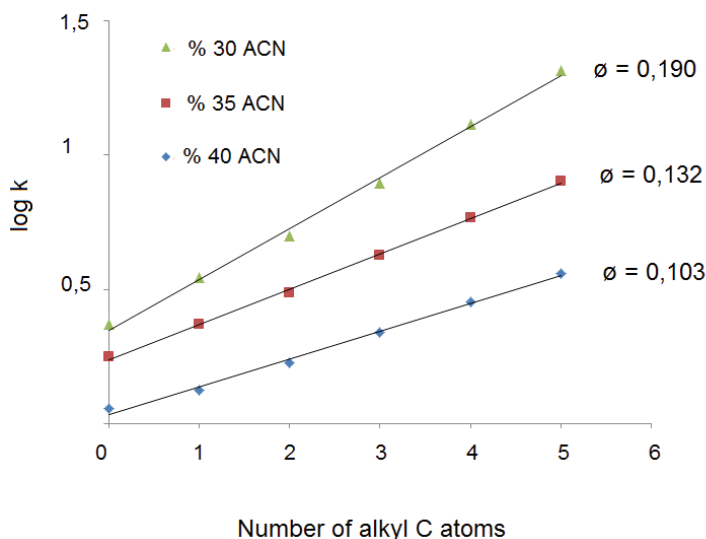

Fig. 5. The variation of logarithm of retention factor by the number of alkyl carbon atom (benzene to pentylbenzene) for the mobile phases prepared with different ACN concentrations. Separation conditions are the same as *Fig. 4*
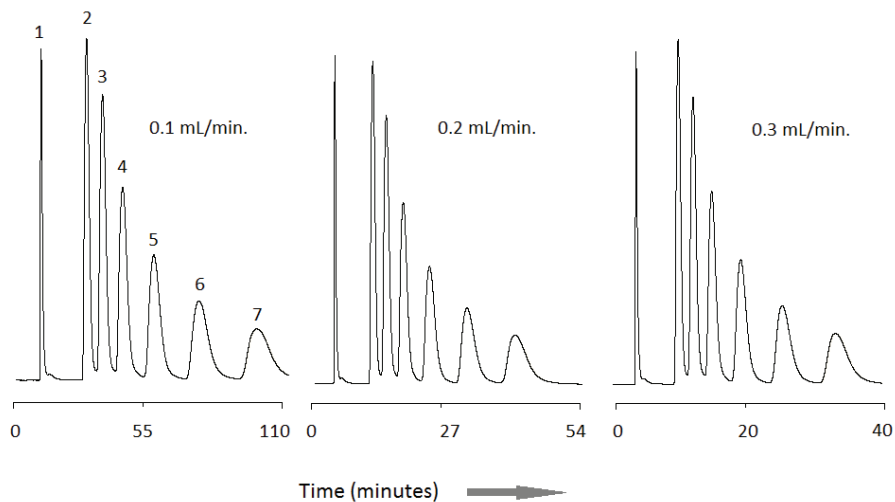
Fig. 6. The liquid chromatogram of alkylbenzenes obtained with different mobile phase flow rates for the column packed with stearoyl chloride attached-poly(GDMA-co-GDGDA) particles. Column: 300 × 2 mm i.d., ACN concentration: % 35 ACN, DAD at 254 nm, order of elution: 1. thiourea, 2. toluene, 3. benzene, 4. ethylbenzene, 5. propylbenzene, 6. butylbenzene, 7. pentylbenzene
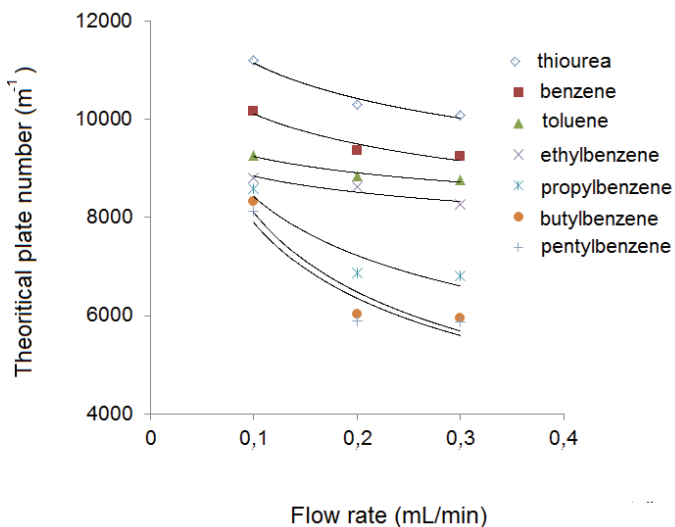

Fig. 7. The effect of mobile phase flow rate on the theoretical plate number for the separation of alkylbenzenes for the column packed with stearoyl chloride attached-poly(GDMA-co-GDGDA) particles. Separation conditions are the same as *Fig.* 6

methylene selectivity range of silica-based and polymer-based reversed-phase columns [31–33]. The results indicated that separation power of poly(GDMA-co-GDGDA) column increased with decreasing ACN concentration in the mobile phase.

The liquid chromatograms of alkylbenzenes obtained with different mobile phase flow rates are shown in *Fig. 6*. The resolution values obtained based on these chromatograms are given in *Table II*. As seen both in *Fig. 6* and *Table II*, satisfactory baseline separation was achieved with all flow rates. The variation of theoretical plate number (TPN) with the mobile phase flow rate is shown in *Fig. 7*. As seen here, the TPN values up to the 12,000 plates m^{-1} were achieved using thiourea as the unretained compound. As expected, TPN moderately decreased with increasing flow rate and decreasing polarity of the analytes.

Table II. Effect of flow rate on peak resolution in the separation of alkylbenzenes

Poly(GDMA-co-GDGDA)	Peak resolutions					
Flow rate (mL min^{-1})	R (2/1)	R (3/2)	R (4/3)	R (5/4)	R (6/5)	R (7/6)
0.1	8.00	1.80	1.76	1.85	2.00	1.79
0.2	7.83	1.74	1.73	1.88	1.92	1.73
0.3	7.54	1.73	1.60	1.83	1.79	1.66

Column: 300 × 2 mm i.d., ACN concentration: % 35 ACN, DAD at 254 nm, order of elution: 1. thiourea, 2. toluene, 3. benzene, 4. ethylbenzene, 5. propylbenzene, 6. butylbenzene, 7. pentylbenzene.

On the other hand, to use mobile phases with low organic solvent concentration in HPLC is an important challenge to reduce the amount of organic wastes from the analytic studies. All these efforts are placed under the title of "green chromatography." The column packed with stearoyl chloride attached-poly(GDMA-co-GDGDA) beads allowed the separation of relatively polar analytes using mobile phases with low organic solvent content. The isocratic separation of relatively polar analytes (caffeine, uracil, aniline, anisole, and toluene as the reference analyte) is shown in *Fig. 8* for different mobile phase ACN concentrations. The flow rate was 0.2 mL min^{-1} in a 300 × 1.5 mm i.d column. As seen in *Fig. 8*, baseline separation of polar analytes was achieved using mobile phase with reasonably low ACN concentration (i.e., 15% *v/v*). The effect of ACN concentration on the peak resolutions is presented in *Table III*. All resolution values were

found to be over the reference value of 1.50, which is important in terms of successful separations. The highest resolutions were obtained when mobile phase contained minimum ACN concentration.

The liquid chromatograms showing the effect of mobile phase flow rate on the separation of polar analytes are given in *Fig. 9*. The peak resolutions
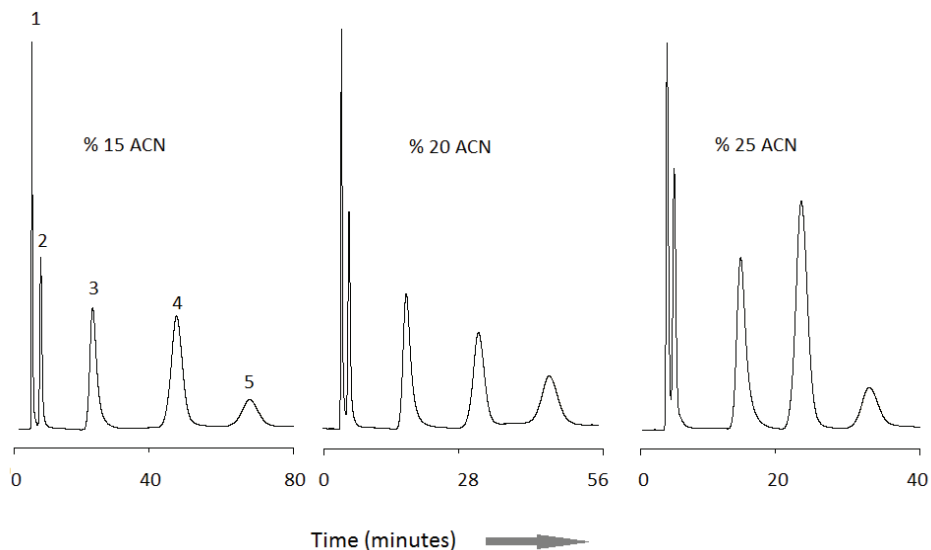

Fig. 8. The liquid chromatograms of polar analytes obtained with different mobile phase ACN concentrations for the column packed with stearoyl chloride-poly(GDMA-co-GDGDA) particles. Column: 300 × 1.5 mm i.d., flow rate: 0.2 mL min^{-1}, DAD at 254 nm, order of elution: 1. caffeine, 2. uracil, 3. aniline, 4. anisole, 5. toluene

Table III. Effect of ACN concentration on peak resolution in the separation of polar analytes

Poly(GDMA-co-GDGDA)	Peak resolutions			
ACN (% *v/v*)	R (2/1)	R (3/2)	R (4/3)	R (5/4)
15	3.96	6.33	4.96	2.34
20	2.05	6.51	4.27	3.53
25	1.50	5.94	3.19	2.46

Column: 300 × 1.5 mm i.d., flow rate: 0.2 mL min^{-1}, DAD at 254 nm, order of elution: 1. caffeine, 2. uracil, 3. aniline, 4. anisole, 5. toluene.
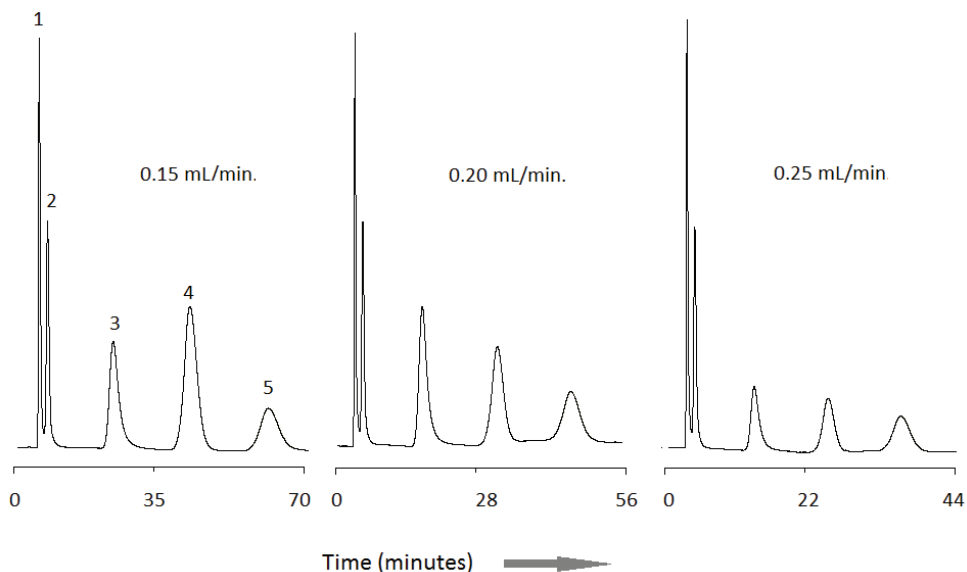
Fig. 9. The liquid chromatograms of polar analytes obtained with different mobile phase flow rates for the column packed with stearoyl chloride attached-poly(GDMA-co-GDGDA) particles. Column: 300 × 1.5 mm i.d., ACN concentration: % 20 ACN, DAD at 254 nm, order of elution: 1. caffeine, 2. uracil, 3. aniline, 4. anisole, 5. toluene
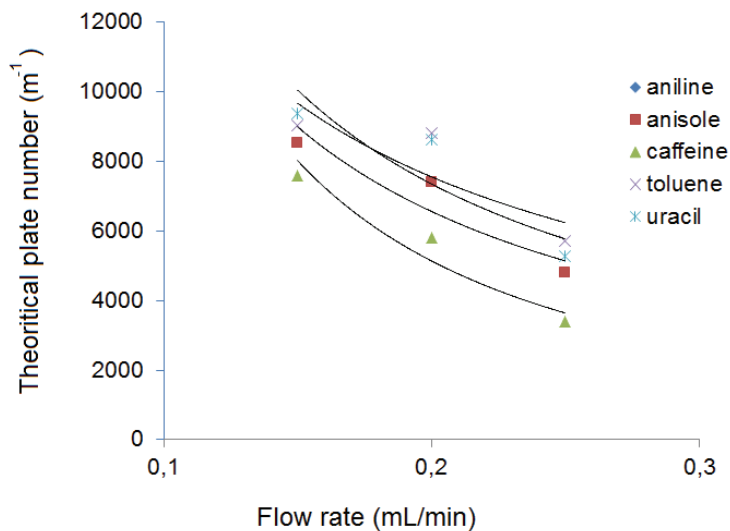

Fig. 10. The effect of mobile phase flow rate on the theoretical plate number for separation of polar analytes for the column packed with stearoyl chloride attached-poly(GDMA-co-GDGDA) particles. Separation conditions are the same as *Fig. 9*

Table IV. Effect of flow rate on peak resolution in the separation of polar analytes

Poly(GDMA-co-GDGDA)	Peak resolutions			
Flow rate (mL min^{-1})	R (2/1)	R (3/2)	R (4/3)	R (5/4)
0.15	2.07	6.12	3.92	2.71
0.20	2.05	5.50	4.46	2.54
0.25	1.89	4.93	3.54	2.27

Column: 300 × 1.5 mm i.d., ACN concentration: % 20 ACN, DAD at 254 nm, order of elution: 1. caffeine, 2. uracil, 3. aniline, 4. anisole, 5. toluene.

calculated for different flow rates are given in *Table IV*. These resolutions show that a successful separation was performed in the flow rate range of 0.15–0.25 mL min^{-1}. The total analysis time decreased from 70 to 40 min with increasing flow rate. The variation of TPN with the flow rate is given in *Fig. 10*. As seen here, TPN values up to 10,000 plates m^{-1} were obtained under the conditions used for the separation of polar analytes. The results showed that the column proposed could be used for the satisfactory separation of relatively polar analytes using mobile phases with low ACN content and by studying in the flow rate range of 0.15–0.25 mL min^{-1}.

Conclusion

A highly hydrophilic monodisperse macroporous beads synthesized by the copolymerization of glycerol dimethacrylate (GDMA) and glycerol-1,3-diglycerolate diacrylate (GDGDA) hydrogel beads were used as starting material for the synthesis of a stationary medium. Octadecylated-poly(GDMA-co-GDGDA) beads were used successfully for separation of small molecules for micro reversed-phase chromatography with less acetonitrile content. TPN values up to 12,000 for alkylbenzenes and 10,000 for polar analytes were achieved. By means of hydroxyl functionality, hydrophilic poly(GDMA-co-GDGDA) can be used as starting material with a single-step surface modification for a number of chromatographic applications like reversed-phase (RP) chromatography, HILIC, ion-exchange chromatography (IEC), and affinity chromatography.

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