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ORIGINAL

A New Stationary Phase for Hydrophilic Interaction Chromatography: Polyacrylate-Based Hydrophilic, Monosized-Porous Beads with Zwitterionic Molecular Brushes

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Abstract Hydrophilic, polyacrylate-based, monosized-porous beads with zwitterionic molecular brushes were synthesized as a new stationary medium for hydrophilic interaction chromatography. Monosized-porous poly(glycerol-1,3-diglycerolate diacrylate-co-glycerol dimethacrylate), poly(GDGDA-co-GDMA), beads 5 μm in size were obtained by a staged-shape template polymerization. As an initiator for surface-initiated atom transfer radical polymerization (SI-ATRP), bromine functionality was obtained on the beads by reacting their hydroxyl groups with 3-(aminopropyl)triethoxy silane and α-bromoisobutyryl bromide, respectively. Zwitterionic molecular brushes on the hydrophilic poly(GDGDA-co-GDMA) beads were generated by SI-ATRP of a sulfobetaine monomer, [2-(methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl) ammonium hydroxide (MESH). Poly(MESH)-grafted poly(GDGDA-co-GDMA), poly(MESH)g-poly(GDGDAco-GDMA), beads were slurry packed into the microbore columns with 2 mm i.d. and evaluated as stationary medium for the separation of organic acids, nucleosides and peptides using microbore columns in hydrophilic interaction chromatography with the plate numbers up to 30,000 plates m^{-1}.

Keywords Hydrophilic interaction chromatography · HPLC · Monodisperse porous particles · Chromatographic packing · Peptides · Ethylene dimethacrylate

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Introduction

In the case of polar compounds, the mobile phases containing organic modifiers at very low concentrations should be used to achieve satisfactory retention and separation in reversed-phase chromatography [1, 2]. However, the use of mobile phases dominantly containing water may lead to the collapse of hydrophobic alkyl (C8 or C18) ligands on the reversed-phase stationary media. The ligand collapse can lead to the poor retention and selectivity for polar analytes in this chromatographic mode [1]. Hydrophilic interaction chromatography (HILIC) is a relatively new chromatographic mode allowing the effective separation of polar compounds on polar stationary phases. For this purpose, HILIC uses mobile phases containing a mixture of water and organic modifier, in most cases acetonitrile with the polar stationary phase. The polar compounds are more strongly retained under these conditions [3]. Hence, HILIC is a more appropriate chromatographic mode for the better separation of polar compounds with respect to the reversed-phase chromatography [4]. HILIC is particularly used for the separation of polar compounds with biological importance such as amino acids, peptides, proteins, nucleotides, nucleosides, carbohydrates, oligosaccharides, etc. [4].

Various monolithic- and particle-based hydrophilic stationary media were developed for HILIC applications. Mostly silica-based materials were preferred for the preparation of HILIC stationary media with different properties. Graft polymerization is one of the most widely used methods for the generation of ligands in the form of molecular brushes. A phosphorylcholine type zwitterionic stationary phase was prepared by graft polymerization of 2-methacryloyloxyethyl phosphorylcholine onto the porous silica particles and successfully evaluated for peptide separation in HILIC [5]. Graft polymerization of sorbitol methacrylate on

the surface of porous silica particles was used for the preparation of a hydrophilic stationary medium for HILIC [6]. Imidazoline-type molecular brushes were generated on the porous silica particles via graft polymerization for the preparation of stationary media for HILIC and reversed-phase chromatography [7]. The mixed sulfated/methacryloyl polysaccharide derivative was covalently attached onto the surface of porous silica beads by polymerization for the preparation of a separation medium for HILIC [8].

Living polymerization techniques, particularly surface-initiated atom transfer radical polymerization (ATRP) and reversible addition fragmentation chain transfer polymerization (RAFT) were preferred for the synthesis of particle-based stationary media carrying hydrophilic ligands in the form of molecular brushes. A separation medium both reversed-phase and HILIC was prepared by the surface-initiated atom transfer radical polymerization of 3-[*N,N*-dimethyl-*N*-(methacryloyloxyethyl) ammonium] propane sulfonate on porous silica particles [9]. Aqueous reversible addition fragmentation chain transfer polymerization (RAFT) was evaluated for grafting of sulfobetaine polymers onto the porous silica beads as a stationary phase in HILIC [10]. Poly(glycidyl methacrylate) grafted porous silica beads were reacted with tris (hydroxymethyl) aminomethane and evaluated as a chromatographic separation medium for nucleic bases, organic acids, and nucleotides under mixed hydrophilic interaction chromatography and weak anion exchange conditions [11]. Poly(vinyltetrazole) chain-grafted poly(glycidymethacrylate-co-ethylenedimethacrylate) beads were also prepared by surface-initiated atom transfer radical polymerization for the use in weak cation exchange and hydrophilic interaction chromatography [12]. Hydrophilic polymer-grafted polystyrene beads were also synthesized via surface-initiated atom transfer radical polymerization as a stationary phase for hydrophilic interaction chromatography [13]. The poly(acrylic acid) grafted beads obtained with surface-initiated atom transfer radical polymerization of acrylic acid were also used as chromatographic packings in the separation of proteins by ion-exchange chromatography [14]. Recently, a more specific route, "thiol-ene" click chemistry was successfully applied for the preparation of a cysteine-bonded zwitterionic HILIC stationary phase [15].

In this study, reactive, monosized-porous poly(glycerol-1,3-diglycerolate diacrylate-co-glycerol dimethacrylate), poly(GDGDA-co-GDMA), beads were obtained by a modified staged-shape template polymerization method [16]. Zwitterionic molecular brushes in the form of poly(2-methacryloxyethyl) sulfobetaine, poly(MESH), were generated via surface-initiated ATRP of respective monomer on the poly(GDGDA-co-GDMA) beads. Monosized-porous poly(MESH)-attached poly(GDGDA-co-GDMA) beads were evaluated as a stationary medium in microbore columns for the separation of polar compounds by HILIC.

Experimental

Materials

Glycerol dimethacrylate (GDMA), and glycerol-1,3-diglycerolate diacrylate (GDGDA) were supplied from Aldrich Chem. Co., USA and used without further purification. Cyclohexanol (Cyc-OH), dibutylphthalate (DBP), ethanol (Et-OH, HPLC grade), tetrahydrofuran (THF, HPLC grade), acetonitrile (AcN, HPLC grade) were also obtained from Aldrich. Glycidyl methacrylate (GMA), poly(vinyl pyrrolidone) (PVP K-30, Mr:40.000) were supplied from Sigma Chemical Co., St. Louis, USA Sodium lauryl sulfate (SLS) and polyvinylalcohol (PVA, 87–89 % hydrolysed, molecular weight: 85,000–1,46,000) were also supplied from Sigma. The initiator, 2,2′-azobisizobutyronitrile (AIBN, Merck A.G. Darmstad, Germany) was recystallized from methanol before use. Benzoyl peroxide was obtained from Merck and dried in vacuo at 30 °C. The sulfobetaine monomer, [2-(methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl) ammonium hydroxide, (MESH), ($H_2C{=}C(CH_3)CO_2CH_2CH_2N(CH_3)_2(CH_2)_3SO_3$, MW: 279.35 Da) was used without further purification. The reagents used for SI-ATRP, 3-(aminopropyl) triethoxysilane (APTES), α-bromoisobutyryl bromide (BrIBuBr), CuBr and 2,2′-bipyridyl (BiPy) were supplied from Aldrich and used as received. The organic acids, *n*-decanoic acid, acetic acid, *p*-toluic acid, benzoic acid, 4-chlorobenzoic acid, the nucleosides, thymidine, uridyne, inosine, guanosine; and the peptides, Met-Leu, Gly-Ser, NCBZ-β-Ala-Val were supplied from Sigma and used as analytes in HILIC runs.

Synthesis and Characterization of Poly(glycerol-1,3-diglycerolate Diacrylate-co-glycerol Dimethacrylate), Poly(GDGDA-co-GDMA) Beads

Poly(glycidyl methacrylate), [poly(GMA)] seed late × 2.1 μm in size was synthesized by dispersion polymerization of GMA (3 mL) in a continuous medium containing ethanol (30 mL), PVP K-30 (0.45 g) and AIBN (0.24 g). The polymerization was performed in a glass-sealed polymerization reactor placed in a temperature-controlled shaking water bath kept at 70 °C for 24 h [17]. Monodisperse porous poly(GDGDA-co-GDMA) beads were obtained by a modified form of two-step swelling and polymerization protocol. Typically, the diluent mixture containing Cyc-OH (3.1 mL) and DBP (1.8 mL) was emulsified in water (60 mL) containing SLS (0.15 g) by ultrasonication (Bandelin, Sonoplus, Germany) for 8 min. The aqueous dispersion containing poly(GMA) seed particles (4 mL, solid content: 0.2 g) was added and the emulsion was stirred with 250 rpm, at room temperature for 24 h. The monomer phase containing GDGDA (2.6 mL), GDMA (2.6 mL) and BPO (0.12 g) was emulsified in water

(60 mL) including SLS (0.15 g) by sonication for 5 min. The monomer emulsion was then mixed with the aqueous dispersion of diluent-swollen seed particles. The resulting emulsion was magnetically stirred at 250 rpm for another 24 h. The aqueous solution (10 mL) of PVA (0.8 g) was added and the polymerization in the swollen seed particles was performed at 80 °C, with 120 cpm shaking rate for 24 h. Monodisperse porous poly(GDGDA-co-GDMA) particles were washed with ethanol several times and extracted with THF. The particles were then extensively washed with ethanol and DDI water and finally dispersed in DDI water.

The size distribution and the surface morphology of poly(GDGDA-co-GDMA) beads were examined by scanning electron microscope (SEM, JEOL, JEM 1200EX, Japan). The porosity properties (i.e., average pore size, pore-size distribution, porosity, pore volume) of beads were determined by inverse-size exclusion chromatography (ISEC) according to the procedure described elsewhere [18]. The specific surface area of the poly(GDGDA-co-GDMA) beads was measured by surface area and pore-size analyzer using nitrogen adsorption–desorption method (Quantachrome, Nova 2200E, UK).

Table 1 Surface derivatization and SI-ATRP conditions for poly(GDGDA-co-GDMA) beads

Ingredients	SI-ATRP1	SI-ATRP2
APTES attachment		
Poly(GDGDA-co-GDMA) beads (g)	1.0	1.0
APTES (mL, mmol)	2.0, 8.6	2.0, 8.6
TEA (mL)	0.1	0.1
Toluene (mL)	30	30
Conditions	120 °C, 4 h, 250 rpm	
BrIBuBr attachment		
Poly(GDGDA-co-GDMA) beads (g)[a]	1.0	1.0
BrIBuBr (mL)	2.0, 16.2	2.0, 16.2
THF (mL)	20	20
Conditions	60 °C, 8 h, 120 cpm	
SI-ATRP		
Poly(GDGDA-co-GDMA) beads (g)[b]	1.0	1.0
MESH (g, mmol)	2.794, 10	2.794, 10
Water (mL)	10	10
BiPy (mmol)	0.2	0.5
CuBr (mmol)	0.5	1.0
Conditions	80 °C, 6 h, 120 cpm	

[a] APTES-attached beads. The mass was given for plain beads

[b] BrIBuBr-attached beads. The mass was given for plain beads

Attachment of ATRP Initiator onto Poly(GDGDA-co-GDMA) Beads

Typically, poly(GDGDA-co-GDMA) beads (1 g) were dried in vacuo at 70 °C for 24 h and dispersed in dry toluene (20 mL). APTES (4 mL) and triethylamine (0.1 mL) were added into the medium. The resulting dispersion was stirred magnetically at 250 rpm for 24 h at 120 °C under reflux. The dispersion was allowed to cool to room temperature and centrifuged at 5000 rpm for 15 min for the isolation of APTES-attached poly(GDGDA-co-GDMA) beads. The beads were washed with toluene, acetone and THF by following a washing protocol including successive centrifugation–decantation stages.

APTES-attached poly(GDGDA-co-GDMA) beads were dispersed in dry THF (20 mL) in a Pyrex glass reactor placed in an ice-bath at 0 °C. BrIBuBr (2 mL) was slowly added into the dispersion. The reactor was sealed under nitrogen blanket and transferred to a temperature-controlled water bath. The reaction was performed at 60 °C for 8 h with a shaking rate of 120 cpm. The resulting beads were extensively washed with THF ethanol and DDI water several times by following successive centrifugation–decantation stages.

Surface-Initiated ATRP of MESH on Poly(GDGDA-co-GDMA) Beads

In a typical synthesis, MESH (10 mmol) was dissolved in a water (2 mL) in a Schlenk tube. A freshly prepared Cu-BPy stock solution including CuBr (0.20 mmol), and BiPy (0.5 mmol) in water (2 mL) was then added. The resulting medium was sonicated for 5 min and degassed with nitrogen for 10 min. An aqueous dispersion (6 mL) of BrIBuBr-attached poly(GDGDA-co-GDMA) beads (1.0 g) was added into the polymerization medium. The reactor was sealed, and the vacuum was applied for 10 min. The polymerization was conducted at 80 °C for 6 h in a shaking water bath. The beads were extensively washed with ethanol, and distilled deionized water and dried in vacuo at 70 °C and subjected to sulfur analysis. The grafting yield (moles of monomer attached to the particles/moles of monomer initially charged, % mol) and the polyionic ligand content of particles after heterogeneous ATRP (mmol MESH/g dry particles) were calculated based on the elemental analysis of poly(MESH)-g-poly(GDGDA-co-GDMA) beads. Two different SI-ATRP formulations (SI-ATRP1 and SI-ATRP2) were applied for the synthesis of poly(MESH)-g-poly(GDGDA-co-GDMA) beads. In these formulations, only the concentration of CuBr-BiPy complex was changed. The SI-ATRP conditions for both formulations are given in Table 1.

Chromatography

A micro-liquid chromatography system (Dionex, Ultimate 3000, USA) containing a ternary gradient pump

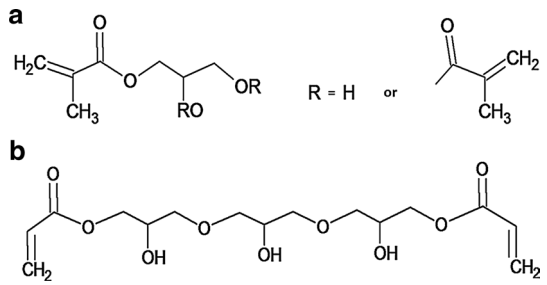

Fig. 1 The molecular structures of GDMA and GDGDA

and a diode array detector was used for HILIC experiments. Poly(MESH)-attached poly(GDGDA-co-GDMA) beads were slurry packed into the stainless steel columns (300 mm in length and 2 mm i.d.) under 25 MPa pressure. HILIC runs were done under isocratic conditions, using mobile phases containing AcN and aqueous buffer with an appropriate volume ratio.

For a certain analyte mixture, first the AcN/water volume ratio of the mobile phase was changed to find its appropriate value for a satisfactory chromatographic separation of the selected analyte mixture. Then, the effect of mobile phase flow rate on the chromatographic separation was investigated using the predetermined value of AcN/water volume ratio. In the HILIC runs, different types of analyte mixtures, nucleosides, organic acids, peptides and acrylic acid–acrylamide mixture were used. After connecting to the micro-liquid chromatography system, the column packed with poly(MESH)-g-poly(GDGDA-co-GDMA) beads was first washed with DDI water at a flow rate of 200 μL min^{-1} for 6 h and conditioned using a mobile phase prepared with a certain AcN/water volume ratio at a flow rate of 100 μL min^{-1} for 1 h. The volumetric flow rate of the same mobile was set to a prescribed value and the analyte mixture was automatically injected via a closed loop with a volume of 0.5 μL. The chromatograms were recorded at isocratic mode, by a DAD detector operated at 260 nm for the nucleosides and at 214 nm for the other analytes. The chromatograms were evaluated by the determination of retention factor, peak resolution and theoretical plate number as described before [17, 18].

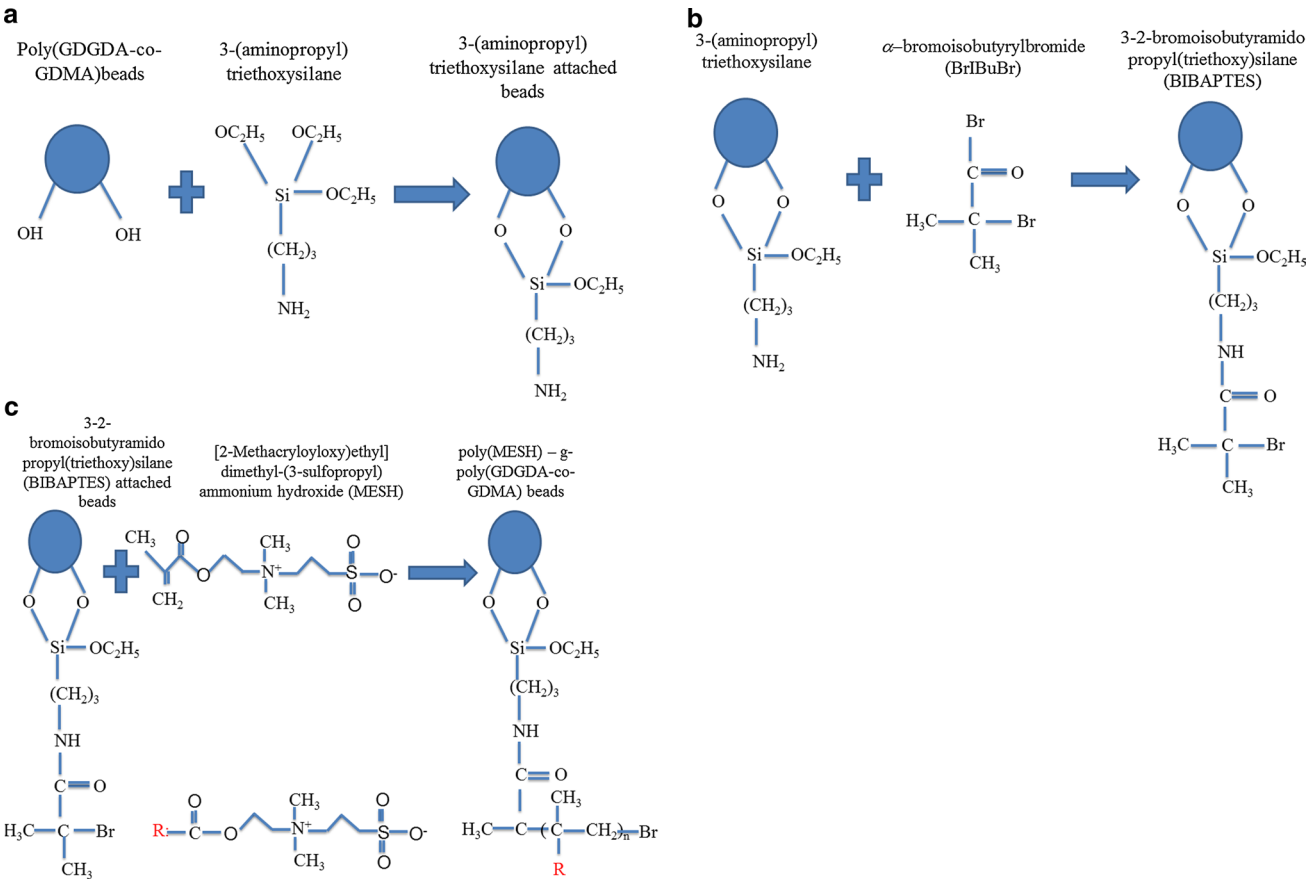

Fig. 2 The chemical route for obtaining zwitterionic molecular brushes on the poly(GDGDA-co-GDMA) beads. **a** The generation of primary amine functionality on the beads, **b** The attachment of ATRP initiator onto the amine functionalized beads, **c** The formation of zwitterionic poly(MESH) brushes on the poly(GDGDA-co-GDMA) beads

Results and Discussion

A new hydrophilic polyacrylate structure, monodisperse porous poly(GDGDA-co-GDMA) beads were selected as starting material for the synthesis of chromatographic support. The molecular structures of GDMA and GDGDA are given in Fig. 1. As seen here, both components of the plain beads, GDGDA and GDMA contain two acrylate and methacrylate groups, respectively. Hence, these monomeric units also act as crosslinking agent. The hydroxyl functionality present in both monomeric units provides both hydrophilicity and also reactivity to the polymeric structure. Hence, hydrophilic beads with highly crosslinked structure were obtained as the product in the seeded microsuspension copolymerization of GDMA and GDGDA. Zwitterionic molecular brushes on the poly(GDGDA-co-GDMA) beads were obtained by the SI-ATRP of MESH according to the chemical route given in Fig. 2. Hydroxyl functionality allows easy derivatization of poly(GDGDA-co-GDMA) beads. Using this advantage, a spacer-arm, APTES was covalently attached onto the plain beads via the reaction between hydroxyl and trietoxysilane groups (Fig. 2a). An ATRP initiator, BrIBuBr was covalently linked onto the APTES-attached beads via the reaction between amino and bromine moieties (Fig. 2b). In the last stage, zwitterionic poly(MESH) molecular brushes were generated on BrIBuBr-attached poly(GDGDA-co-GDMA) beads via SI-ATRP of corresponding monomer in an aqueous medium (Fig. 2c).

Particle Characterization

The SEM photographs showing size distribution and surface morphology of poly(GDGDA-co-GDMA) beads are given in Fig. 3. The properties of plain poly(GDGDA-co-GDMA) and poly(MESH)-g-poly(GDGDA-co-GDMA) beads are given in Table 2. The average size and coefficient of variation for size distribution were calculated as 6.6 μm and 7.9 % with the SEM photograph in Fig. 3a. As seen in Fig. 3b, the particles have a sponge-like, macroporous surface. The specific surface area of poly(GDGDA-co-GDMA) beads determined by nitrogen adsorption–desorption method and using BET equation was 26 m^2 g^{-1} (Table 2). The pore-size distribution curve of poly(GDGDA-co-GDMA) beads obtained by mercury porosimeter is given in Fig. 4. As seen here, the pore size lies between 10 and 50 nm. Two maxima were observed on the pore-size curve at 21 and 34 nm. By the evaluation of pore-size distribution curve in Fig. 4, the mean pore size of poly(GDGDA-co-GDMA) beads was calculated as 32 nm (Table 2).

Two different SI-ATRP formulations were tried for the generation of poly(MESH) molecular brushes on the BrIBuBr-attached poly(GDGDA-co-GDMA) beads (Table 1). As seen in Table 1, the weight ratio of APTES/

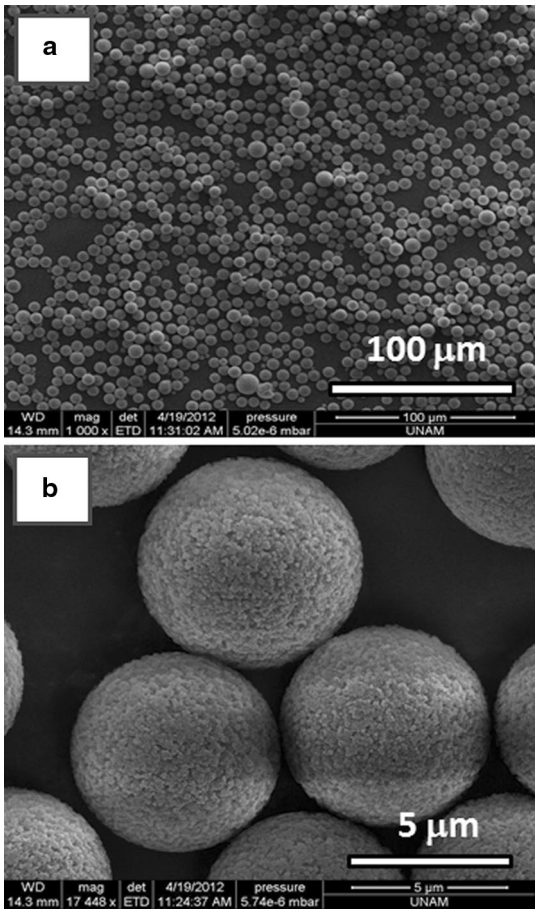

Fig. 3 The SEM photographs showing **a** the average size and size distribution and **b** the surface morphology of poly(GDGDA-co-GDMA) beads. Magnification: 1000X for (**a**) and 17448X for (**b**)

poly(GDGDA-co-GDMA) beads was kept constant for the generation of primary amine groups on the poly(GDGDA-co-GDMA) beads. The concentration of CuBr-BiPy complex was changed by keeping the amount of ATRP initiator attached onto the beads as constant. The amount of poly(MESH) grafted onto the beads is given in Table 2. As seen here, higher amount of MESH was grafted onto the beads by increasing the concentration of CuBr-BiPy complex during SI-ATRP.

HILIC with Poly(MESH)-g-Poly(GDMA-co-GDGDA) Beads

Poly(MESH)-g-poly(GDGDA-co-GDMA) beads were slurry packed into the stainless steel microbore columns with 300 mm length and 2 mm i.d. The variation of column back pressure with the mobile phase flow rate is shown in Fig. 5. As seen here, the back pressure linearly increased with the flow rate for both columns. This behavior showed the suitability of these columns for the micro/semi-micro

Table 2 The properties of plain and poly(MESH)-g-poly(GDGDA-co-GDMA) beads

Properties of poly(GDGDA-co-GDMA) beads		
Average size (μm)	6.6	
Coefficient of variation (%)	7.9	
Median pore size (nm)	32	
Specific surface area ($m^2\ g^{-1}$)	26	
Properties of poly(MESH)-g-poly(GDGDA-co-GDMA) beads	**SI-ATRP1**	**SI-ATRP2**
Sulfur content (mg S/g dry beads)	11	17
Poly(MESH) grafted [(mg MESH)/g dry beads]	96.0	148.4
Poly(MESH) grafted [(mmol MESH)/g dry beads]	0.34	0.53
Grafting yield (% w/w)	3.4	5.3

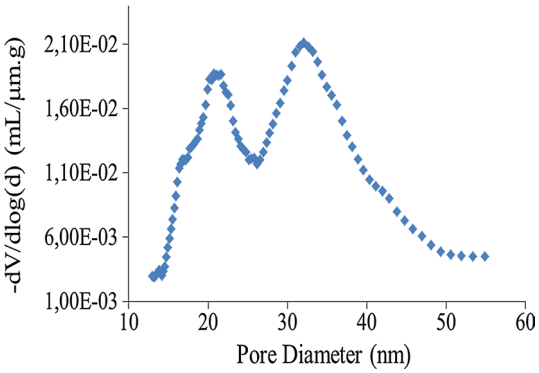

Fig. 4 The pore-size distribution curve of poly(GDGDA-co-GDMA) beads determined by mercury porosimeter

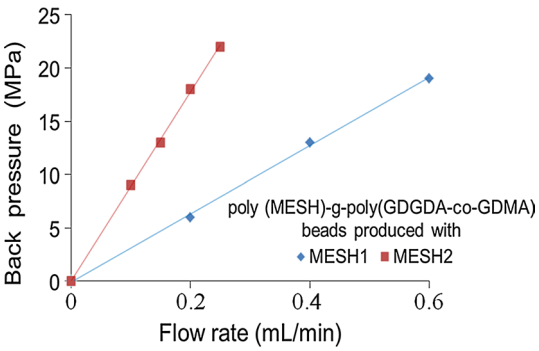

Fig. 5 The variation of column back pressure with the mobile phase flow rate

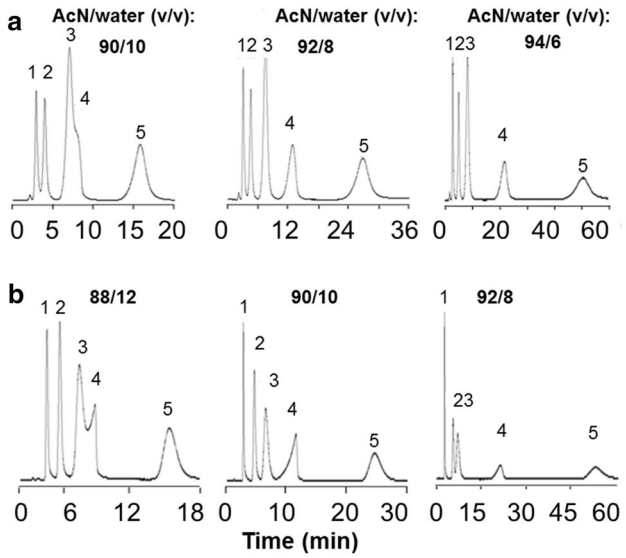

Fig. 6 Separation of test mixture containing various hydrophilic molecules in the columns packed with poly(MESH)-g-poly(GDGDA-co-GDMA) beads using the mobile phases prepared with different AcN/water ratios. Column: 300 × 2 mm i.d., Mobile phase: AcN + water + 0.05 % (v/v) AAc, pH:4.5, Flow rate: 0.3 mL min^{-1}, DAD at 254 nm, AcN/water volume ratio (mL mL^{-1}) was given on each chromatogram. Column packed with the beads obtained with **a** SI-ATRP1 **b** SI-ATRP2, Order of elution: *1* acrylamide, *2* uracil, *3* *p*-toluic acid, *4* cytosine, *5* guanine

liquid chromatography system in the studied range of the mobile phase flow rate.

The separation of different analyte groups was studied in HILIC mode with the columns packed with both types of poly(MESH)-g-poly(GDGDA-co-GDMA) beads. The separation of a test mixture containing various hydrophilic molecules in the columns prepared with two different SI-ATRP formulations is exemplified in Fig. 6. The peak resolutions are given in Table 3. Here, the AcN/water volume ratio was varied in a suitable range for each column. As seen both in Fig. 6 and Table 3, the separation performances obtained with both columns were similar. The representative chromatograms showing the effect of mobile phase flow rate on the separation of test mixture in the column prepared with SI-ATRP1 are given in Fig. 7. As seen from the resolution values given in Table 4, satisfactory separations were achieved up to 0.6 mL min^{-1}. The variation of TPN with the flow rate is exemplified for the packing material prepared by SI-ATRP1 and given in Fig. 8. Sharper peaks leading to higher TPN values were obtained with the polar analytes like uracil. Less polar analytes (i.e.,

Table 3 Effect of AcN/water ratio of the mobile phase on the peak resolutions obtained in the separation of test mixture

AcN/water (v/v)	Peak resolution			
	R_{21}	R_{32}	R_{43}	R_{54}
Poly(MESH)-g-poly(GDGDA-co-GDMA) beads produced with SI-ATRP1				
90/10	1.83	2.39	0.49	3.37
92/8	2.26	2.40	2.53	3.76
94/6	2.48	2.20	4.49	4.07
Poly(MESH)-g-poly(GDGDA-co-GDMA) beads produced with SI-ATRP2				
88/12	2.96	0.82	4.65	3.61
90/10	4.65	1.92	3.07	4.53
92/8	5.44	1.29	5.53	5.21

Column: 300 × 2 mm i.d. DAD at 254 nm. Mobile phase: AcN + water + 0.05 % (v/v) AAc, pH:4.5. Flow rate: 0.3 mL min^{-1} , Order of elution: (1) acrylamide, (2) uracil, (3) *p*-toluic acid, (4) cytosine (5) guanine

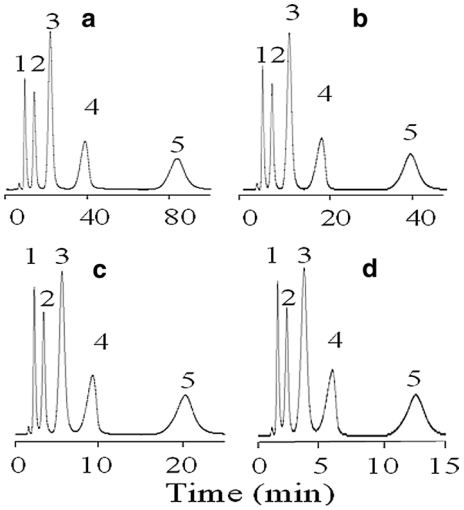

Fig. 7 Effect of mobile phase flow rate on the separation of test mixture containing various hydrophilic molecules in the column packed with poly(MESH)-g-poly(GDGDA-co-GDMA) beads produced with SI-ATRP1. Column: 300x2 mm i.d., Mobile phase: AcN + water + 0.05 % (v/v) AAc, pH:4.5, AcN/water volume ratio (mL mL^{-1}): 92/8, DAD at 254 nm, Flow rate (mL min^{-1}): **a** 0.1, **b** 0.2, **c** 0.4, **d** 0.6, Order of elution: *1* acrylamide, *2* uracil, *3* *p*-toluic acid, *4* cytosine, *5* guanine

p-toluic acid, cytosine and guanine) had broader peaks with higher retention times. In other words, higher TPN values were observed for the analytes with more polar character. As anticipated, TPN decreased with increasing flow rate for all analytes (Fig. 8).

The separation of nucleosides using the columns packed with both types of poly(MESH)-g-poly(GDGDA-co-GDMA) beads was investigated by changing the AcN/water ratio in the mobile phase. The AcN/water ratio of 90/10 (v/v) was determined as the most appropriate value (data not shown). In these runs, the column packed with the beads obtained by SI-ATRP2 showed better chromatographic performance. Hence, the separation of nucleosides using the column prepared by SI-ATRP2 was investigated with different flow rates ranging between 0.2 and 1.0 mL min^{-1}. The chromatograms obtained with different flow rates are given in Fig. 9. As seen here, four nucleosides were satisfactorily separated with all flow rates. The variation of TPN with the flow rate is shown for different nucleosides in Fig. 10. Here, the data obtained with thymidine in the column packed with the beads produced with SI-ATRP1 were also included for comparison. For the column prepared by SI-ATRP2, the highest TPNs were obtained with the most polar nucleoside tried (i.e., uridine). Similar to the results obtained with the test mixture, lower TPNs were obtained with the less polar nucleosides.

Table 4 Effect of mobile phase flow rate on the peak resolutions obtained in the separation of test mixture using the microbore column prepared with SI-ATRP1

Flow rate (mL min^{-1})	Peak resolution			
	R_{21}	R_{32}	R_{43}	R_{54}
0.1	2.51	2.52	2.90	4.07
0.2	2.32	2.51	2.54	3.77
0.3	2.26	2.40	2.53	3.76
0.4	2.09	2.25	2.18	3.62
0.5	1.98	2.17	2.17	3.29
0.6	1.83	1.97	1.89	3.10

Column: 300 × 2 mm i.d. DAD at 254 nm. Mobile phase: AcN + water + 0.05 % (v/v) AAc, pH:4.5, AcN/water volume ratio (mL mL^{-1}): 92/8, Order of elution: (1) acrylamide, (2) uracil, (3) *p*-toluic acid, (4) cytosine (5) guanine

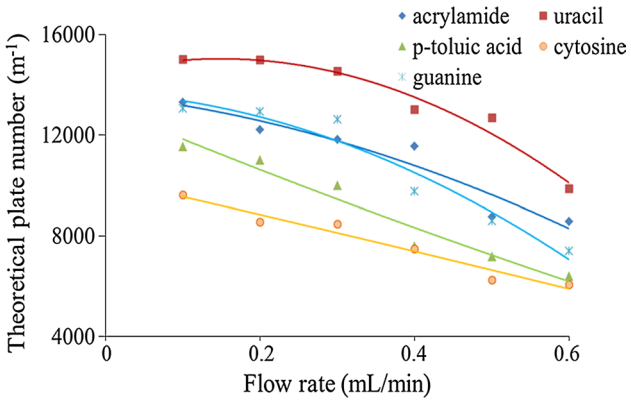

Fig. 8 The variation of TPN with the flow rate for the separation of test mixture in the column packed with poly(MESH)-g-poly(GDGDA-co-GDMA) beads produced with SI-ATRP1. Chromatographic conditions are given in Fig. 7

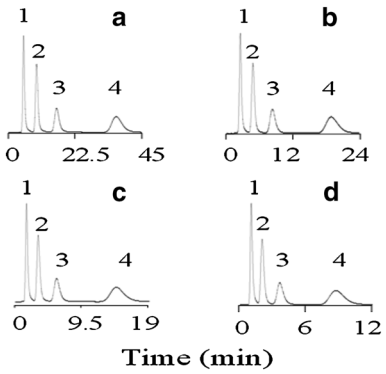

Fig. 9 Effect of mobile phase flow rate on the separation of nucleosides in the column packed with poly(MESH)-g-poly(GDGDA-co-GDMA) beads obtained with SI-ATRP2. Column: 300 × 2 mm i.d., Mobile phase: AcN + water + 0.05 % (v/v) AAc, pH:4.5, AcN/water volume ratio (mL mL^{-1}): 90/10, DAD at 260 nm, Flow rate (mL min^{-1}): **a** 0.2, **b** 0.4, **c** 0.6, **d** 1.0, Order of elution: *1* thymidine, *2* uridine, *3* inosine, *4* guanosine

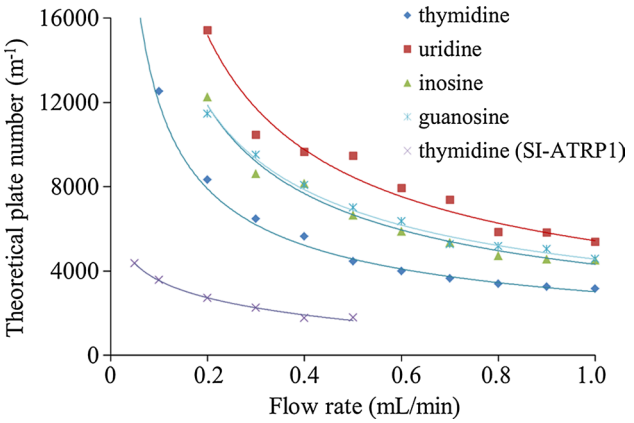

Fig. 10 The variation of theoretical plate number with the flow rate in the separation of nucleosides in the columns packed with poly(MESH)-g-poly(GDGDA-co-GDMA) beads obtained with SI-ATRP2. Chromatographic conditions are given in Fig. 9

(i.e., thymidine, inosine and guanosine). TPN showed a sharper decrease particularly in the flow rate range of 100–400 μL min^{-1} (Fig. 10).

A sample chromatogram showing the separation of three peptides in the column packed with poly(MESH)-g-poly(GDGDA-co-GDMA) beads prepared by SI-ATRP1 is shown in Fig. 11a. The variation of theoretical plate number for different peptides is given in Fig. 11b. As seen here, three peptides were satisfactorily separated without observing a significant decrease in the separation efficiency with the increasing flow rate.

The column packed with poly(MESH)-g-poly(GDGDA-co-GDMA) beads was used for the separation of acrylic acid–acrylamide mixture in HILIC mode. This analysis is

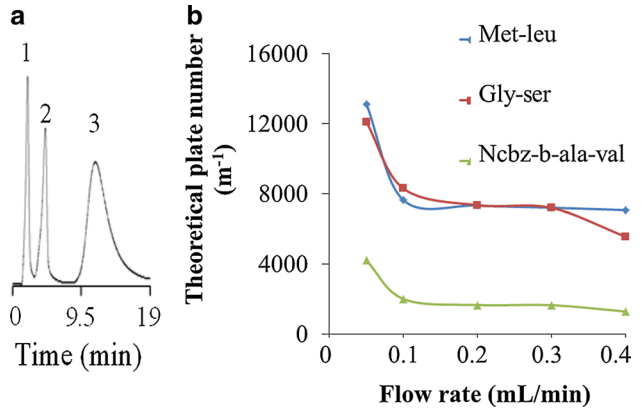

Fig. 11 Separation of peptides in the column packed with poly(MESH)-g-poly(GDGDA-co-GDMA) beads obtained with SI-ATRP1. **a** A sample chromatogram obtained with the flow rate of 0.3 mL min^{-1}, **b** The variation of theoretical plate number with the flow rate. Column: 300 × 2 mm i.d., Mobile phase: AcN + water + 0.05 % (v/v) AAc, pH:4.5, AcN/water volume ratio (mL mL^{-1}): 75/25, DAD at 260 nm, Order of elution: *1* Met-Leu, *2* Gly-Ser, *3* *N*-cbz-*β*-Ala-Val

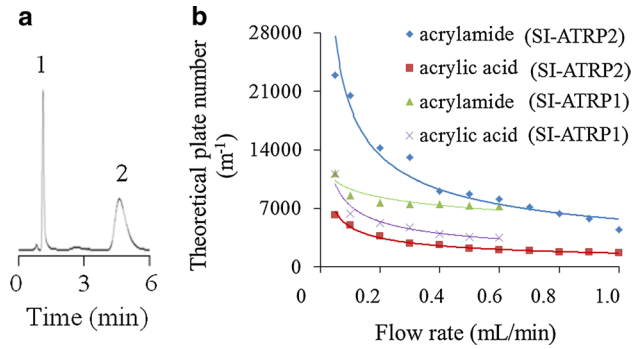

Fig. 12 Separation of acrylic acid–acrylamide mixture in the columns packed with poly(MESH)-g-poly(GDGDA-co-GDMA) beads obtained with SI-ATRP2. **a** A sample chromatogram obtained with the flow rate of 0.8 mL min^{-1}, **b** the variation of theoretical plate number with the flow rate. Column: 300 × 2 mm i.d., Mobile phase: AcN + water + 0.05 % (v/v) AAc, pH:4.5, AcN/water volume ratio (mL mL^{-1}): 92.5/7.5, DAD at 260 nm, Order of elution: *1* Acrylamide, *2* Acrylic acid

practically important since the chromatographic separation of these analytes is relatively difficult by reversed-phase chromatography [1, 19]. A sample chromatogram recorded with the most appropriate AcN/water ratio determined by the preliminary experiments (i.e., 92.5/7.5 v/v) using the column prepared by SI-ATRP2 is shown in Fig. 12a. As seen here, both acrylic compounds mostly found together in most samples were successfully resolved within 6 min. The comparison of TPN values obtained with both columns indicated that better chromatographic performance was observed with the column prepared by SI-ATRP2 in this analysis (Fig. 12b).

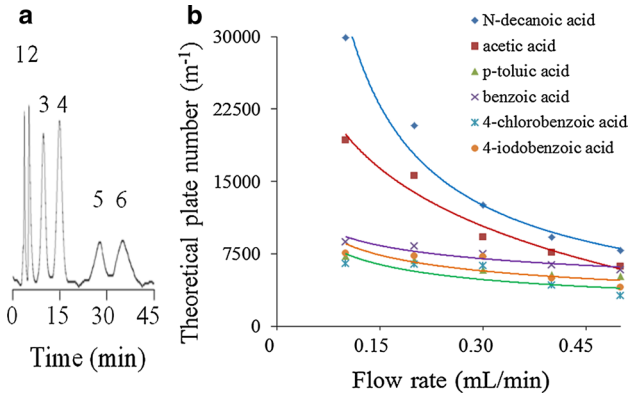

Fig. 13 Separation of organic acids in the column packed with poly(MESH)-g-poly(GDGDA-co-GDMA) beads obtained with SI-ATRP1. **a** A sample chromatogram obtained with the flow rate of 0.2 mL min^{-1}, **b** The variation of theoretical plate number with the flow rate. Column: 300 × 2 mm i.d., Mobile phase: AcN + water + 0.05 % (v/v) AAc, pH:4.5, AcN/water volume ratio (mL mL^{-1}): 92.5/7.5, DAD at 214 nm, Order of elution: *1* *N*-decanoic acid, *2* acetic acid, *3* *p*-toluic acid, *4* benzoic acid, *5* 4-chlorobenzoic acid, *6* 4-iodobenzoic acid

The column packed with poly(MESH)-g-poly(GDGDA-co-GDMA) beads encoded by SI-ATRP1 was used for the separation of organic acids. A sample chromatogram recorded with the appropriate AcN/water ratio determined by the preliminary experiments (i.e., 92.5/7.5 v/v) is shown in Fig. 13a. As seen here, the organic acid was satisfactorily separated. The variation of theoretical plate number of the column for different organic acids is given in Fig. 13b. As seen here, TPNs up to 30,000 m^{-1} were achieved using *n*-decanoic acid as the analyte in the column encoded as SI-ATRP1. Lower TPN values were obtained with the less polar (i.e., aromatic ring containing) analytes.

The column efficiencies obtained with the commercial silica-based HILIC columns or polymer-based HILIC columns recently appeared in the literature are given in Table 5. As seen here, silica-based commercial HILIC columns exhibited TPN values ranging between 26.315 and 43,480 plates m^{-1}. These values were relatively higher with respect to the TPN values achieved by our columns. When the analytes given in Table 5 were considered, TPN values ranging between ca 6.500 and 11,500 plates m^{-1} were obtained in our column using a benzoic acid derivative, *p*-toluic acid as the analyte (Fig. 8). On the other hand, the nucleotides, uracil and cytosine provided TPN values ranging between 10,000–15,000 and 6,250–9,600 plates m^{-1}, respectively, with the our column (Fig. 8). On the other hand, a nucleoside, guanosine gave TPN values between 5.500 and 11.500 plates m^{-1} (Fig. 10). These values were relatively higher with respect to those achieved with the polymer-based HILIC columns selected for Table 5 [1, 4, 11–13]. Based on this comparison, one can conclude that polymer-based HILIC columns synthesized by SI-ATRP process in our case exhibited better separation performance with respect to the similar polymer-based HILIC columns. Hence, the use of a hydrophilic zwitterionic molecular brush on a hydrophilic porous polymer core resulted in a

Table 5 The column efficiencies for different particle-based HILIC columns

Stationary phase	Dimension (mm × mm)	Analyte	TPN (plates m^{-1})	Refs.
ZIC HILIC	250 × 4.6	Cytosine	26,315	[4]
	250 × 4.6	Guanosine	30,300	[4]
YMC-Pack NH_2	250 × 4.6	Cytosine	43,480	[4]
	250 × 4.6	Guanosine	29,400	[4]
TSKgel Amide-80	250 × 4.6	Cytosine	29,410	[1]
	250 × 4.6	Guanosine	26,315	[1]
THMAM-attached poly(GMA)-g-silica beads	50 × 4.6	3,4-Dihydroxy benzoic acid	66,000	[11]
	50 × 4.6	Cytosine	15,625	[11]
	50 × 4.6	Toluene	9,700	[11]
Poly(VT)-g-poly(GMA-co-EDMA) beads	100 × 4.6	Cytidine	2,680	[12]
	100 × 4.6	Cytosine	2,165	[12]
	100 × 4.6	Adenosine	1,630	[12]
Poly(HTMA)-g-poly(CMS-co-DVB) beads	100 × 4.6	Guanosine	7,460	[13]
	100 × 4.6	Cytosine	3,115	[13]
	100 × 4.6	Uracil	2,725	[13]
	100 × 4.6	Salicylic acid	3,550	[13]

THMAM Trishydroxymethyaminomethane, *HTMA* 2-hydroxyl-3-[4-(hydroxymethyl)-1H-1,2,3-triazol-1-yl] propyl 2-methylacrylate, *VT* Vinyl tetrazole

satisfactory separation performance for the polar compounds in HILIC mode.

Conclusion

A new hydrophilic chromatographic support was synthesized by surface-initiated ATRP of a zwitterionic monomer on monodisperse porous poly(GDGDA-co-GDMA) beads obtained by a newly proposed staged-shape template polymerization protocol. The synthesis method for zwitterionic molecular brush-functionalized silica beads as a separation medium for HILIC applications was first developed by Irgum's group [9]. In our study, a newly synthesized polymer-based hydrophilic support (i.e., poly(GDGDA-co-GDMA) beads) was successfully used instead of a silica gel based column packing. Polymer-based packings have been used in a broader pH range and more resistant against environmental conditions with respect to silica-based column materials. This property is required for the chromatographic modes using acidic or basic mobile phases. The polymer-based support exhibited a satisfactory chromatographic performance for the separation of polar compounds in HILIC mode. Various analyte groups (i.e., nucleosides, peptides, polar organic compounds and organic acids) were separated by the column packing synthesized in this study.

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