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Preparation of an Ion-Exchange Chromatographic Support by A "Grafting From" Strategy Based on Atom Transfer Radical Polymerization

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A new "grafting from" strategy based on surface-initiated atom transfer radical polymerization (ATRP) was first used for the preparation of a polymer-based ion-exchange support for HPLC. The most important property of the proposed method is to be applicable for the synthesis of any type of ion exchanger in both the strong and the weak forms. Monodisperse, porous poly(glycidyl methacrylate-*co*-ethylene dimethacrylate), poly(GMA-*co*-EDM) particles 5.8 μm in size were synthesized by "modified seeded polymerization". Poly(dihydroxypropyl methacrylate-*co*-ethylene dimethacrylate), poly(DHPM-*co*-EDM) particles were then obtained by the acidic hydrolysis of poly(GMA-*co*-EDM) particles. The ATRP initiator, 3-(2-bromoisobutyramido)propyl(triethoxy)silane was covalently attached onto poly(DHPM-*co*-EDM) particles via the reaction between triethoxysilane and diol groups. In the next stage, the selected monomer carrying strong cation exchanger groups, 3-sulfopropyl methacrylate (SPM), was polymerized on the initiator-immobilized particles via surface-initiated ATRP. The degree of polymerization of SPM (i.e., length of polyionic ligand) on the particles was precisely controlled by adjusting ATRP conditions. Poly(SPM)-grafted poly(DHPM-*co*-EDM) particles obtained with different ATRP formulations were tried as chromatographic packing in the separation of proteins by ion-exchange chromatography. The proteins were successfully separated with higher column yields with respect to the previously proposed materials. The plate heights between 100 and 150 μm were achieved with the column packed with the particles carrying the shortest poly(SPM) chains. The plate height showed no significant increase with increasing flow rate in the range of 0.5−16 cm/min.

Supports in the form of monodisperse polymer particles have attracted significant attention in ion-exchange chromatography. The "activated swelling method" was developed for the synthesis of monodisperse particles in the range of 1−20 μm.[1,2] The monodisperse, porous poly(glycidyl methacrylate−ethylene dimethacrylate) (poly(GMA-*co*-EDM)) particles were produced by "staged shape template polymerization".[3,4] The particles modified by a pore size-specific functionalization process were used as separation media for the complete separation of complex samples that require a combination of ion exchange with reversed-phase chromatography.[5,6] Monodisperse poly(glycidyl methacrylate−divinylbenzene) microspheres carrying strong anion and cation exchanger groups were used in the separation of macrolide antibiotics and proteins by capillary electrochromatography.[7,8] The strong cation-exchange packings based on monodisperse poly(GMA-*co*-EDM) particles were also used in the separation and purification of combinant human interferon.[9] The ion-exchange polymeric stationary phases presenting amino acids and amine units were prepared by the surface grafting of glycidyl methacrylate onto a silica gel surface and subsequent amination.[10] All of these packings were obtained by the surface derivatization methods involving the use of conventional activation agents or free radical polymerization techniques for the covalent attachment of ion exchanger ligands onto the particles.

Atom transfer radical polymerization (ATRP) was proposed as a relatively new technique for the surface derivatization of silica- or polymer-based particles.[11−14] Polymer-grafted silica nanoparticles carrying homopolymers, block copolymers, or hyperbranched polymers were synthesized by the surface-initiated ATRP of vinyl- or acrylate-based monomers.[15−20] Poly(divinyl-

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benzene) particles with 1-phenylethyl bromide groups on the surface layer were used as a macroinitiator for ATRP of styrene.[21] The graft polymerization of various monomers by ATRP from lightly cross-linked poly(divinylbenzene-*co*-hydroxyethyl methacrylate) microspheres modified with 2-bromopropionyl bromide was investigated.[22,23] Initiator attached latex particles were used for the synthesis of thermoresponsive block copolymer brushes by surface-initiated aqueous ATRP.[24,25]

Although surface-initiated ATRP was used for the derivatization of both silica- and polymer-based particles, no report was found in the literature for the synthesis of a HPLC packing material prepared by surface-initiated ATRP techniques. In this study, we attempted to synthesize a polymer-based ion-exchange chromatographic packing by surface-initiated ATRP. For this purpose, monodisperse, porous poly(GMA-*co*-EDM) particles were used as the starting material. The selected ion-exchange ligand in the polymeric form, poly(sulfopropyl methacrylate) (poly(SPM)), was grafted from the particles by a surface-initiated ATRP technique proposed in this study. The strong cation exchanger packing prepared by this new "grafting from" strategy was successfully used in the separation of proteins by ion-exchange chromatography.

EXPERIMENTAL SECTION

Materials. The monomers, glycidyl methacrylate (GMA), ethylene dimethacrylate (EDM), and 3-sulfopropyl methacrylate potassium salt (SPM) were supplied from Aldrich Chemical Co. and used without further purification. Cyclohexanol (Cyc-OH), dibutyl phthalate (DBP), methanol (MeOH, HPLC grade), dimethylformamide (DMF), tetrahydrofuran (THF, HPLC grade), 2,2′-azobisizobutyronitrile (AIBN), (3-aminopropyl)triethoxysilane (APTES), 2-bromoisobutryl bromide (BrIBuBr), copper(I) chloride, copper(II) chloride, and 2,2′-bipyridyl (BPy) were supplied from Aldrich Chemical Co. Sodium lauryl sulfate (SLS) and poly(vinyl alcohol) (PVA, 87−89% hydrolyzed, molecular weight 85.000−146.000) and poly(vinylpyrrolidone) (PVP K-30, average molecular weight 40.000) were obtained from Sigma Chemical Co. The proteins (cytochrome *c*, myoglobin, ribonuclease a, lysozyme) were supplied by Sigma Chemical Co. The properties and the suppliers of other chemicals used in this study were given elsewhere.[26,27] *CAUTION: 2,2′-Bipyridyl is toxic. Glycidyl methacrylate and 3-sulfopropyl methacrylate potassium salt are irritant for eyes, respiratory system, and skin. These chemicals should be handled in a well-ventilated hood with the utmost care.*

Synthesis and Characterization of Poly(2,3-dihydroxypropyl methacrylate-*co*-ethylene dimethacrylate) (Poly(DHPM-*co*-EDM)) Particles. The polystyrene (PS) seed latex, 2.1 μm in size, was synthesized by dispersion polymerization.[27] Typically, styrene (15 mL) was dissolved in a relatively polar medium containing ethanol (69 mL), 2-methoxyethanol (48 mL), water (3 mL), AIBN (0.44 g), and PVP K-30 (2.1 g). The polymerization was conducted at 70 °C for 24 h with a shaking rate of 120 cpm.

The monodisperse poly(GMA-*co*-EDM) particles were obtained by a modified form of one-step swelling and polymerization protocol in the literature.[28] Typically, the organic phase containing Cyc-OH (14 mL), DBP (5.4 mL), GMA (6.4 mL), EDM (4.2 mL), and AIBN (0.32 g) was emulsified in water (360 mL) including SLS (0.60 g) and PVA (2.8 g) by sonication for 30 min. The dispersion containing PS seed particles (0.7 g) was added to the emulsion. The resulting dispersion was stirred at room temperature for 24 h. Polymerization in the swollen seed particles was conducted at 70 °C and 120 counts/min shaking rate for 24 h. The monodisperse, porous poly(GMA-*co*-EDM) particles were obtained as the product. The isolated particles were washed with ethanol several times and extracted with THF. The hydrolysis of poly(GMA-*co*-EDM) particles was performed according to the literature in 0.5 M H_2SO_4 solution at 60 °C for 4 h.[3,4,28] The hydrolyzed particles were extensively washed with ethanol and dried in vacuo at 80 °C. The hydrolysis was confirmed by FT-IR spectroscopy by the appearance of hydroxyl band at 3500 cm^{-1}.[29]

The size distribution properties and the surface morphology of poly(DHPM-*co*-EDM) particles were examined by scanning electron microscopy (SEM; JEOL, JEM 1200EX).[30] Four separate SEM photographs taken with the magnification of 2000× from different fields were evaluated in the determination of average particle size and coefficient of variation for size distribution. The pore size distribution and the specific surface area of poly(DHPM-*co*-EDM) particles were determined by Brunauer−Emmet−Teller (BET) apparatus (Quantachrome, Nova 2200E). The distribution of hydroxyl groups in the particle structure was investigated by confocal laser scanning microscopy (CLSM) by following an imaging protocol described elsewhere.[31] For this purpose, the hydroxyl groups of poly(DHPM-*co*-EDM) particles were converted into the primary amine form by treating particles (1 g) with APTES (2 mL) in toluene (20 mL) for 24 h at 60 °C. The primary amine groups on the particles were then reacted with fluorescein isothiocyanate (FITC) in an acetone−water mixture at pH 9.[31] FITC-labeled particles were then imaged under CLSM (Leica SP2, Heidelberg, Germany) equipped with He−Ne laser operated at an excitation wavelength of 489 nm.

Synthesis of 3-(2-Bromoisobutyramido)propyl(triethoxy)-silane (BIBAPTES). The ATRP initiator BIBAPTES was prepared by the reaction of APTES with BrIBuBr according to the

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literature.[32] In a typical synthesis, APTES (9.4 mL, 40 mmol) and triethylamine (7.0 mL, 50 mmol) were dissolved in anhydrous THF (60 mL). To this solution, BrIBuBr (6.3 mL, 50 mmol) was added slowly under ice-cooling. The solution was allowed to warm to room temperature, and the stirring was continued for 4 h under nitrogen. The salt triethylammonium bromide (TEABr) was removed by filtration, and the filtrate was evaporated to 1/4 of its initial volume. The residual TEABr precipitated by evaporation was again removed by centrifuging the solution, and the resulting solution was evaporated to dryness. The oily residue was stirred under vacuum at 50 °C and stored at +4 °C under a nitrogen blanket. The product, BIBAPTES, was obtained in the form of a brown solid (13.48 g, 36.4 mmol, 91% w/w).

Synthesis of Initiator Carrying Poly(DHPM-*co*-EDM) Particles. The initiator (BIBAPTES) was covalently attached onto the poly(DHPM-*co*-EDM) particles via the reaction between hydroxyl and triethoxysilane groups. A typical reaction was conducted in a 205 mM solution of BIBAPTES in dry THF (25 mL) including poly(DHPM-*co*-EDM) particles (3.5 g) at room temperature for 24 h at a stirring rate of 250 rpm. After reaction, the particles were extensively washed with THF, ethanol, and finally with distilled water by using a centrifugation−decantation protocol. The washed particles were dried in vacuo at 60 °C for 24 h and weighed. The amount of BIBAPTES covalently attached to the particles was calculated from the difference between the dry weights of particles after and before the attachment of BIBAPTES.

Synthesis of Ion-Exchange Support by ATRP. The recipe proposed for homogeneous ATRP of SPM elsewhere was adopted for surface-initiated ATRP in our study.[33] In a typical synthesis, SPM (8.1 mmol) was dissolved in a DMF/water mixture (50/50% v/v, 7 mL) in a Schlenk tube. A freshly prepared Cu−BPy stock solution including CuCl (0.40 mmol), $CuCl_2$ (0.4 mmol), and BPy (2 mmol) in a DMF/water mixture (50/50% v/v, 1 mL) was then added. The resulting medium was sonicated for 5 min and degassed with nitrogen for 10 min. BIBAPTES-attached poly(DHPM-*co*-EDM) particles (1.8 g) dispersed in a degassed DMF/water mixture (50/50% v/v, 2 mL) were added to the polymerization medium. The reactor was sealed, and the vacuum was applied for 10 min. The polymerization was conducted at room temperature for 6 h at 250 rpm stirring rate. The particles were extensively washed with DMF, methanol, and then with distilled−deionized water. The washed particles were dried in vacuo at 70 °C for 48 h and weighed. The amount of poly(SPM) covalently attached to the particles was calculated from the difference between the dry weights of particles after and before the surface-initiated ATRP. In the heterogeneous ATRP runs, the polymerization conditions were changed to have polyionic ligands with different lengths on the poly(DHPM-*co*-EDM) particles.

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 SPM/g dry particles) were calculated based on the gravimetric data. The degree of polymerization was calculated as the ratio of mole of monomer polymerized on the particles to the mole of initiator covalently attached to the particles.

Chromatographic Study. The particles were slurry packed into 100 × 4.6 mm i.d. or 50 × 4.6 mm i.d. HPLC columns under high pressure (25 MPa) by following the protocol given elsewhere.[26,27] Each column was first washed with phosphate buffer at pH 7 containing 1 M NaCl and then with the phosphate buffer at the same pH with a flow rate of 1 mL/min for 24 h. The separation of proteins by ion-exchange chromatography was studied in gradient mode in an HPLC system (LC-10 ADVP, Shimadzu). The chromatograms were recorded by a UV detector (SPD-10 AVVP) at 280 nm.

The chromatographic runs with the columns packed with poly(SPM)-attached particles were performed by using a protein mixture including myoglobin (pI 7.2), ribonuclease A (pI 9.5), cytochrome *c* (pI 10.2), and lysozyme (pI 10.5). The gradient runs were performed by using mobile phases with pHs ranging between 6 and 8. Then 20 mM buffer solutions of 4-morpholinoethanesulfonic acid hemisodium salt (MES; pH 6), sodium phosphate (pH 7), and *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) hemisodium salt (HEPES; pH 8) were used as mobile phase A. Mobile phase A + 1 M NaCl was used as mobile phase B. The peak resolution for a selected protein, $R(n + 1/n)$ was calculated based on eq 1,[34]

$$R(n + 1/n) = 2(t_{n+1} - t_n)/(W_{n+1} + W_n) \quad (1)$$

where $R(n + 1/n)$ is the resolution between the selected peak ($n + 1$) and the preceding peak (n). t_{n+1} and t_n are the retention times from the point of injection for peak $n + 1$ and peak n, respectively. W_{n+1} and W_n are the widths of the base for the peaks $n + 1$ and n, respectively.

The plate heights for different columns were calculated by the chromatographic runs performed in isocratic mode by using cytochrome *c* as the analyte with 20 mM sodium phosphate buffer + 1 M NaCl (pH 7) as the mobile phase. In a column with a length of L, the theoretical plate number (N) was calculated by eq 2,

$$N = 5.54(t_r/t_w)^2 \quad (2)$$

where t_r and t_w are the retention times and the peak width at half-height, respectively. The plate height (h) was calculated according to eq 3,

$$h = L/N \quad (3)$$

The protein recovery experiments with the columns packed with the particles carrying poly(SPM) ligands in different lengths were done in isocratic mode with 20 mM sodium phosphate + 1 M NaCl solution (pH 7) with a flow rate of 0.75 mL/min and a protein concentration of 1 mg/mL. The protein recovery was calculated as the percentage of the peak area of protein leaving the column with respect to the peak area of the same amount of protein injected into the HPLC system with a closed loop (i.e., the column was removed and the inlet and outlet tubes were connected).[5]

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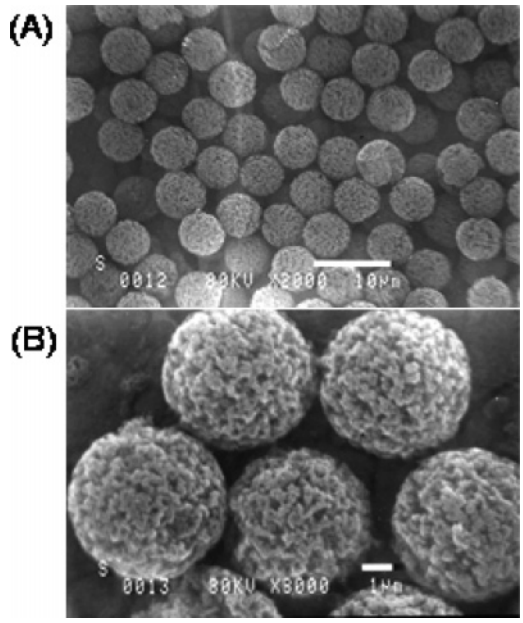

Figure 1. Representative SEM photographs showing size distribution and surface morphology of poly(DHPM-*co*-EDM) particles, Magnification: (A) 2000×; (B) 8000×.

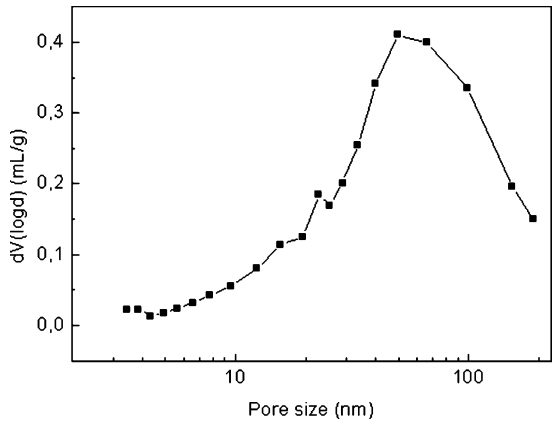

Figure 2. Pore size distribution curve of poly(DHPM-*co*-EDM) particles obtained by BET.

RESULTS AND DISCUSSION

Particle Characterization. Poly(DHPM-*co*-EDM) particles were obtained by the acidic hydrolysis of poly(GMA-*co*-EDM) particles according to the literature.[5,6,28] The SEM photographs showing the size distribution and surface morphology of poly(DHPM-*co*-EDM) particles are given in Figure 1. The average diameter and coefficient of variation for size distribution were calculated as 5.8 μm and 6.1% by using the SEM photograph in Figure 1A. As seen in Figure 1B, the particle surface was highly porous. The average pore size and the specific surface area of poly(DHPM-*co*-EDM) particles determined by BET were 40 nm and 37.1 m^2/g, respectively. The pore size distribution curve of poly(DHPM-*co*-EDM) particles is given in Figure 2. The maximum point on this curve was observed at ~50 nm. Note that the contribution of pores larger than 200 nm could not be taken into account in the calculation of mean pore size since the measurement was performed by BET.

FITC-labeled poly(DHPM-*co*-EDM) particles were examined under CLSM by performing serial optical sections in the vertical direction. The optical section at midplane of the particles and the fluorescent intensity profile obtained for the selected optical section are given in Figure 3. The fluorescent intensity at a certain point is directly proportional to the concentration of FITC bound to the primary amine groups.[31] For this reason, the fluorescent intensity should be considered as a measure of hydroxyl concentration at a certain point since the primary amine groups were generated by the reaction of hydroxyl groups with the selected reagent (i.e., APTES).[31] This intensity profile clearly showed the homogeneous distribution of hydroxyl groups in the particle structure (Figure 3B).

Attachment of Polyionic Ligand by ATRP. The entire grafting process is given in Figure 4. In the first stage, the ATRP initiator, BIBAPTES, was covalently attached onto poly(DHPM-*co*-EDM) particles. Note that the distribution of hydroxyl groups in the particles should probably affect the concentration profile of BIBAPTES obtained by covalent attachment. Hence, a homogeneous distribution for bound BIBAPTES is also expected. The effect of initial BIBAPTES concentration on the variation of BIBAPTES content of particles (i.e., millimoles of BIBAPTES covalently attached onto per gram of particles) is given in Supporting Information. The BIBAPTES content of particles increased up to 0.221 mmol of BIBAPTES/g of particles by increasing the initial BIBAPTES concentration in the reaction medium up to 205 mM (Figure S1, Supporting Information). For the attachment of polyionic ligand by ATRP, poly(DHPM-*co*-EDM) particles with two different BIBAPTES contents (i.e., 0.042 and 0.221 mmol of BIBAPTES/g of particles) obtained with the initial BIBAPTES concentrations of 32 and 205 mM were used.

The spacer arm length was an effective variable in manipulating retention properties in the ion-exchange chromatography of proteins on a stationary phase carrying sulfonic acid groups.[35] In the studies on the synthesis of ion-exchange stationary phases based on monodisperse, porous poly(DHPM-*co*-EDM) particles, a spacer arm (i.e., epichlorohydrin) was mostly utilized for obtaining an effective interaction between the ion exchanger ligand and the analyte.[5,6,9,28,36] Alternatively, the ion-exchange ligands in the polymeric form serve a large number of ionic groups on a long-chain, flexible backbone. Such type of structure provides higher ionic ligand density and more effective interaction with the analytes with respect to the ionic groups attached onto the support with a relatively shorter spacer arm. As known, ATRP is a powerful tool for controlling the molecular weight and the molecular weight distribution in a narrow range.[17,18,24,25] The control of molecular weight and the molecular weight distribution was also shown for many heterogeneous ATRP systems initiated with the particles carrying immobilized initiators.[15−20,24,25] In principle, the cation or anion exchanger polyionic ligands in weak or strong forms can be synthesized on the support materials by the selection of appropriate monomers (i.e., SPM, acrylic acid, or 2-dimethylaminoethyl methacrylate) via surface-initiated ATRP. Hence, ATRP seems a new tool for the synthesis of a polyionic ligand with well-defined chemical structure and length on a solid support.

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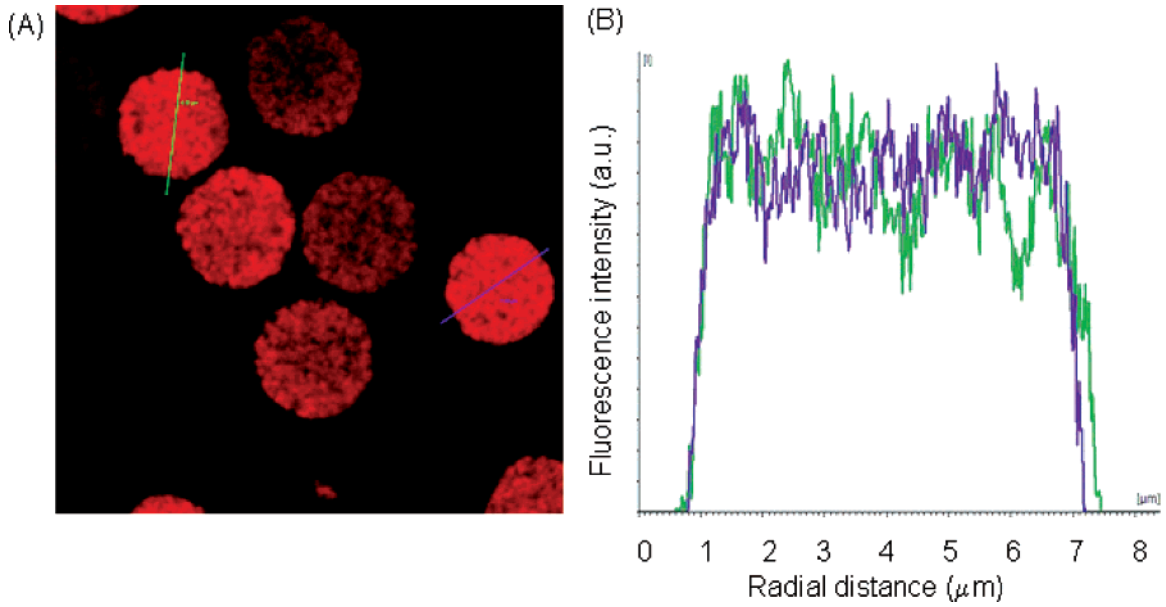

Figure 3. CLSM image showing (A) the optical section at midplane of poly(DHPM-*co*-EDM) particles and (B) the fluorescent intensity distribution in the optical section.

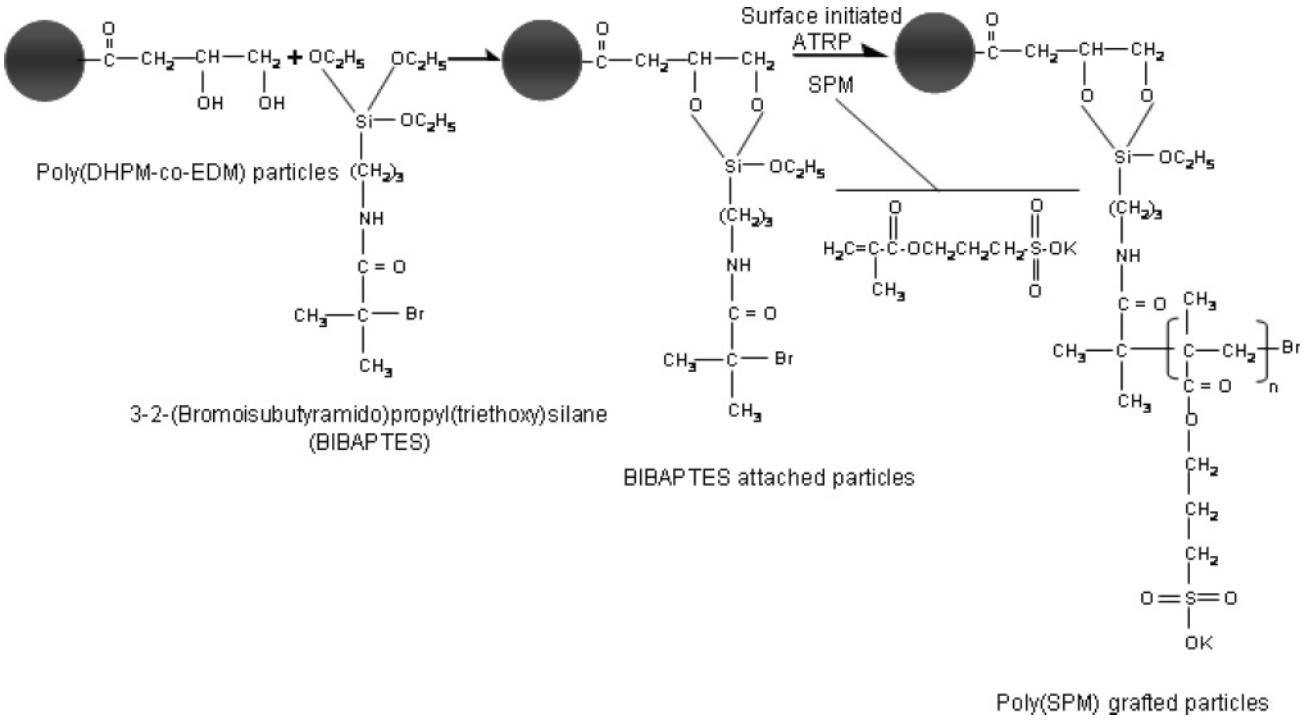

Figure 4. Entire "grafting from" process for poly(DHPM-*co*-EDM) particles.

By considering these reasons, a new "grafting from strategy" is proposed based on surface-initiated ATRP for the synthesis of polymer-based, ion-exchange supports carrying polymeric ligands in the form of weak or strong ion exchangers. The most important property of this strategy is to be applicable for the synthesis of any type of ion exchanger in both the particulate and monolithic forms.

To exemplify the proposed strategy, a monomer carrying strong cation exchanger groups (i.e., SPM) was grafted from the surface of BIBAPTES-attached poly(DHPM-*co*-EDM) particles by surface-initiated ATRP. The chemical structure of poly(SPM)-grafted particles is given in Figure 4. To have polyionic ligands with different lengths on the surface of particles, three surface-initiated ATRP formulations were tried. These recipes are given in Table 1. In the first recipe, [SPM]/[initiator]/[CuCl]/[$CuCl_2$]/[BiPy] ratios were selected as 20:1:1:1:5. Hence, relatively shorter poly(SPM) chains on the particles were achieved by using low monomer/initiator ratios. In the second one, [SPM]/[initiator]/[CuCl]/[$CuCl_2$]/[BiPy] ratios were selected as 108:1:1:1:5. So the synthesis of particles with longer poly(SPM) chains was achieved by using a high monomer/initiator ratio. In the third formulation, the concentrations of CuCl and $CuCl_2$ were doubled with respect

Table 1. ATRP Conditions for Polyionic Ligand Grafting from the Initiator-Attached Poly(DHPM-*co*-EDM) Particles

ingredients	ATRP1	ATRP2	ATRP3
poly(DHPM-*co*-EDM)-Br (g)	1.8 (0.4)[a]	1.8 (0.075)[a]	1.8 (0.075)[a]
SPM (mmol)	8.12	8.12	8.12
DMF (mL)	5.0	5.0	5.0
water (mL)	5.0	5.0	5.0
BiPy (mmol)	2.0	0.375	0.75
CuCl (mmol)	0.4	0.075	0.15
$CuCl_2$ (mmol)	0.4	0.075	0.15

[a] The amount of ATRP initiator, BIBAPTES, attached to the particles is given in parentheses in terms of millimoles.

Table 2. Properties of Poly(SPM) Grafted-Poly(DHPM-*co*-EDM) Particles[a]

recipe	grafting yield (% mol)	polyionic ligand content (mmol SPM/g dry particles)	degree of polymerization on the particles
ATRP1	10.8 ± 0.8	0.487 ± 0.036	2.2 ± 0.2
ATRP2	14.3 ± 1.8	0.646 ± 0.079	15.5 ± 1.9
ATRP3	23.1 ± 1.5	1.043 ± 0.068	25.1 ± 1.6

[a] The results are given as mean ± standard deviation calculated by three parallel runs for each ATRP formulation.

to the second recipe. In this one, [SPM]/[initiator]/[CuCl]/[$CuCl_2$]/[BiPy] ratios were 108:1:2:2:5. Hence, further increase in the length of poly(SPM) chains was attempted.

The grafting yield and polyionic ligand content and degree of polymerization for poly(SPM)-grafted particles are given in Table 2 for different ATRP formulations. As seen here, the particles with different poly(SPM) contents were obtained by the ATRP formulations in Table 1. As mentioned above, the degree of polymerization calculated based on the polyionic ligand content of particles could be effectively changed by the selected "grafting from" protocol based on surface-initiated ATRP. It should be noted that complete consumption of initiator attached to the particles is assumed in the calculation of degree of polymerization. The lowest polyionic ligand content was obtained by ATRP1 by starting with the highest amount of immobilized initiator. Hence, the shortest poly(SPM) chains on the particles were synthesized. Higher polyionic ligand contents were achieved with ATRP2 and ATRP3 by starting with the particles having lower initiator content with respect to ATRP1. Therefore, higher degrees of polymerization (i.e., longer poly(SPM) chains) were obtained. The standard deviation values for degree of polymerization showed that it was possible to repeat each ATRP formulation by obtaining poly(SPM) chains with reproducible length on the poly(DHPM-*co*-EDM) particles (Table 2).

Chromatographic Performance of Poly(SPM)-Grafted Particles. The variation of column back pressure with the flow rate is given in Figure 5 for the columns prepared with different ATRP formulations. As seen in Table 2 and Figure 5, the column packed with the particles with higher poly(SPM) content exhibited higher back pressure at constant flow rate. This behavior should be probably explained by the presence of poly(SPM) chains in the pores forming a resistance against the flow of mobile phase. In other words, greater amount of poly(SPM) ligand in the pores led to higher flow resistance. The back pressure linearly increased with increasing flow rate for the columns prepared with the formulations of ATRP1 and ATRP2. However, the column including the particles prepared by ATRP3 (i.e., the particles with the highest poly(SPM) content and the longest poly(SPM) ligand) provided a parabolic back pressure curve. This behavior should be probably explained by the deformation of particles in the column due to the high flow resistance particularly occurring at flow rates higher than 0.75 mL/min.

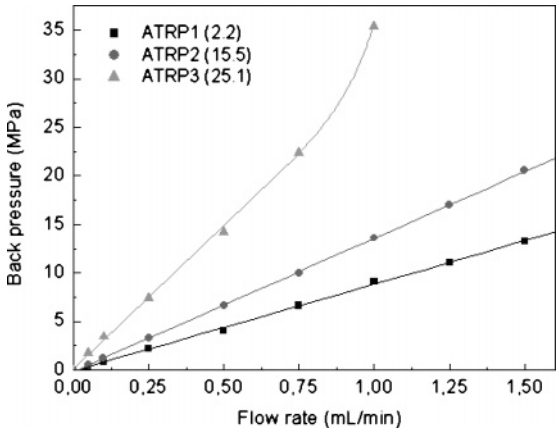

Figure 5. Effect of flow rate on the back pressure of the columns packed with the poly(SPM)-grafted poly(DHPM-*co*-EDM) particles obtained with different ATRP formulations. Mobile phase: 20 mM phosphate buffer at pH 7. Column size: 50 × 4.6 mm i.d. Degree of polymerization of polyionic ligand on the particles is given in parentheses.

The chromatographic performance of poly(SPM)-grafted poly(DHPM-*co*-EDM) particles prepared with different ATRP formulations was investigated by the ion-exchange separation of proteins in gradient mode. In the first set, the columns obtained by the recipes given in Table 1 were examined by using mobile phases at different pHs ranging between 6 and 8. The liquid chromatograms obtained at pH 7 are given in Figure 6 for the packings produced with different ATRP formulations. As seen here, the proteins were eluted from the columns with reasonably sharp peaks in an order obeying their isolectric points. The peak resolutions calculated from the chromatograms obtained at different pHs are given in Table 3. As seen here, the most satisfactory resolutions were obtained at pH 7 for all columns.

The effect of flow rate on the gradient separation of proteins at pH 7 was investigated by the ion exchangers prepared by different ATRP formulations. The chromatograms obtained with the poly(SPM)-grafted poly(DHPM-*co*-EDM) particles prepared with ATRP1 (i.e., the particles with the shortest poly(SPM) ligand) are exemplified in Figure 7. For all columns, the resolutions calculated based on the chromatograms obtained with different flow rates are given in Table 4. As seen here, the protein mixture was successfully resolved with flow rates between 0.25 and 1.5 mL/min by using the packings with relatively shorter ligands (i.e., ATRP1 and ATRP2). For these columns, the resolutions with all flow rates are higher with respect to 1.5, which is commonly accepted as a threshold value for satisfactory separation. Note that the flow rates higher than 0.75 mL/min could not be tried for the packing with the highest poly(SPM) content since the back pressure at these flow rates was higher than the filling pressure

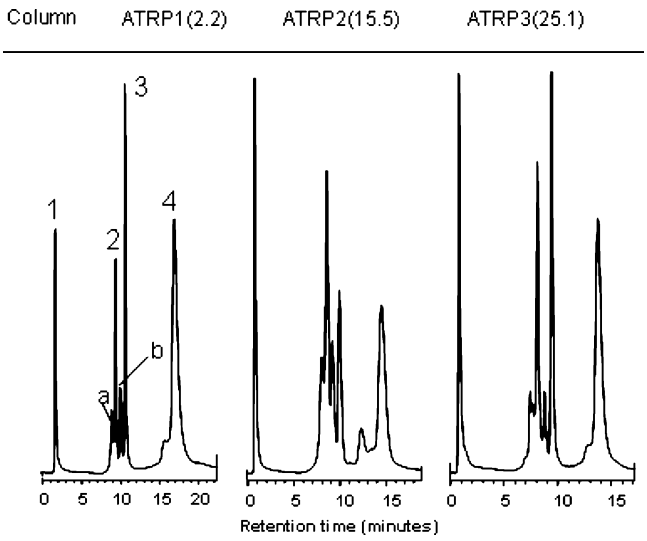

Figure 6. Sample liquid chromatograms obtained for the gradient separation of proteins at pH 7 in the columns packed with poly(SPM)-grafted poly(DHPM-*co*-EDM) particles produced with different ATRP formulations, Conditions: formulation, (A) ATRP1, (B) ATRP2, and (C) ATRP3; degree of polymerization of polyionic ligand on the particles is given in parentheses. Mobile phases, (A) 20 mM sodium phosphate buffer at pH 7, (B) 20 mM sodium phosphate buffer at pH 7 + 1 M NaCl; flow rate, 0.75 mL/min; gradient conditions from 100% A to 100% B in 20 min; column size, 100 × 4.6 mm i.d. for the particles produced by ATRP1 and 50 × 4.6 mm i.d. for the particles produced by ATRP2 and ATRP3; UV detection at 280 nm. Order of peaks: (1) myoglobin, (2) ribonuclease a, (3) cytochrome *c*, and (4) lysozyme. The peaks a, b belong to the impurities of ribonuclease a and cytochrome *c*, respectively.

Table 3. Effect of Mobile-Phase pH on Peak Resolution for the Columns Packed with the Particles Prepared with Different Surface-Initiated ATRP Formulations[a]

pH	R(2/1)	R(3/2)	R(4/3)
	Column Prepared by ATRP1 (DP 2.2)		
6	15.0	0.6	4.3
7	20.6	2.7	6.4
8	13.3	2.5	3.8
	Column Prepared by ATRP2 (DP 15.5)		
6	20.3	1.1	4.6
7	16.7	2.6	4.7
8	12.8	2.9	2.9
	Column Prepared by ATRP3 (DP 25.1)		
6	11.7	-	3.9
7	20.9	3.4	5.9
8	14.0	2.4	2.4

[a] Conditions: mobile phases, (A) 20 mM sodium phosphate buffer at pH 7, (B) 20 mM sodium phosphate buffer at pH 7 + 1 M NaCl, flow rate, 0.75 mL/min, gradient conditions, from 100% A to 100% B in 20 min; column size, 100 × 4.6 mm i.d. for the particles produced by ATRP1 and 50 × 4.6 mm i.d. for the particles produced by ATRP2 and ATRP3; UV detection at 280 nm. Order of peaks: (1) myoglobin, (2) ribonuclease a, (3) cytochrome *c*, and (4) lysozyme.

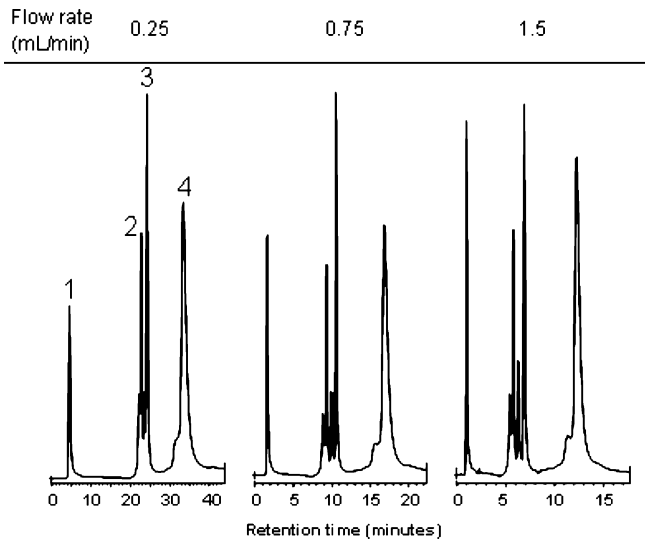

Figure 7. Sample liquid chromatograms obtained for the gradient separation of proteins with different flow rates in the columns packed with poly(SPM)-grafted poly(DHPM-*co*-EDM) particles produced with ATRP1, Conditions: flow rate (mL/min), (A) 0.25, (B) 0.75, and (C) 1.50; mobile phase, (A) 20 mM phosphate buffer at pH 7, (B) 20 mM phosphate buffer at pH 7 + 1 M NaCl; gradient conditions, from 100% A to 100% B in 20 min; column size 100 × 4.6 mm i.d.; UV detection at 280 nm. Order of peaks: (1) myoglobin, (2) ribonuclease a, (3) cytochrome *c*, and (4) lysozyme.

Table 4. Effect of Mobile-Phase Flow Rate on Peak Resolution for the Columns Packed with the Particles Prepared with Different Surface-Initiated ATRP Formulations[a]

flow rate (mL/min)	R(2/1)	R(3/2)	R(4/3)
	Column Prepared by ATRP1 (DP 2.2)		
0.50	17.0	2.3	7.0
0.75	20.6	2.7	6.4
1.00	17.5	1.7	6.5
1.50	16.6	2.9	5.6
	Column Prepared by ATRP2 (DP 15.5)		
0.25	18.4	1.5	3.5
0.50	18.0	2.0	4.1
0.75	15.3	2.0	4.1
1.00	16.7	2.5	4.7
1.50	14.9	2.4	4.3
	Column Prepared by ATRP3 (DP 25.1)		
0.25	17.4	1.4	3.2
0.50	22.6	2.8	5.3
0.75	20.9	3.4	5.9

[a] Conditions: mobile phase, (A) 20 mM phosphate buffer at pH 7, (B) 20 mM phosphate buffer at pH 7 + 1 M NaCl; gradient conditions. from 100% A to 100% B in 20 min; column size, 100 × 4.6 mm i.d. for the particles produced by ATRP1 and 50 × 4.6 mm i.d. for the particles produced by ATRP2 and ATRP3; UV detection at 280 nm. Order of peaks: (1) myoglobin, (2) ribonuclease a, (3) cytochrome *c*, and (4) lysozyme.

of this column (i.e., 25 MPa). As seen in Figure 7, no significant change was observed in the resolutions with increasing flow rate although the total analysis time was shortened ~3-fold by increasing the flow rate from 0.25 to 1.5 mL/min (Figure 7).

A trace amount of copper was found in the ion-exchange packings by ICPMS after microwave digestion of washed particles. The highest copper content was determined as 0.0355 μg of Cu/g of dry particles for the ion-exchange packing produced by ATRP1 (i.e., the formulation with the highest Cu(I) and Cu(II) concentrations). This value was too low to cause an appreciable chromatographic problem like a baseline shift or low protein recovery in the ion-exchange separation of proteins.

The variation of plate height with linear flow rate is given in Figure 8 for the columns prepared with different ATRP formulations. The column packing carrying the shortest poly(SPM) chains

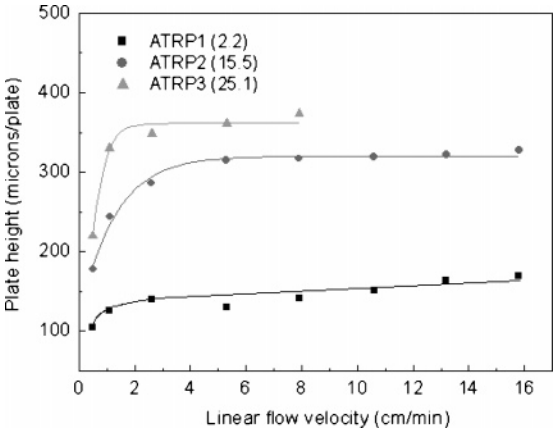

Figure 8. Variation of plate height with linear flow velocity for the columns packed with poly(SPM)-grafted poly(DHPM-*co*-EDM) particles produced with different ATRP formulations. Conditions: analyte, cytochrome *c* (0.25 mg/mL); column size, 100 × 4.6 mm i.d. for the particles produced by ATRP1 and 50 × 4.6 mm i.d. for the particles produced by ATRP2 and ATRP3; UV detection at 280 nm. Degree of polymerization of polyionic ligand on the particles is given in parentheses.

(i.e., ATRP1) provided the lowest plate height at constant flow rate. For this column, the plate heights around 125−150 μm were obtained in the linear flow rate range of 1−10 cm/min. In other words, no significant increase occurred in the plate height with increasing flow rate. A similar monomer, acrylamidopropanesulfonic acid (AMPS), was grafted onto the poly(GMA-*co*-EDM) monoliths via cerium(IV)-initiated free radical polymerization, and the resulting monoliths were used for the ion-exchange separation of proteins.[37] In the referred study, plate height values between 140 and 370 μm were obtained. A clear increase from 140 to 370 μm was observed in the plate height by increasing the linear flow rate from 0.5 to 4.0 cm/min.[37] In our case, both the plate height and the increase in the plate height with increasing flow rate were significantly lower, particularly for the column prepared with ATRP1 (Figure 8). The packings with longer poly(SPM) chains gave higher plate heights at constant flow rate. This result should be explained by band broadening, depending upon the excessive interaction of longer poly(SPM) chains on the particles with the proteins (not shown here). However, the plate height also showed no significant increase with increasing flow rate for the packings with longer polyionic ligands (i.e., ATRP2 and ATRP3). All these results indicated that the columns packed with poly(SPM)-grafted poly(DHPM-*co*-EDM) particles exhibited good performance in protein separation by ion-exchange chromatography. Hence, the grafting of a polymeric ligand onto the polymeric particles via surface-initiated ATRP is a new, efficient strategy in the preparation of an ion-exchange support.

The protein recoveries in the columns packed with poly(SPM)-grafted poly(DHPM-*co*-EDM) particles prepared with different ATRP formulations are given in Table 5. As seen here, the protein recovery was higher and almost quantitative for most of the proteins for the column including the particles with the shortest polyionic ligand. The slight decrease occurred in protein recovery for the packings prepared by ATRP2 and ATRP3 should be probably explained by the increasing number of interaction sites on the longer poly(SPM) chains with the proteins.

Table 5. Protein Recoveries for the Columns Packed with Poly(SPM),Grafted Poly(DHPM-*co*-EDM) Particles Obtained with Different ATRP Formulations[a]

protein	ATRP1 (DP 2.2)	ATRP2 (DP 15.5)	ATRP3 (DP 25.1)
myoglobin	94	87	88
ribonuclease a	97	90	90
cytochrome *c*	100	96	94
lysozyme	102	99	94

[a] Conditions: pH 7, 20 mM phosphate buffer + 1 M NaCl; protein concentration, 1 mg/mL; flow rate, 0.75 mL/min.

Table 6. Reproducibility of Ion Exchangers Synthesized with Different ATRP Formulations[a]

column reproducibility	analyte	run-to-run reproducibility RSD (%)	day-to-day RSD (%)
ATRP1 (DP 2.2)	myoglobin	0.66	0.18
	ribonuclease a	0.69	0.47
	cytochrome *c*	0.55	0.35
	lysozyme	0.38	0.60
ATRP2 (DP 15.5)	myoglobin	0.87	0.82
	ribonuclease a	0.40	0.73
	cytochrome *c*	0.22	0.89
	lysozyme	0.32	0.40
ATRP2 (DP 25.1)	myoglobin	0.30	0.33
	ribonuclease a	0.22	0.56
	cytochrome *c*	0.10	0.60
	lysozyme	0.37	0.43

[a] Conditions: mobile phase:, (A) 20 mM phosphate buffer at pH 7, (B) 20 mM phosphate buffer at pH 7 + 1 M NaCl; gradient conditions. from 100% A to 100% B in 20 min; column size, 100 × 4.6 mm i.d. for the particles produced by ATRP1 and 50 × 4.6 mm i.d. for the particles produced by ATRP2 and ATRP3; UV detection at 280 nm; flow rate, 0.75 mL/min for the particles produced by ATRP1 and 0.5 mL/min for the particles produced by ATRP2 and ATRP3.

To determine run-to-run and day-to-day precision in retention time, the protein mixture containing each protein at a concentration of 1 mg/mL was injected into the each column 10 times/day during a period of one week. The relative standard deviation (RSD) values of run-to-run and day-to-day reproducibilities obtained in the gradient separation of proteins are given in Table 6. Here, run-to-run reproducibility values were given for the runs performed in the third day. As seen in Table 6, both run-to-run and day-to-day reproducibility values were lower than 1% for all columns. Hence the ion-exchange stationary phases carrying poly(SPM) chains with different lengths exhibited satisfactory reproducible retention behavior in the gradient separation of proteins.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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