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Chromatographic Performance of Weak Cation Exchanger Carrying Molecular Brushes Synthesized by Atom Transfer Radical Polymerization

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Abstract – The column materials in the form of monodisperse and porous particles were synthesized with regard to test their column performances in Ion Exchange Chromatography (IEC) mode. Particles in the form of poly(glycidil methacrylate-co-ethyleneglycol dimethacrylate) poly(GMA-co-EDM) copolymer used for starting materials in the synthesis, were maintained by "multi-stage microsuspension polymerization". Particle characterization was assessed by Scanning Electron Microscopy (SEM), BET surface area and porosity measurement system and FTIR spectroscopy. Weak cation-exchange carboxyl (-COOH) group on monodisperse and porous poly(DHPM-co-EDM) particles, used for ion-exchange chromatography, was synthesized by "Atom Transfer Radical Polymerization" (ATRP) method. The investigation of column performance concluded that weak cation-exchanged columns, synthesized by ATRP (150-300 µm) showed low and stable theoretical plate heights and high peak resolution values compared to literature knowledge. Protein recovery data in ion-exchange column, which is synthesized by ATRP (94-100%) polymerization technique were determined as similar as quantitative values for all proteins. *"Nano-scale change"* of ligand length determined the chromatographic performance of functional based ion-exchanged stable phase. Chromatographic studies demonstrated that the length of polymeric ligand, which can be controlled by ATRP polymerization, affected decrease of retention time without any change of column reverse pressure values, chromatographic separation ability (peak resolution and plate height) and analyte recovery from column.

Keywords – Atom Transfer Radical Polymerization (ATRP), High Performance Liquid Chromatograpy (HPLC), Ion Exchange Chromatography (IEC), Monodisperse and porous polymeric particles

I. INTRODUCTION

High Performance Liquid Chromatography (HPLC) is one of the most widely used chromatographic separation techniques. This technique is based on the principle that the analytes dissolved in the liquid phase diffuse at the end of the interaction with the column packing material at different times. In HPLC columns, microparticles prepared in silica or polymeric form are used as packing material. Research on HPLC column packing materials in recent years has concentrated on polymeric particles due to the fact that the synthesis of spherical, porous and monodisperse forms is more varied and easier than the particle derivatization method and processes. Generally, commercially produced HPLC columns are composed of porous particles of different size distribution (polydisperse). This causes channeling in the column and decreases the chromatographic performance. In the presence of monodisperse porous particles, which are described as a new generation of chromatographic packing materials, high chromatographic separation ability can be obtained due to the regular flow profile formed in the column. The method of producing these particles is generally referred to as Multi Step Polymerization [1-5]. In many types of HPLC applications (ion exchange chromatography, hydrophobic interaction chromatography, normal phase chromatography, affinity chromatography, reverse phase chromatography), columns of polar or different polarity are used. In practice, polar columns are needed in situations where analytes are difficult to leave the column, shortening the analysis period, preventing non-specific interactions [6-10]. Ion-Exchange Chromatography (IEC) columns are produced by ion exchange groups on a silica or polymer based support, where polar particles are required. The most preferred ionic groups at constant phases are strong cation exchange sulfonic acid ($-SO_3H$), weak cation exchange carboxylate (-COO-), strong anion exchange quaternary ammonium and weak anion exchange diethylamino ($-N(C_2H_5)_2$). The ligands containing these groups are either directly attached to the silica or polymer-based stationary phase bound by a spacer arm. Particularly in the presence of high molecular weight analytes, the limited analyt-ligand interaction resulting from steric or electrostatic hindrance and the resulting ineffective chromatographic separation is one of the major problems encountered in ion exchange chromatography. In recent years, studies on stationary phase synthesis for ion-exchange chromatography on HPLC have shown that ligand synthesis in polymeric form, which is more easily interacted with analytes to be chromatographically separated in a long chain and flexible structure, is a new alternative method instead of directly linked ion-exchange ligand has emerged. It has been thought that the use of polymeric ligands and constraints arising from ligand-analyte interaction can be eliminated. In recent studies, new surface derivatization methods have been developed on the basis of "Free Radical Polymerization" and "Living / Controlled Polymerization" for the polymeric ligand molecule and the strong cation exchanger "sulphonic acid" and weak anion exchanger " dimethylamino "groups have been successfully

used in protein separation by ion exchange chromatography [10].

In the scope of the present study, a monodisperse porous weak cation-exchange polar column support material suitable for chromatographic analysis of proteins for Ion Exchange Chromatography (IEC) was synthesized by Atom Transfer Radical Polymerization (ATRP).

II. Materials and Method

IEC column packed materials were synthesized with "Atom Transfer Radical Polymerization" (ATRP) which is one of the "Living Polymerization" methods. Poly (methacrylic acid) poly(MAA) ion-exchange ligand synthesis was carried out in the weak cation-exchange form which can adjust the molecular length with ATRP on poly (DHPM-co-EDM) microspheres. The final particles were tested for protein separation as IEC column packing material in High Performance Liquid Chromatography.

A. Production of Monodisperse and Porous HPLC Column Packed Materials

First, polystyrene (PS) seed latex was produced by dispersion polymerization method for poly (DHPMA-EDM) monodisperse particle synthesis. In the production of the seed latex by dispersion polymerization, styrene (S, Yarpet, Kocaeli, Turkey) as the monomer, absolute ethyl alcohol (Et-OH, Merck AG, Germany) and 2-methoxyethanol (HPLC grade, Met-OH, Aldrich Chemical Co 2-azobisisobutyronitrile (AIBN, BDH Chemicals LTD., UK) as the initiator and polyvinylprolidone K-30 (Mr: 40000, PVP K-30, Sigma Chemical Co., USA) as the stabilizer. A total of 120 ml of a solution containing 60% ethanol and 40% 2-methoxyethanol was prepared as the dispersion medium for polymerization carried out in a sealed cylindrical glass reactor with a volume of 250 ml. PVP K-30 dissolved in the dispersing medium. AIBN and styrene was added to the dispersion medium. Dispersion polymerization was carried out at a polymerization temperature of 70 ° C for 24 hours at a shaking speed of 120 cpm.

Secondly, Cyclohexanol (Cyc-OH, Aldrich Chemical Co., USA) and dibutylphthalate (DBP, Aldrich Chemical Co., USA) were used as a diluent for the production of monodisperse-porous poly (GMA-co-EDM) microspheres by the Multi-step Polymerization method. (GMA, Aldrich Chemicals Co., USA) and ethyleneglycol dimethacrylate (EDM, Aldrich Chemicals Co., USA) as monomeric and crosslinking agents, respectively, as anionic emulsifying agent, sodium dodecyl sulfate (SDS, Sigma Chemicals Co., used. 2-2'-azobisisobutyronitrile (AIBN, BDH Chemicals Ltd., England) as a initiator, and polyvinylalcohol (PVA, MA: 87000-146000, 87% hydrolyzed, Aldrich Chemicals Co., USA) as a stabilizer. A homogeneous organic phase eluent is provided by stirring Cyc-OH (14 mL), DBP (5.4 mL), GMA (6.4 mL), EDM (4.2 mL) and AIBN (0.32 g) for the synthesis of the particles. This phase is dispersed in aqueous medium (360 mL) containing SDS (0.60 g) and PVA (2.8 g) for 30 minutes. Polystyrene latex (0.70 g) is added to the resulting emulsion. The mixture is stirred at room temperature for 24 hours at a magnetic speed of 250 rpm. At the end of this time, the polymerization is completed in a shaking water bath for 24 hours at a shaking speed of 120 cpm at 70 ° C. The particles were extracted with THF in a shaking water bath at 50 ° C for 4 hours.

As the third step, the poly (GMA-co-EDM) copolymer obtained as a result of the polymerization is hydrolyzed with sulfuric acid (H2SO4, Merck A.G., Germany). The particles are first dispersed in 50 ml of 0.5 M 95% H2SO4 solution in 500 ml of pyrex. The hydrolysis is carried out in a shaker water bath at 60 ° C for 12 hours.

As a final step, 3- (2-bromoisobutyramido) propyl (triethoxy) silane (BIBAPTES) was used as initiator in polyionic ligand synthesis with ATRP on poly (DHPM-co-EDM) particles. The following method was used for BIBAPTES synthesis [10]. At the end of the reaction period, BIBAPTES bound poly (DHPM-co-EDM) particles were washed first by THF followed by sequential centrifugation-decantation with ethanol and water. While methacrylic acid (MAA, Aldrich) was in Schlenk tubular, the pH of the reaction medium was adjusted to 9 with sodium hydroxide solution. This was added with CuBr (Aldrich) and bipyridine (BPy, Aldrich). In the medium, poly (DHPM-co-EDM) particles were added and a final pH of 9 was achieved. The reaction medium was washed with N_2 for 5 minutes then vacuumed for 10 minutes and ligated with poly (MAA) ligand ATRP on poly (DHPM-co-EDM) particles, pyrex at 85 ° C in a Schlenk reactor for 6 hours in a water bath at 120 cpm agitation is still in progress during the polymerization. After polymerisation, the particles were washed by sequential centrifugation-decantation with ethanol and distilled water.

B. Characterization of Monodisperse and Porous HPLC Column Packing Materials

The poly (gylcidyl methacrylate-co-ethyleneglycidyl dimethylacetate) (poly (GMA-co-EDM)) particles obtained by the "Multi-Step Suspension Polymerization" technique are hydrolyzed in an acidic medium and transformed to poly (2,3-dihydroxypropylmethacrylate-ethyleneglycoldimethacrylate) -co-EDM) particles were formed. The formation of this form is evidenced by the hydroxyl (-OH) band of dihydroxypropylmethacrylate (DHPM) units in the FTIR spectrum of the structure obtained before and after hydrolysis.

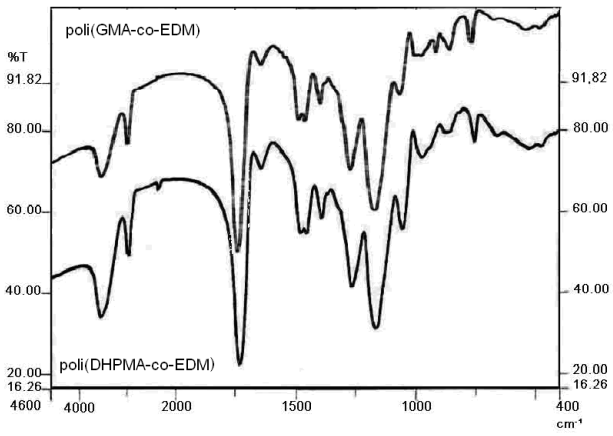

Fig. 1 The FTIR spectrum of poly (GMA-co-EDM) and poly (DHPM-co-EDM)

The mean particle size and size distribution of monodisperse and porous particles were determined by scanning electron microscope (SEM) (JEOL, JEM 1200EX, Japan).

(BET) device (Quantachrome, Nova 2200E, UK) was used to determine the surface area and average pore size and distribution of the particles.

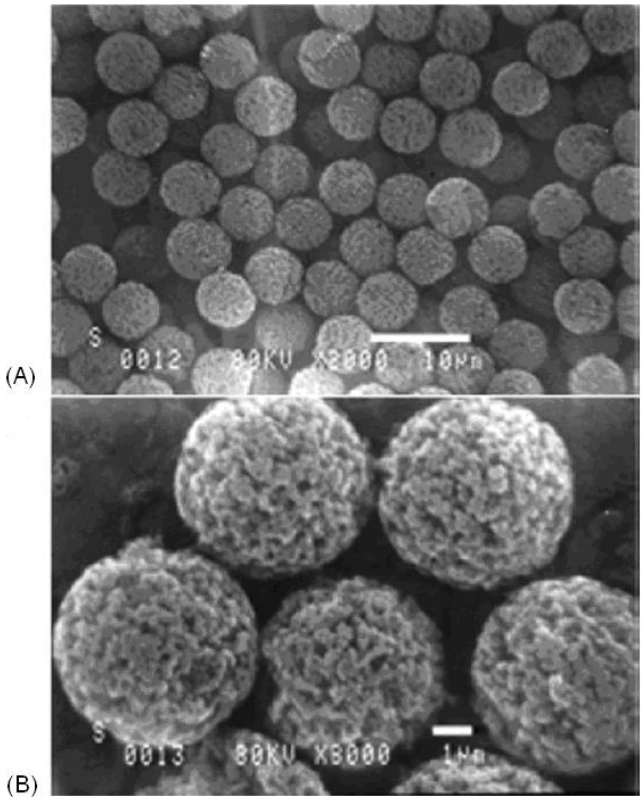

Fig. 2 The SEM photos of poly (DHPM-co-EDM) (A) 2000X, (B) 8000X.

C. Chromatographic performance tests

Steel columns (Schimadzu, Japan) having an inner diameter of 4.6 mm and a length of 100 mm were used in the study. The columns are filled at about 200 atm.

A sample mixture containing 4 different proteins (Myoglobin, Ribonuclease A, Cytochrome C and Lysozyme) (Sigma) was used as an analyte in chromatographic performance tests. The isoelectric point (pI) values are taken into consideration in the selection of these proteins. It is aimed that the prepared columns can perform chromatographic separation at a wide range of pI as possible. In the experiments performed in cation exchange chromatography mode, different buffers were used at different pH as the mobile phase.

D. Determination of Chromatographic Parameters

The peak resolution, $R_{(n+1/n)}$, for a given protein in the resulting chromatograms is calculated according to equation (1). Where $R_{(n+1/n)}$ is the resolution between the selected peak (n + 1) and the previous peak (n). t_{n+1} and t_n are the retention times for peak n + 1 and peak n, respectively, from the injection point. W_{n+1} and W_n represent the base width for peak n + 1 and peak n, respectively.

$$R_{(n+1)/(n)} = 2\,[(t_{n+1} - t_n) / (W_n + W_{n+1})] \qquad (1)$$

Theoretical plate number and height values were calculated in isocratic mode and the chromatographic experiments using cytochrome C as the analyte were calculated.

III. Results and Discussion

Identification by FTIR: The poly (GMA-co-EDM) particles obtained by the "Multi-Step Suspension Polymerization" technique are converted into poly (DHPM-co-EDM) by hydrolysis in acidic medium. The formation of this form is evidenced by the strong hydroxyl (-OH) band observed in the FTIR spectrum of the hydrolysed particles at a wavelength of 3500 cm-1 belonging to DHPM units. The FTIR analysis of the structure obtained before and after hydrolysis is given in Figure 1.

Identification by Scanning Electron Microscopy: Size and surface morphology of poly (DHPM-co-EDM) particles were investigated by scanning electron microscope (SEM). SEM photographs showing the size distribution of the particles and surface morphology are given in Figure 2. Using the SEM photographs given, the coefficient of variation (CV) for average particle size (5.8 μm) and size distribution (5.8%) was calculated. In the SEM photographs of Figure 2B, it is clear that the particle surface and the surface have a porous structure.

Identification with BET Surface Area and Pore Size Measuring Device: The average pore size, pore volume, and specific surface area for poly (DHPM-co-EDM) particles were measured using a BET device. The average pore size is 40 nm, the pore volume is 0.34 mL/g and the specific surface area is 37 m^2/g.

Poly(MAA) grafted-poly(DHPM-co-EDM) particles by ATRP as Column Support Material in IEC: Chromatograms obtained with different flow rates for poly (MAA) grafted particles produced by ATRP are given in Figure 3. Experiments showed that the column was capable of separating all analytes in as little as 12 minutes at a flow rate of 1 mL / min. Higher performance of the column is an indication of the increased flow rate and the pixels do not overlap each other and maintain their resolution.

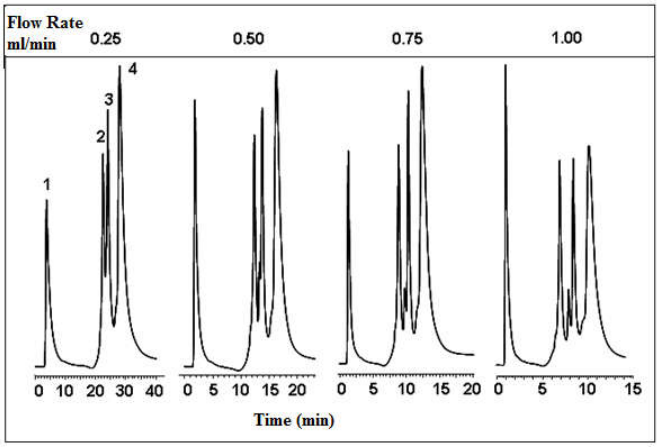

Fig. 3 Sample chromatograms showing protein separation under gradient conditions by ion exchange chromatography using column containing poly (MAA) -grafted poly (DHPM-co-EDM) particles synthesized by ATRP Mobile phase: A: 20 mM HEPES buffer (pH 8), B: 20 mM HEPES buffer (pH 8) + 1 M NaCl, Gradient conditions: 100% A to 100% B in 20 min. Column dimensions: 100x4.6 mm i.d., UV-detector 280 nm. Peak order: (1) Myoglobin, (2) Ribonuclease A, (3) Cytochrome C, (4) Lysozyme.

The peak resolution values obtained at different flow rates of the column are given in Table 1. The time of analysis could be shortened by about three times while maintaining the chromatographic separation ability by increasing the flow rate from 0.25 mL/min to 1.0 mL/min.

Table 1. Effect of mobile phase flow rate on chromatographic behavior of poly (MAA) grafted poly (DHPM-co-EDM) column produced by ATRP

Flow Rate (ml/min)	R(2/1)	R(3/2)	R(4/3)
0.25	9.1	0.8	1.1
0.50	9.2	1.1	1.3
0.75	9.4	1.3	1.4
1.00	10.4	1.7	1.4

As is known, two important criteria for chromatographic performance and separation power for HPLC columns are the theoretical number of plate and the plate height. The lower value of the layer height indicates that the separation efficiency of the column is higher. With the column produced by ATRP a sufficiently low layer height (high separation yield) values were obtained. When the linear flow rate value is changed in the range of 1-10 cm/min, the layer height value for this column varies between 150-280 µm (Figure 4).

Protein recovery values of poly (MAA) grafted poly (DHPM-co-EDM) particles produced by ATRP range from 94 to 100%. The protein recovery rate is important for repeated use for a column used in ion exchange chromatography. If this ratio is low, the injected analyte is adsorbed by the column and regeneration becomes difficult. The unregulated analyte in the pores causes the back pressure of the column to increase and the column to become clogged. Currently, the protein recovery rates of commercial columns are generally above 90%.

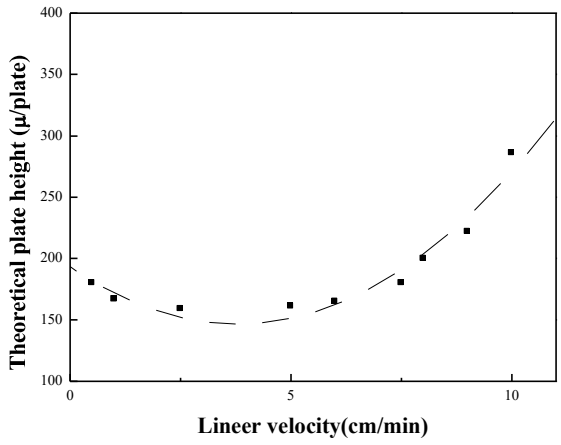

Fig. 4 Alteration of plate height by linear flow rate for columns containing poly (MAA) -grafted poly (DHPM-co-EDM) particles obtained by ATRP. Analyt: Cytocrom C (0.25 mg / mL), Column dimensions: 100x4.6 mm i.d., UV detector, 280 nm. Mobile phase: pH 8, 20 mM HEPES buffer + 1 M NaCl

In the last part of the chromatographic studies, analytical run-to-run and day-to-day reproducibility tests of the analytes for the column produced by ATRP were performed. The relative standard deviation values (BSS) obtained from the analysis "day to day" and "analysis to analysis" range from 0.17-0.57% and 0.14-0.59%, respectively. BSS values below 1% are an indication that this column can be reliably used several times.

IV. CONCULISION

These results show that ATRP-synthesized column-packed materials can be used for ion exchange chromatography.

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