ISMSIT 2017
1st International Symposium on Multidisciplinary Studies and Innovative Technologies Proceedings
(November 2-4, 2017, Tokat, Turkey)
Symposium homepage: www.ismsitconf.org

Chromatographic Performance of Weak Cation Exchanger Synthesized by a Simple Method

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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 a simple method. The particles were hydrolyzed with trifloroacetic acid (TFA) in order to generate carboxyl groups on their surfaces. By use of this approach, a new column type having small ion-exchange ligands, are maintained. The investigation of column performances concluded that weak cation-exchanged column, synthesized by acidic hydrolysis (200-350 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 acidic hydrolysis (AH) (94-99%) polymerization technique, was determined as similar as quantitative values for all proteins. Reproducibility tests of analytes of poly(MAA) column was assessed "during analysis and day by day". The BSS values in column, synthesized by acidic hydrolysis, were determined as 2.51% and 1.75%, respectively. Results showed that synthesized column material can be successfully used in ion-exchange chromatography.

Keywords – Acidic hydrolysis (AH), 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 [6-9]. 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, 11]. However, these methods are time consuming, costly and difficult methods.

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 a simple method such as Acidic hydrolysis (AH).

II. Materials and Method

IEC column packed materials were synthesized with "Acidic hydrolysis" (AH) as a simple method. 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 AH 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 (H_2SO_4, Merck A.G., Germany). The particles are first dispersed in 50 ml of 0.5 M 95% H_2SO_4 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, synthesized Poly (DHPM-co-EDM) particles (1.50 gr) were reacted in methylene chloride (10 mL) with TFA (0.6 mL) in the second hydrolysis step for 24 hours at room temperature. Interaction with TFA, a strong acid, provided that the diol-containing ester form was converted to the carboxylic acid form. This reaction was first used in the literature for the synthesis of ion-exchange column material.

After polymerization, the particles were washed by sequential centrifugation-decantation with ethanol, then 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.

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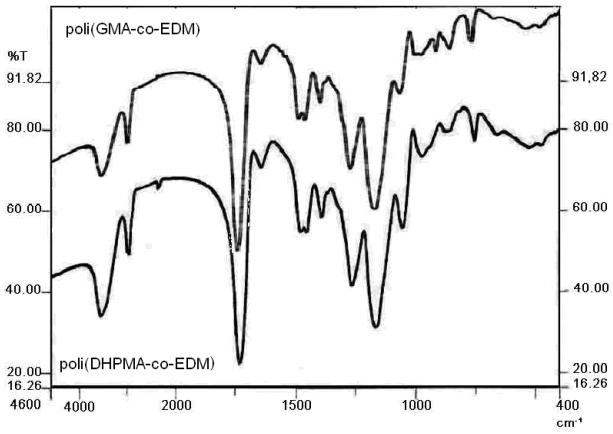

Fig. 1 The FTIR spectrum of poly (GMA-co-EDM) and poly (DHPM-co-EDM)

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.

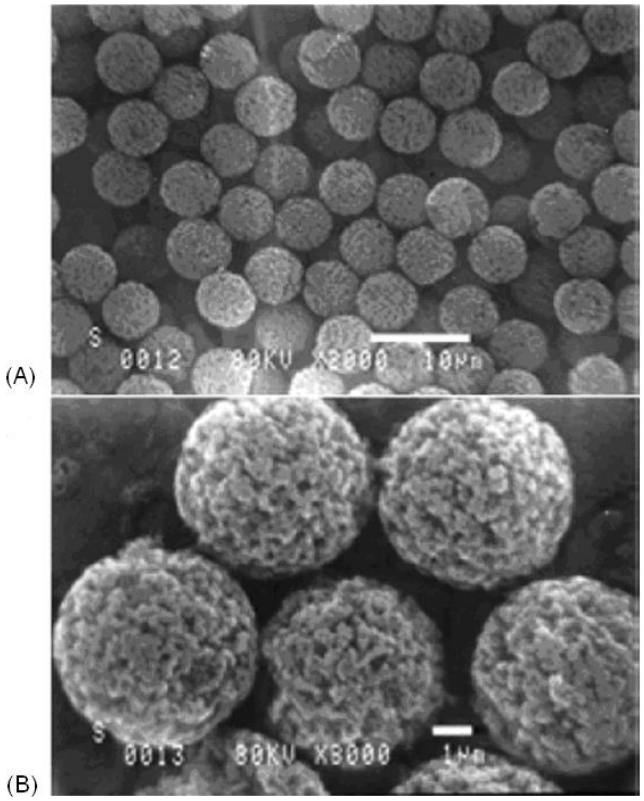

Fig. 2 The SEM photos of poly (DHPM-co-EDM) (A) 2000X, (B) 8000X.

C. Chromatographic performance tests

Steel columns (Schimadzu, Japan) having an inner diameter of 4.6 mm and a length of 50 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 AH as Column Support Material in IEC: A reference column material was synthesized for comparison with the ion exchange support materials bearing the polymeric ligand in the weak cation exchanger form. For this purpose, monodisperse-porous poly (DHPM-co-EDM) particles of 5.8 μm size were reacted with TFA and carboxyl groups were formed by hydrolysis of DHPM units on the particle surface.

In the literature, there are methods developed for the synthesis of ion exchangers from poly (GMA-co-EDM) particles [10-11]. But the methods and forms of implementation are quite complex. However, the method proposed in this study ensures that the carboxyl group is formed directly on the carrier by a single reaction. After hydrolysis, the carboxyl content of the porous particles was determined as 3.81 mmol/g dry particle by potentiometric titration.

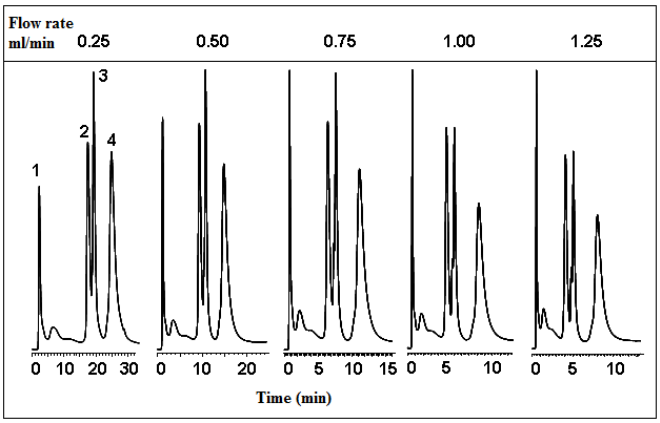

Fig. 3 Sample chromatograms showing protein separation under gradient conditions by ion exchange chromatography using column containing poly (MAA) -grafted poly (DHPM-co-EDM) particles synthesized by AH Mobile phase: A: 20 mM HEPES buffer (pH 8), B: 20 mM HEPES buffer (pH 8) + 1 M NaCl, Gradient conditions: 100% A to 100% B in 20 min. Column dimensions: 50x4.6 mm i.d., UV-detector 280 nm. Peak order: (1) Myoglobin, (2) Ribonuclease A, (3) Cytochrome C, (4) Lysozyme.

Chromatograms obtained with different flow rates for poly (MAA) grafted particles produced by AH are given in Figure 3. Experiments showed that the column was capable of separating all analytes in as little as 10 minutes at a flow rate of 1.25 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.

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 1.25 mL / min

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

Flow Rate (ml/min)	R(2/1)	R(3/2)	R(4/3)
0.25	10.5	1.1	2.1
0.50	10.3	1.2	2.4
0.75	9.1	1.3	2.5
1.00	8.7	1.4	2.5
1.25	8.1	1.4	2.6

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 AH a sufficiently low layer height (high separation yield) values were obtained. The layer height value for this column varies between 200-350 μm (Figure 4).

Protein recovery values of poly (MAA) grafted poly (DHPM-co-EDM) particles produced by AH range from 94 to 99%. 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%.

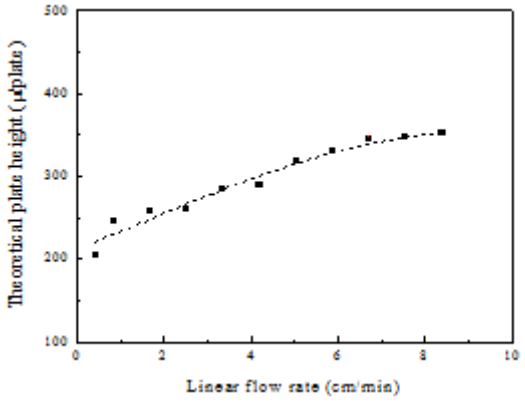

Fig. 4 Alteration of plate height by linear flow rate for columns containing poly (MAA) -grafted poly (DHPM-co-EDM) particles obtained by AH. Analyt: Cytocrom C (0.25 mg / mL), Column dimensions: 50x4.6 mm i.d., UV detector, 280 nm. Mobile phase: pH 8, 20 mM HEPES buffer + 1 M NaCl

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 AH were performed. The relative standard deviation values (BSS) obtained from the analysis "day to day" and "analysis to analysis" range from 0.92-1.75% and 0.95-2.31%, respectively. BSS values below 1% are an indication that this column can be reliably used several times.

IV. Conclusion

These results show that AH-synthesized column-packed materials can be used for ion exchange chromatography. However, it is thought that the column packing material can not be used efficiently in the long run.

References

[1] Ellingsten T., Aune O., Ugelstad J., Hagen S., "Monosized Stationary Phases For Chromatography", *Journal of Chromatography*, 535, 147-161, 1990.

[2] Wang j., Matyjaszewski K., "Living"/Controlled Radical Polymerization. Transition-Metal-Catalyzed Atom Transfer Radical Polymerization in the Presence of a Conventional Radical Initiator", *Macromolecules*, 28, 7572-7573, 1995.

[3] Galia M., Svec F., Frechet J. M. J., "Monodisperse Polymer Beads As Packing Material For High Performance Chromatography: Effect of Divinylbenzene Content on the Porous and Chromotagraphic Properties of Poly(styrene-co-divinylbenzene) Beads Prepared in the Presence of Lineer Polystyrene as a Porogen", *Journal of Polymer Science: Part A: Polymer Chemistry*, 32, 2169-2175, 1994.

[4] Svec F., Frechet J. M. J., "New Designs of Macroporous Polymers and Supports: From Seperation to Biocatalysis", *Science,* 273, 205-211, 1996.

[5] Hossoya K., Teramachi M., Tanaka N., Kobayashi A., Kanda T., Ohtsu Y., "Preparation Strategy for Uniformly Sized, Polymer-Beads HPLC Packing Materials Having Practically Acceptable Column Efficiency. 1. Copolymerization Technique", *Anal. Chem*., 73, 5852, 2001.

[6] Smigol V., Svec F., Frechet J.M.J., "2-Dimensional High-Performance Liquid-Chromatography Using Monodisperse Polymer Beads Containing Segregated Chemistries Prepared By Pore-Size Specific Functionalization - Single-Column Combinations Of Size Exclusion Or İon-Exchange With Reversed-Phase Chromatography", *Analytical Chemistry* 66 (23), 4308-4315, 1994.

[7] Smigol V.; Svec, F., Frechet, J.M.J., "High-Performance Liquid Chromatography of Complex mixtures Using Monodisperse Dual-Chemistry Polymer Beads Prepared by a Pore-Size-Specific Functionalization Process. A Single Column Combination of Hydrophobic Interaction and Reversed-Phase Chromatography", *Analytical Chemistry*, 66, 2129, 1994.

[8] Lewandowski K.; Svec F., Frechet J.M.J., "Polar, A Novel Polar Seperation Medium For The Size Exclusion Chromatography of Small Molecules: Uniformly Sized, Porous Poly(vinylphenol-co-divinylbenzene) Beads", *J. Liq. Chrom. & Rel. Technol*., 20, 227, 1997.

[9] Lewandowski K.; Svec F., Frechet J.M.J., "Polar, Monodisperse, Reactive Beads from Functionalized Metacrylate Monomers by staged Templated Suspension Polymerization", *Chem. Mater*., 10, 385, 1998.

[10] Unsal E., Elmas B., Çağlayan B., Tuncel M., Patır S., Tuncel A., "Preparation of An Ion Exchange Chromatographic Support By A New "Grafting From" Strategy Based on Atom Transfer Radical Polymerization", Analytical Chemistry, 78, 1-8, 2006.

[11] Unsal E., uguzdogan E., Patır S., Tuncel A., "Ion-exchanger synthesis using reversible addition-fragmentation chain transfer polymerization", Journal of Separation Science, 32, 1791-1800, 2009.