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Modified poly(glycidyl methacrylate–co-ethylene dimethacrylate) continuous rod columns for preparative-scale ion-exchange chromatography of proteins

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Abstract

A continuous rod of porous poly(glycidyl methacrylate–co-ethylene dimethacrylate) has been prepared by a free radical polymerization within the confines of a 300 × 8 mm I.D. chromatographic column. The epoxide groups of the rod have been modified by a reaction with diethylamine that affords ionizable functionalities required for the ion-exchange chromatographic mode. The properties of this rod column have been characterized and the column has been used successfully for the chromatographic separation of proteins. The column exhibits a dynamic capacity that exceeds 300 mg at a flow velocity of 200 cm/min. An excellent selectivity allows the separation of up to 300 mg of a protein mixture in a single run.

1. Introduction

The inherent problem of all of particulate separation media is the inability to completely fill the space within the chromatographic column. Generally, interparticular porosity contributes to peak broadening and decreases column efficiency. The limited utilization of column space also affects the capital investment costs particularly in preparative-scale separations because the column volume must be much larger than the volume of the separation medium itself.

The column void volume can be decreased or even eliminated when media with a higher degree of continuity are used. Polymerized porous discs [1] or stacked cellulose membranes [2] placed in a cartridge that simulates the function of a chromatographic column exhibit almost no interstitial porosity. However, their efficiency is reduced as a result of non-uniform flow velocity across the membranes due to the cartridge geometry. Rolled cellulose sheets [3] or woven matrices [4] placed in the tube of a chromatographic column can be used successfully for the separations of proteins that can be fractionated using an on–off mode but they have only limited efficiencies. The open-pore silica or polyurethane foams formed directly within a column did not prove to provide sufficient chromatographic properties for normal-phase separations [5,6]. Swollen polyacrylamide gels were compressed in the shape of a column and used successfully for the HPLC separation of proteins and peptides [7–9].

We have introduced HPLC separation media with no discontinuity that consist of a continuous "molded" rod of rigid, highly porous polymer. These rods, that are essentially the equivalent of

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a very large single cylindrical particle, are prepared in a single step by a free-radical polymerization directly within the confines of a chromatographic column acting as a mold [10–13]. The concept of molded separation media was verified with relatively short, 50–100 mm long columns that contained modified poly(glycidyl methacrylate–co-ethylene dimethacrylate) [11] or poly(styrene–co-divinylbenzene) rods [12,13]. These proved to be very efficient for the extremely fast reversed-phase HPLC separation of proteins [12] and for the separation of small and mid-size molecules [13].

Subsequently, Matsui et al. [14] used our approach for the preparation of continuous rods of molecularly imprinted polymers and showed their capabilities for molecular recognition in a series of separations of positional isomers and enantiomers. Most recently, Sellergren [15] imitated this approach.

The most important feature of the continuous media is the absence of interparticular volume. Therefore, *all* of the mobile phase is forced to flow *through* the large pores of the separation medium that substitute the column porosity and allow the mobile phase to flow through. According to theory [16–18], mass transport is enhanced by such a convection and has a positive effect on the chromatographic separation [19–21].

The separations achieved with the short columns as well as the low pressure drops along the column and ease of the preparation of the rods led us to prepare a longer rod column for use in the preparative-scale separation of larger amounts of proteins. This communication reports preliminary results obtained with this continuous rod column.

2. Experimental

2.1. Materials

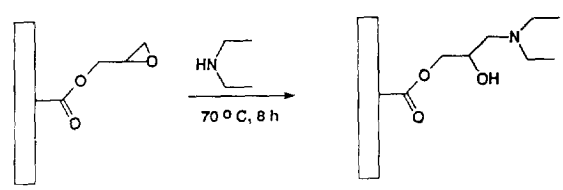
The glycidyl methacrylate and ethylene dimethacrylate (Sartomer, Exton, PA, USA) were distilled under vacuum. Azobisisobutyronitrile (AIBN) was obtained from Kodak, cyclohexanol and dodecyl alcohol from Aldrich. The proteins were purchased from Sigma. All solvents were HPLC grade.

2.2. Preparation of the continuous column

The continuous column was prepared by an in situ polymerization within the confines of the stainless-steel tube of a 300×8 mm I.D. chromatographic column. The 40:60 (v/v) mixture of monomers (glycidyl methacrylate and ethylene dimethacrylate, 60:40, v/v) and porogenic diluents (cyclohexanol and dodecanol, 90:10, v/v) in which AIBN (1%, w/v, with respect to monomers) was dissolved, was purged with nitrogen for 15 min. The stainless-steel tube was filled with the above mixture and then sealed at both ends with pieces of polyethylene foil covering rubber septa. The polymerization was allowed to proceed at 55°C for 12 h. The seals were removed, the column was provided with fittings, attached to the HPLC system and heated to 45°C. Tetrahydrofuran (THF) (100 ml) was pumped through the column at a flow-rate of 0.5 ml/min to remove the alcohols and other soluble compounds present in the polymer rod after the polymerization was completed.

2.3. Preparation of amino-functionalized porous rod

The epoxide groups of the polymerized glycidyl methacrylate were allowed to react with diethylamine according to the reaction:



Diethylamine (20 ml) was pumped through the column for 1 h at a flow-rate of 0.33 ml/min. The column was then removed from the chromatographic system, sealed at both ends with plugs and heated in a bath to 70°C for 8 h. The modified column was then reattached to the chromatograph and washed at a flow-rate of 0.5 ml/min first with water for 14 h and then with a

0.01 *M* Tris–HCl buffer solution at pH 7.6 for another 3 h. The content of diethylamino groups determined by elemental analysis was 3.24 mmol/g.

2.4. Characterization of pore properties.

After all of the chromatographic experiments has been completed, the rod was washed with water (2 h at 0.5 ml/min), the bottom column fitting was removed and the polymer rod was pushed out of the tube using pressure of the mobile phase. About 5 cm of the 30 cm long cylinder was cut into small pieces and dried at 70°C. The specific surface area of the macroporous polymer rod was calculated from the BET isotherm of nitrogen; the pore size distribution in the dry state was determined by mercury porosimetry using an automated custom-made combined BET-Sorptometer and mercury porosimeter from Porous Materials, Ithaca, NY, USA.

2.5. Chromatography

A Waters HPLC system consisting of two 501 HPLC pumps, a 707 plus autosampler, and a 486 UV detector, was used to carry out all the chromatography. The data were acquired and processed with Millenium 2010 software (Waters). A solution of chicken egg albumin in 0.01 *M* Tris–HCl buffer (15 mg/ml) was used for frontal analysis and dynamic capacity determination.

3. Results and discussion

The ion-exchange chromatographic mode is often used in large-scale processes for the initial and intermediate purification of proteins. Glycidyl methacrylate-based polymers can be easily functionalized to afford separation media with ionizable groups that are well suited for ion-exchange chromatography. Though the chemistry itself is very important, it is not the only feature required for a successful separation. There are many other requirements that must also be met when designing a separation medium, particularly for large-scale chromatography. These are related to both the material, including porosity, pore size distribution, rigidity and resistance to alkaline solutions, and the chromatographic properties, including dynamic capacity, selectivity, equilibration rate, etc.

3.1. Porous properties of the continuous rod column

Based on an extensive study of the polymerization mechanism of monomer–porogen mixtures in closed molds that will be published elsewhere, we have chosen polymerization conditions that afford a rod with a bimodal pore size distribution typical for the short molded columns. Fig. 1 shows the pore size distribution profile for the rod prepared by a polymerization at 55°C. The total pore volume of the porous polymer is 1.0 ml/g and translates to a porosity of about 55%. According to the mercury porosimetry, the pores in the range of from 50 to 500 nm and those smaller than 50 nm represent 12 and 13% of the total pore volume, respectively. More than 75% of all of the pores have a diameter in the range of 500–2200 nm. These pores are the channels through which the mobile phase flows. Their volume and size are sufficiently large to ensure a modest flow resistance

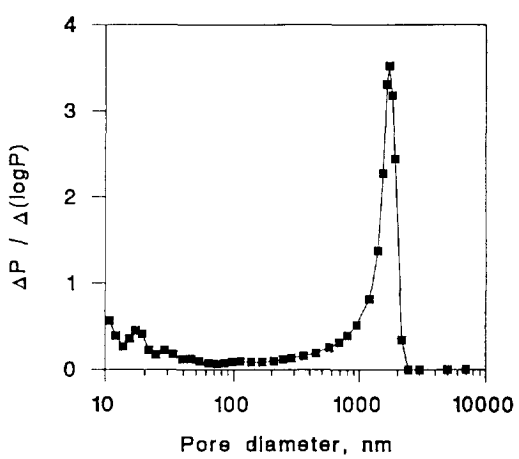


Fig. 1. Differential pore size distribution curve of the modified poly(glycidyl methacrylate–co-ethylene dimethacrylate) rod measured by mercury intrusion porosimetry.

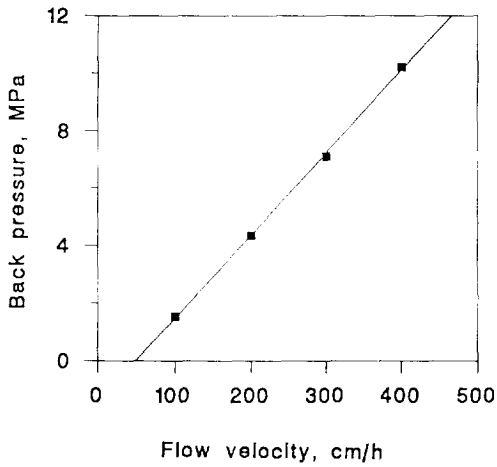


Fig. 2. Effect of flow velocity on back pressure in the continuous poly(glycidyl methacrylate–co-ethylene dimethacrylate) column. Conditions: column 300 × 8 mm I.D.; mobile phase 0.01 *M* Tris–HCl buffer.

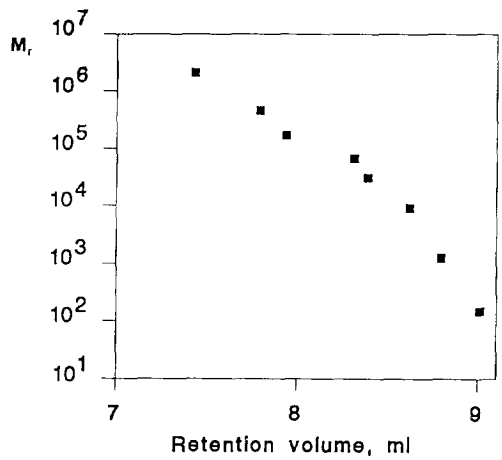


Fig. 3. Size-exclusion chromatography calibration curve of the continuous poly(glycidyl methacrylate–co-ethylene dimethacrylate) 300 × 8 mm I.D. rod column. Conditions: flow-rate 0.5 ml/min; mobile phase tetrahydrofuran; UV detection at 254 nm.

for the 300 mm long column. For example, a back pressure of only 10.2 MPa is obtained at a linear flow velocity of 400 cm/h (Fig. 2). The linearity of the back pressure vs. flow velocity dependency as measured using a buffer solution as the mobile phase clearly documents the rigidity of the long rod that easily withstands pressures higher than 10 MPa.

The size-exclusion calibration curve for the rod as measured with amylbenzene and polystyrene standards in THF shown in Fig. 3 is another tool used for the description of the porous structure in the range of smaller pores. It has a shape similar to that typically found for porous separation media. The mercury porosimetry data measured in the dry state indicate that the volume of pores with a diameter of 10–300 nm represents only about 0.2 ml/g of the polymer or about 1.2 ml for the entire 300 mm long column. This correlates well with the column effective pore volume of 1.6 ml in which the molecules of standards with molecular masses from 140 (amylbenzene with an equivalent sphere diameter of about 1 nm) to 2 100 000 (polystyrene with the diameter of about 300 nm) have been separated. The somewhat larger pore volume determined from the calibration curve includes also pores smaller than 10 nm that are not accessible during measurements by mercury porosimetry.

The specific surface area measured by nitrogen adsorption was found to be 295 m^2/g. This surface area is mainly due to the smallest pores of the rod.

3.2. Chromatographic properties

Breakthrough curve and dynamic capacity

The frontal analysis, which provides the breakthrough curve, serves as a tool for the evaluation of mass transport kinetics. In an ideal case, the breakthrough curve, that shows the solute concentration at the column outlet vs. the total volume passed through the column, is almost vertical. If the saturation rate of the separation medium with the solute of interest is slower than the flow-rate, the curve is less steep. The short rod columns have proved to have excellent mass transfer characteristics [12] with sharp breakthrough profiles independent of the flow velocity within a very broad range. The 300 mm long modified poly(glycidyl methacrylate–co-ethylene dimethacrylate) column also has a breakthrough curve with a sharp front that confirms the fast mass transport kinetics of the rod (Fig. 4). The dynamic capacity of the column for ovalbumin at

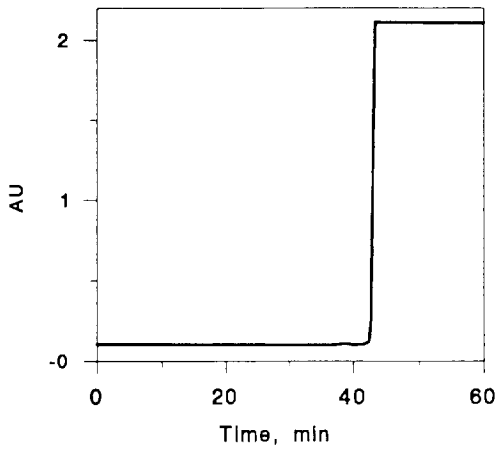


Fig. 4. Breakthrough curve for chicken egg albumin. Conditions: column 300 × 8 mm I.D.; mobile phase 0.01 *M* Tris–HCl buffer pH 7.6; flow-rate 0.5 ml/min; albumine concentration 15 mg/ml, UV detection at 280 nm.

1% breakthrough is 324 mg. Since the column contains 8.1 g of a polymer, the specific capacity is 40.0 mg/g of separation medium or 21.6 mg/ml of column volume.

Effect of loading

In an ideal case, the chromatographic properties of a column are supposed to be independent of the loading within the limits of the total column capacity. Fig. 5 shows peaks for three different injections of chicken egg albumin eluted by a salt gradient. The loadings cover more than two orders of magnitude from 0.33 mg, to 3.75 mg to 62.5 mg. Despite the breadth of this loading range, the retention times for the three peaks are 14.4, 14.6 and 14.5 min, respectively. These almost identical retention times document the invariability of the retention characteristics of the rod column with loadings that span a very broad range of protein concentrations.

It should also be emphasized that the peak asymmetry at 10% height is very good for all of the elutions and does not exceed a value of 1.1. This is well demonstrated with the middle peak of Fig. 5 corresponding to 3.75 mg loading. Obviously, the peak for the highest loading is not shown in its entirety because it exceeds the

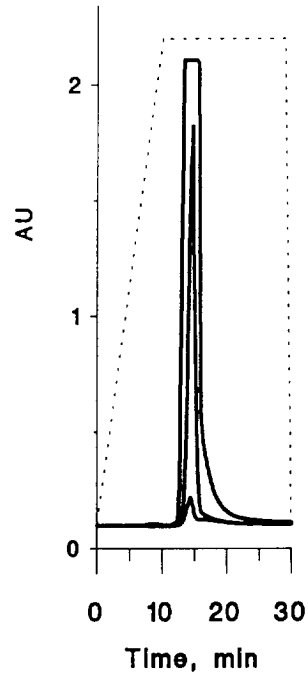


Fig. 5. Effect of column loading on the gradient elution of albumin. Conditions: column 300 × 8 mm I.D.; mobile phase gradient from 0.01 *M* Tris–HCl buffer pH 7.6 to 1 *M* NaCl in the buffer in 10 min; flow-rate 0.5 ml/min; albumin injections 0.3, 3.75 and 62.5 mg, UV detection at 280 nm.

set limits of the UV detector. Therefore, the major part of the tailing, that appears extensive at first glance, is actually in an acceptable range well below the 10% height.

Chemical stability

One of the major perceived limitations of methacrylate-based separation media is the alleged limited stability of their ester bonds to the alkaline conditions that are typically used for the cleaning of columns between operation cycles. It was shown elsewhere [22] that the ester groups can only be hydrolyzed using highly concentrated alkali metal hydroxide solutions at elevated temperatures. However, the column cleaning conditions are relatively mild when compared to those required for the hydrolysis. Therefore, column cleaning with dilute hydroxide is not expected to cause any change in the chemical structure of the modified rods or in their chromatographic properties. This was confirmed by a

series of experiments during which the column was washed four times with 0.1 *M* NaOH at room temperature for 12 h every other night while ovalbumin was used as a probe of column properties. During this period of time, no changes in retention time of ovalbumin were observed.

Chromatographic separations

The ultimate goal in the development of a separation medium is its use for separations. Fig. 6 shows the gradient elution of a protein mixture consisting of lysozyme, soybean trypsin inhibitor and conalbumin (2.5 mg of each). All of the proteins are baseline separated within less than 18 min and the symmetry of the peaks is again excellent. For example, the calculated peak asymmetry for trypsin inhibitor at 10% height is 1.04.

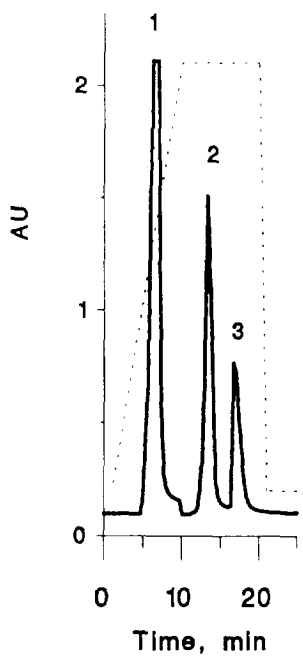


Fig. 6. Separation of lysozyme (1), soybean trypsin inhibitor (2) and conalbumin (3) by ion-exchange chromatography on modified continuous poly(glycidyl methacrylate–co-ethylene dimethacrylate) rod column. Conditions: column 300 × 8 mm I.D.; mobile phase gradient from 0.01 *M* Tris–HCl buffer pH 7.6 to 1 *M* NaCl in the buffer in 10 min; flow-rate 0.5 ml/min; total protein injection 7.5 mg, UV detection at 280 nm.

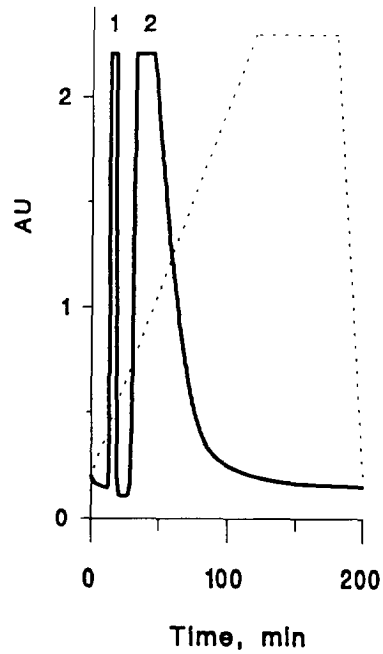


Fig. 7. Separation of bovine serum albumin (1) and chicken egg albumin (2) by ion-exchange chromatography on modified continuous poly(glycidyl methacrylate–co-ethylene dimethacrylate) rod column. Conditions: column 300 × 8 mm I.D.; mobile phase gradient from 0.01 *M* Tris–HCl buffer pH 7.6 to 1 *M* NaCl in the buffer in 120 min; flow-rate 0.5 ml/min; total protein injection 300 mg, UV detection at 280 nm.

High throughputs of the products are generally required for preparative separations. The use of high loadings is one of the options that leads to an increase in the productivity of the separation process. Fig. 7 shows the separation of a protein mixture that contains 66% chicken egg albumin and 33% bovine serum albumin. The total amount of proteins injected (300 mg) is close to the total capacity of the column as determined earlier with ovalbumin. However, baseline separation of both albumins is still achieved within 60 min using a shallow salt gradient (120 min from 100% buffer A to 100% buffer B).

4. Conclusions

We have demonstrated that "molded" columns can be used in a preparative mode for the

separation of larger quantities of proteins. Thus, rod columns with extended dimensions provide a viable alternative to the packed preparative columns. In addition to their ease of preparation, the major advantages of the continuous media are their better exploitation of the space within the column, their good efficiency even at high flow velocities, and their low pressure drop.

Although much remains to be done in the development of larger continuous rod columns, this early work shows their promising chromatographic properties and their great potential for large-scale separations. Further development of the molded rod columns is aimed at the introduction of new chemistries. Since the polymerization mixture involves only one phase, several limitations that restrict the use of some monomers for classical suspension polymerization do not apply for the preparation of the rods and numerous chemistries can be accessed directly by a polymerization. These will complement and expand significantly the chemistries currently available through the polymerization-and-modification process.

5. Acknowledgement

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