Synthesis and Separation Characteristics of Strongly Basic Anion-Exchange Resins Prepared from Different Monomers

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SYNOPSIS

Chloromethylstyrene–ethylene glycol dimethacrylate (EGDMA) copolymer beads (PCMS) and dimethylaminomethylstyrene–EGDMA copolymer (PDAMS) were synthesized by suspension polymerization as noble polymer matrices. The chemical and physical properties of the quaternized resins (PCMS-Q and PDAMS-Q) were approximately equal. The characteristics of both resins were examined by high-performance liquid chromatography using columns packed with the resins. These resins served as conventional anion-exchange resins for small organic anions, but indicated markedly different retention for large molecular anions, namely, PDAMS-Q retained proteins having a negative net charge, but PCMS-Q did not. It was suggested that the chemical and physical structures on the external surfaces of ion exchangers are very important in ion-exchange chromatography of proteins.

INTRODUCTION

Ion-exchange liquid chromatography has been widely used in the separation and purification of proteins. This method can be conducted in an aqueous buffer system, which prevents denaturation and/or inactivation of their biological functions. Presently, many ion-exchangers with outstanding properties suitable for protein separation are commercially available.[1-3] However, to gain further separation efficiency, development of a new ion-exchanger that can recognize a small difference between the structures related to one another is required.

Previously, we reported that a 4-vinylpyridinium polymer (4VP) column in high-performance liquid chromatography (HPLC) is useful in separating human/bovine serum albumin components, such as mercaptalbumin and nonmercaptalbumins.[4,5] It is postulated that these proteins interact with only the functional groups on the outer surface of the resin, because the pore sizes of the resin are too small to be penetrated by proteins. The retention of proteins on 4VP is substantially based on an approach comparable to that of the nonporous type or pellicular supports.[6-8] On the basis of this idea, we intended to synthesize new, highly cross-linked resins with ion-exchange groups, which are used in the conventional-type strongly basic anion-exchange resins, in quantities at their limited surface.

In this study, two resins having the same functional groups and a cross-linking agent were synthesized from different monomers and the retention behavior of some compounds including proteins in HPLC was explored.

EXPERIMENTAL

Materials

All reagents were of commercial reagent grade. Chloromethylstyrene (CMS), a mixture of *meta*- and *para*-isomers containing about 90% of the latter (Tokyo Kasei Kogyo Co., Japan) was purified by distillation (bp_5 76–80°C) before use. Ethylene glycol dimethacrylate (EGDMA) (Tokyo Kasei Kogyo Co.) was washed with 10% sodium hydroxide solution and 10% sodium chloride solution to remove inhibitors and dried over anhydrous calcium chloride. Other reagents were used without further purification. Sodium benzenesulfonate (SBS) and trimethylbenzylammonium chloride (TMBA) were obtained from Tokyo Kasei Kogyo Co. All proteins

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used were commercial products. Bovine serum albumin (BSA), human serum albumin (HSA), ovalbumin (OV), β-lactoglobulin (β-LG), and cytochrome C (CYC) were obtained from Sigma (St. Louis, MO) and lysozyme (LY) from Merck (Darmstadt, Germany).

Preparation of Dimethylaminomethylstyrene (DAMS) Monomer

CMS was added dropwise to a dimethylamine–diethylether solution, and the mixture was stirred for 24 h at room temperature. The reaction product was extracted with 10% hydrochloride and washed successively with diethylether, benzene, and diethylether. The extract was neutralized with excess anhydrous sodium carbonate and distilled under reduced pressure.

bp_2 58–64°C (lit.[9] bp_5 72–78°C), IR neat cm^{-1} 2948, 2820, 2784. ^{1}H-NMR($CDCl_2$) 2.14(3H, CH_3), 3.29(2H, CH_2), 4.97–5.26(2H, $-CH=CH_2$), 5.42–5.85(1H, $-CH=CH_2$), 6.37–6.92(1H, Ar—H), 7.22(4H, C_6H_4).

ANAL: Calcd for $C_{11}H_{15}N$: C, 81.99%; H, 9.32%; N, 8.69%. Found: C, 81.44%; H, 9.29%; N, 8.63%.

Preparation of PDAMS-Q

Polymerization was performed under a nitrogen atmosphere. Toluene solution containing DAMS and EGDMA and azobisisobutyronitrile (0.2% monomers) was suspended in a 1% hydroxyethylcellulose aqueous solution containing 20% sodium chloride with rapid stirring (3000 rpm) at 40°C. The temperature of the mixture was raised to 70°C at a rate of 0.5°C/min after 0.5 h of stirring. After stirring for 6 h at 70°C, the resulting resins, PDAMS, were washed with hot water until freed from the adhering stabilizer. Finally, the resins were classified in methanol, and the fraction of particle diameter (10–15 μm) was placed in a pressure bottle and cooled with a mixture of ice and salt. Methyl bromide was added to the cooled mixture and heated at 60°C for 20 h. The product, PDAMS-Q, was filtered off, then washed successively with methanol, water, and acetone.

Preparation of PCMS-Q

PCMS was obtained in a similar manner as PDAMS, except for use of CMS as the monomer and benzoyl peroxide as the initiator. PCMS was allowed to react with trimethylamine in the pressure bottle at 80°C for 20 h. The product, PCMS-Q, was washed with 10% hydrochloric acid, water, and methanol, then dried.

Apparatus

Chromatographic measurements were carried out with a Hitachi L-6200 intelligent pump equipped with a Hitachi L-4000 UV detector or an L-3300 refractive index detector and a Hitachi D-2000 Chromatointegrator.

The specific surface areas were measured with a Monosorb instrument (Yuasa Co., Japan). Infrared spectra (KBr disks) were recorded with a Model 20 DXC FT-IR (Nicolet).

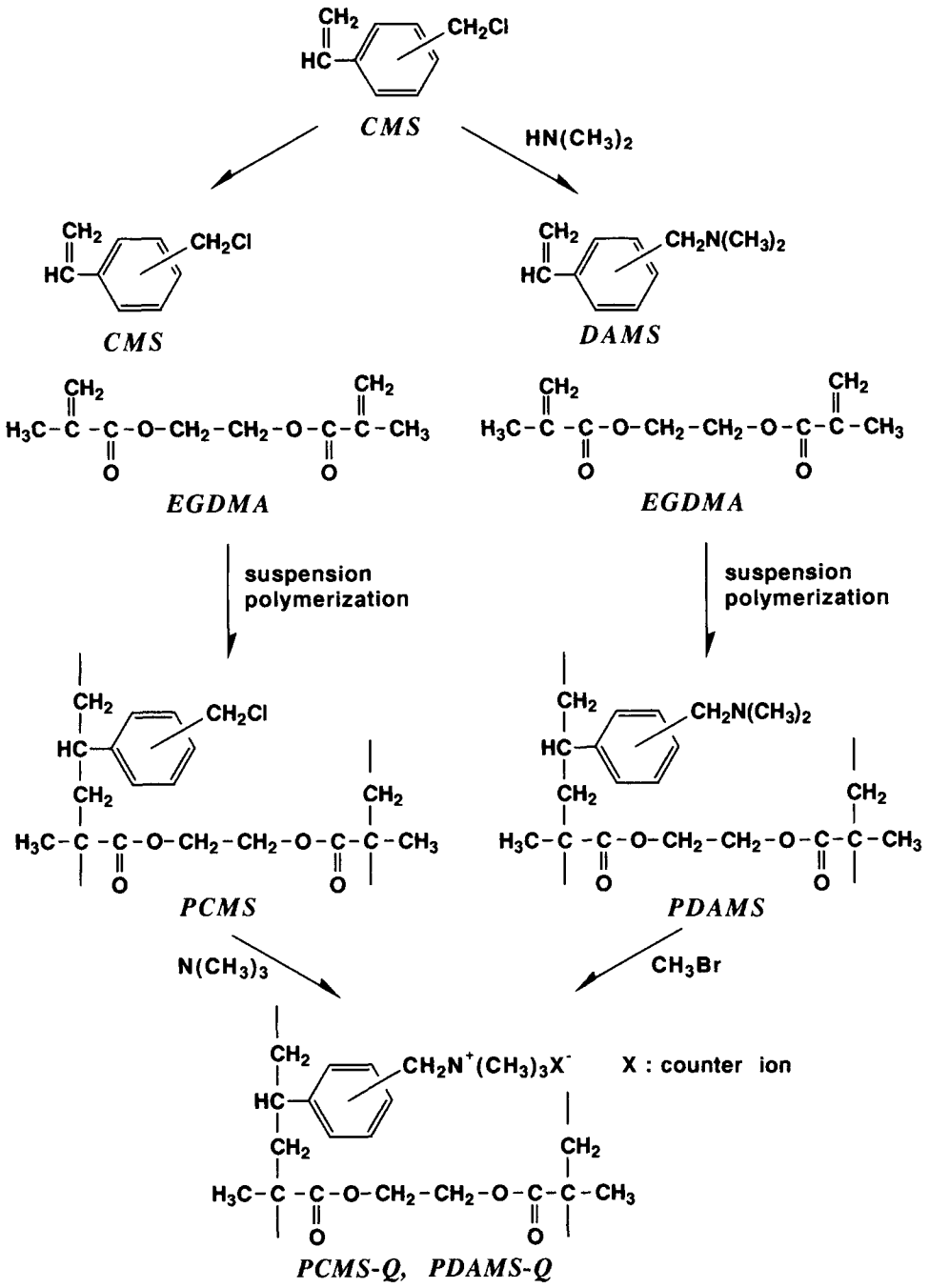
Chromatography

PCMS-Q and PDAMS-Q were packed into a stainless-steel column (150 × 4 mm i.d.) and conditioned with 0.05*M* Tris–HCl buffer (pH 7.0) containing 0.5*M* sodium chloride. SBS and TMBA were eluted under isocratic conditions using 0.05*M* Tris–HCl buffer (pH 7.0) containing 0.5*M* sodium chloride. Sample proteins were eluted under a 30 min linear gradient from 0–0.5*M* sodium chloride in 0.05*M* Tris–HCl buffer (pH 7.0) at a flow rate of 0.5 mL/min. The chromatographic procedure was performed at room temperature, with detection at 254 nm for SBS and TMBA and at 280 nm for proteins. One hundred micrograms of SBS or TMBA and 50 μg of proteins were injected onto the columns. The pore size of the resins in the wet state was evaluated by measuring the relationship between the molecular weight and the elution time in size-exclusion chromatography (SEC) with standard dextrans. The hold-up volume of the column was measured with heavy water. The retention behavior of the carbohydrates was measured using the refractive index detector.

RESULTS AND DISCUSSION

The surface between the organic and aqueous layers in the suspension copolymerization process should reflect the surface properties of the resulting resin. These properties prepared from hydrophilic monomers and a cross-linker ought to be different from those obtained from a hydrophobic monomer and the cross-linker. To obtain matrices for ion-exchange resins having different surface properties, there are many approaches, such as changing the copolymerization conditions including the composition of the monomers and the synthetic method.

In this study, two types of anion-exchange resins having the same functional group and the chemical structure were synthesized using different monomers, as shown in Scheme 1.



Scheme 1

The monomers used were hydrophobic CMS and hydrophilic DAMS. The suspension copolymerizations were performed under the same conditions except for the initiators. Therefore, we surmised that the difference in their hydrophobic/hydrophilic nature exactly reflected the surface properties of the resulting resins. To minimize nonspecific adsorption of proteins, EGDMA was used and highly crosslinked matrices (25, 50, and 75% EGDMA) were synthesized.

The physical properties of PCMS and PDAMS are shown in Table I. Although the copolymerization was carried out in the presence of diluent, the specific surface areas of the resins were very small. The an-

Table I Properties of Synthesized Polymers

Polymer (EGDMA %)	Nitrogen Content % (calcd %)	Chlorine Content % (calcd %)	Specific Surface Area (m^2/g)
PDAMS(25)	5.37 (5.84)		0.01
PDAMS(50)	3.28 (3.92)		0.13
PDAMS(75)	2.03 (1.95)		0.27
PCMS(25)		14.28 (14.60)	0.26
PCMS(50)		10.23 (11.89)	0.51
PCMS(75)		4.86 (6.01)	0.37

Table II Characterization of Quaternized Polymers

Polymer (EGDMA %)	Nitrogen Content (mmol/g)	Ion-Exchange Capacity (mEq/g)
PDAMS-Q(25)	2.92	2.89
PDAMS-Q(50)	2.24	2.04
PDAMS-Q(75)	1.34	1.30
PCMS-Q(25)	3.02	2.81
PCMS-Q(50)	2.30	2.23
PCMS-Q(75)	1.48	1.48

ion-exchange capacities and the data of elemental analysis of their quaternized PCMS-Q and PDAMS-Q are shown in Table II. The nitrogen contents were nearly equal to the total exchange capacities determined by the conventional titration method. Between PCMS-Q and PDAMS-Q having the same degree of cross-linking, the nitrogen contents and capacities were similar. In addition, the difference between chlorine and nitrogen contents (mol/g resin) of each resin is very small. This suggests that all the chlorine atoms are present as the counterions of quaternary ammonium groups.

Figure 1 shows infrared spectra of the resins (75% EGDMA), which clearly demonstrated that both resins synthesized from different monomers have the same chemical structure. In PCMS-Q, a hydroxy group originating from hydrolysis of the chloromethyl groups is not observable.

In the dry states, PCMS-Q and PDAMS-Q had no physical pore structure, similar to their starting resins. However, when resins are used for HPLC column packings, the pore structure in a wet state is more important than that in the dry state. To elucidate the effects of size exclusion on the columns packed with the quaternized resins, standard carbohydrates (mainly dextrans) were chromatographed, and the data are shown in Figure 2. The exclusion limits of both the resins for the carbohydrates were nearly equal and about 5000 Daltons.

The characteristics of these strongly basic anion-exchange resins were evaluated by HPLC using the resins as the column packings. Resins cross-linked with 25% EGDMA were not appropriate for this application column because of their low mechanical strength. Therefore, resins with more cross-linkages were used in the subsequent investigation. SBS and TMBA were used as low molecular weight anionic and cationic solutes, respectively. Figure 3 shows the relationship between retention times and the pH of the mobile phase on both columns. SBS was tightly retained because of its negative charge under the elution conditions (pH 5.0–9.0), but cationic TMBA was not retained on either column. This fact demonstrates that PCMS-Q and PDAMS-Q acted

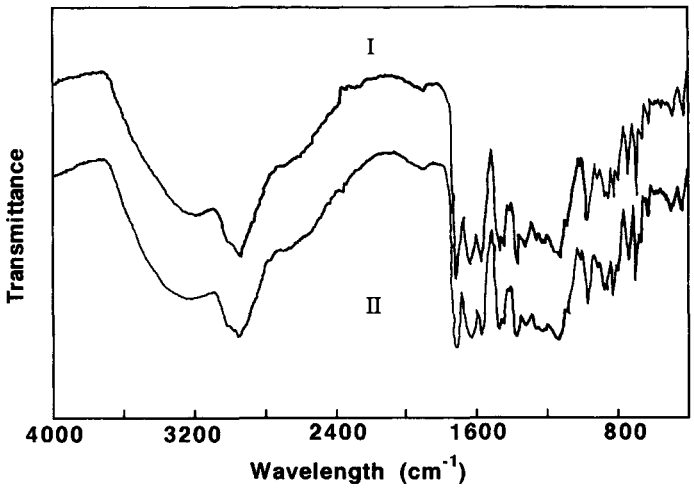


Figure 1 Typical FTIR spectra of PDAMS-Q(75)(I) and PCMS-Q(75)(II).

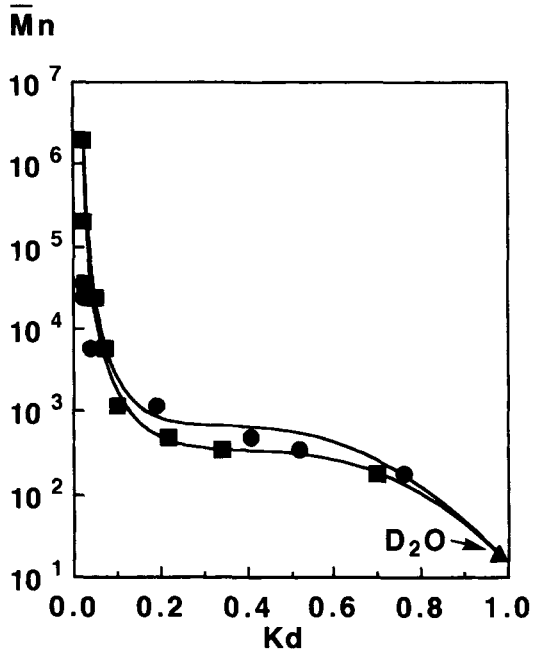


Figure 2 Calibration curves for (■) PCMS-Q(75) and (●) PDAMS-Q(75) columns using carbohydrates.

as typical anion-exchangers under the experimental conditions.

The retention behavior of proteins in the HPLC was subsequently examined. Table III shows the retention times of the proteins determined using a 30 min linear gradient from 0 to 0.5 *M* sodium chloride in Tris–HCl buffer (pH 7.0). On the PDAMS-Q column, the acidic proteins having a lower isoelectric point (pI) than 7, such as HSA, BSA, β-LG, and OV, were retained, but the neutral or basic proteins were eluted in the void volume by the gradient. On the other hand, on the PCMS-Q column, none of the proteins tested were retained under the same conditions. Under isocratic conditions using the Tris–HCl buffer (pH 7.0) containing 0.5 *M* sodium chloride, again, none of the proteins were retained on either column. However, under elution conditions using a Tris–HCl buffer without sodium chloride, the PDAMS-Q column tightly retained the acidic proteins. These were not eluted within 1 h, whereas the PCMS-Q column showed entirely different retention behavior for proteins, i.e., all the proteins

Table III Retention Times of Proteins on Quaternized Polymer Columns

		t_R (min)	
Protein	pI	PCMS-Q(75)	PDAMS-Q(75)
HSA	4.7–5.2	1.76	13.35
BSA	4.7–4.9	1.76	12.96
β-LG	5.1	1.91	17.45
OV	4.6	1.82	13.56
LY	11.0–11.4	1.77	1.73
CYC	10.1	1.81	1.73

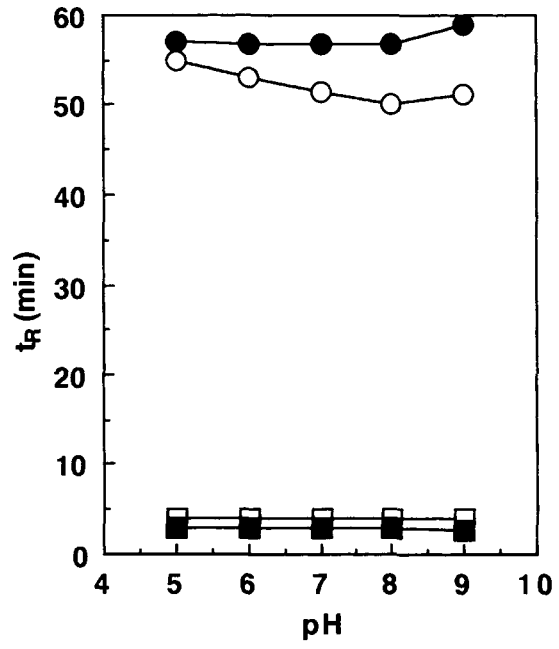


Figure 3 Relationship between pH of eluent and retention time (t_R) on PCMS-Q(75) and PDAMS-Q(75) columns. PCMS-Q column: (□) TMBA; (○) SBS. PDAMS-Q column: (■) TMBA; (●) SBS.

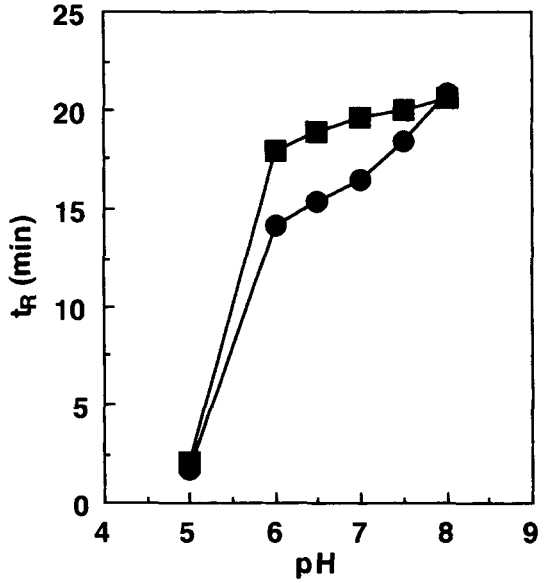


Figure 4 Effect of eluent pH on retention times of (●) BSA and (■) β-LG on PDAMS-Q(75) column.

eluted at the void volume. This behavior is clearly distinct from that for small molecules. It is supposed that PCMS-Q has an anion-exchange group on its internal surface where large molecular weight proteins cannot permeate. In addition, the resins 50 and 75% cross-linked showed the same retention behavior toward the ion of small and large molecules.

Figure 4 shows the relationship between the retention time of BSA and β-LG and the pH of eluents under the gradient conditions. On the PDAMS-Q column, since the pH of the mobile phase was approaching their pI value, the retention times of both proteins became shorter. This fact supports the contention that the retention of proteins is based mainly on the contribution of ion-exchange. It is speculated that the anion-exchange groups in PDAMS-Q are present on both the internal and external surfaces; hence, this resin could interact with all the acidic solutes tested.

In conclusion, in the development of high-performance ion-exchange resins, it is necessary to take into account the surface properties of the resins including the distribution of the ion-exchange groups as well as the nature of exchange groups and physical pore structure.

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