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Journal of Chromatography A, 743 (1996) 15–23

JOURNAL OF CHROMATOGRAPHY A

Study of protein binding to a silica support with a polymeric cation-exchange coating

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Abstract

A silica-based, polyacrylate ion-exchange stationary phase has been prepared using Ce(IV) as the initiator. Analysis of the physical properties of the polymeric layer separated from the silica surface indicates that the polymeric coating is cross linked to some extent. The polymerization carried out at different concentrations of Ce(IV) demonstrated that the effective surface area can be increased by lowering the Ce(IV) concentration at higher monomer concentrations of the reaction mixture. These materials are quite reproducible and of high electrostatic binding capacity; 1.485 μmol/m^2. The electrostatic binding capacity of a non-polymeric stationary phase reached the theoretical limit for a monolayer (0.16 μmol/m^2). However, the covalent binding capacity of the same stationary phase was only 50% of the electrostatic binding capacity. The same trend was observed in all the polymeric stationary phases tested. This shows that the mechanism of protein binding in polymeric and conventional stationary phases is similar, and multilayer electrostatic binding is highly unlikely in these sorbents examined. Z numbers revealed that the contact area of the protein is independent of the polymeric character of the stationary phase and therefore, the increased loading of these polymeric stationary phases is due to the increased surface area.

Keywords: Stationary phases, LC; Coating; Proteins

1. Introduction

Chromatographic sorbents are frequently prepared by attaching a thin layer of stationary phase to the surface of a support. The advantages of this approach are that (i) a single sorbent may be used to prepare many different types of chromatographic media, (ii) pore structure, particle diameter, and physical strength can be more uniform across a family of media and (iii) the presence of a stationary phase will not impact particle synthesis. The most widely used stationary phases in high-performance liquid chromatography have been simple organosilanes which in the ideal case are applied to the support surface as a monolayer. Adsorption/desorption kinetics of a monolayer are generally superior to thicker polymeric coatings, particularly with small molecules.

It is surprising that in recent years polymeric stationary phases are being used increasingly in the preparation of ion-exchange sorbents for proteins. Reported advantages of polymeric stationary phases are that they (i) are more stable, (ii) have higher loading capacity in the case of ion-exchange media and (iii) provide unique selectivity in protein separations [1]. Kinetic limitations with polymeric stationary phases appear to be far less of a problem for macromolecules than for small molecules.

A puzzling issue is how polymeric ion-exchange coatings can increase capacity 2–5 fold. It has been

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0021-9673/96/$15.00
PII S0021-9673(96)00355-X

reported in the case of polyethyleneimine coatings that enhanced loading capacity is the result of increasing the charge per unit volume of sorbent by immobilizing large amounts of polymer within support pores [2]. The higher loading capacity of these materials was hypothesized to be due to the polymer increasing sorbent surface area in some way. In another case of polymer enhancement of loading capacity, it has been proposed that tentacle like polymer structures are responsible for increasing loading capacity [3]. The enhanced loading capacity of these sorbents would apparently arise from tentacles extending away from the support surface a sufficient distance to allow proteins to penetrate the polymer layer and bind in multiple layers. Although differing explanations are given for the mechanism by which the various polymeric ion-exchange phases function to increase capacity, it should be noted that they all produce similar increases in ion-exchange capacity. This means that there are either multiple ways to enhance ion-exchange loading capacity with polymeric phases or the wide variety of molecular constructs currently used function to increase capacity by the same mechanism.

This paper addresses the question of how polymeric ion-exchange phases bind proteins. An important issue in this question is the nature of the polymer bearing the stationary phase. Polymeric coatings used in these studies were generated by cerium catalyzed polymerization of acrylate monomers on a γ-glycerylpropylsilane derivatized silica surface. It has been reported that cerium initiated polymerization at a diol surface generates linear, non-crosslinked tentacles of polyacrylate [3].

2. Materials and methods

2.1. Chemicals

LiChrospher 1000 Diol (10 μm, 1000 Å) was purchased from E. Merck (Darmstadt, Germany). γ-Glycidoxypropyltrimethoxysilane, acrylic acid, acrylamido-2-methyl-1-propanesulfonic acid (AMPS), Ce(IV) sulfate, hydrogen peroxide, potassium fluoride, and dicyclohexyl carbodiimide (DCC) were purchased from Aldrich (Milwaukee, WI). 3-sulfo-N-hydroxysuccinimide (S-NHS) was purchased from Pierce (Rockford, IL). Lysozyme (chicken egg white), cytochrome *c* (horse heart), α-chymotrypsinogen A (bovine pancreas), hemoglobin (bovine) were purchased from Sigma (St. Louis, MO).

2.2. Instrumentation

The particles were slurry packed into stainless steel columns 50×4.6 mm using a high pressure packing pump (Shandon Southern Instrument, Sewickley, PA). Chromatography was performed on BioCad liquid chromatography system (PerSeptive Biosystems, Framingham, MA). Absorbance was monitored at 280 nm.

2.3. Synthesis of stationary phases

2.3.1. Poly(AMPS) stationary phase synthesis

A modification of the method developed by Mino and Kaizerman [4] was used. 1 g of LiChrospher Diol silica (10 μm, 1000 Å) was transferred into a 100 ml three-necked round bottom flask fitted with a mechanical stirrer and 50 ml of water was added. Addition of appropriate amount of AMPS was followed by purging with nitrogen while stirring. The mixture was briefly sonicated to enhance the removal of air from the pores of silica. Then the required amount of Ce(IV) sulfate was transferred into the reaction flask and heated periodically every 3 h to 70 °C for 14 h under nitrogen. The mixture was then cooled to room temperature and filtered in a sintered-glass funnel, washed thoroughly with water and 50 ml quantities of 0.5 *M* H_2SO_4. When the silica beads turned pure white, it was washed again with water to neutrality and finally rinsed with THF and dried under vacuum.

2.3.2. Poly(acrylate) stationary phase synthesis

1 g of LiChrospher Diol silica (10 μm, 1000 Å) was transferred into a 100 ml three-necked round bottom flask fitted with a mechanical stirrer and 50 ml of water was added. Addition of appropriate amount of acrylic acid was followed by purging with nitrogen while stirring. The mixture was briefly sonicated to enhance the removal of air from the pores of silica. Then the required amount of Ce(IV) sulfate was transferred into the reaction flask and

heated to 50 °C for 7 h under nitrogen. The mixture was then cooled to room temperature and filtered in a sintered-glass funnel, washed thoroughly with water and 50 ml quantities of 0.5 *M* H_2SO_4. When the silica beads turned pure white, it was washed again with water to neutrality and finally rinsed with THF and dried under vacuum.

2.3.3. Oxidation of glycol groups on Diol silica

Very mild, but efficient reaction conditions were used [5] for this reaction. 1.0 g of LiChrospher Diol silica (10 μm, 1000 Å) was transferred into a 15 ml round bottom flask, and 2 ml of CCl_4, 2 ml of CH_3CN, and 3 ml of water were added. The solution was then degassed followed by the addition of 877 mg (4.2 equiv.) of $NaIO_4$ and 5 mg (2.2%) $RuCl_3 \cdot H_2O$. This mixture was stirred vigorously for 2 h at room temperature, then filtered and washed with an excess of water to remove $NaIO_4$ and $RuCl_3 \cdot H_2O$. Finally, it was washed with CH_2Cl_2 and THF and dried in vacuo.

2.3.4. Detaching the stationary phase using H_2O_2

A method reported by Tamao et al. [6] was modified and hydrogen peroxide was employed instead of *m*-chloroperbenzoic acid. The poly-(AMPS) coated silica 0.75 g (99 μmol of C–Si bonds) was mixed with 5 ml of dry dimethylformamide (DMF) and degassed under vacuum while sonicating briefly. After stirring for 1 h, 0.028 g (5 equiv.) of KF and 90 μl (4 equiv.) of 30% H_2O_2 was added and stirred for another 16 h. Then deionized water was added to quench the reaction and the solid residue was removed by filtering through a sintered-glass funnel. The resulting yellow solution was evaporated to dryness using high vacuum at 40°C to yield a white, sticky, highly insoluble polymer.

2.4. Static protein binding capacity assays

2.4.1. Ionic lysozyme-binding capacity (LYSiec)

A procedure used by Rounds et al. [2] was employed. A 50 mg sample of coated silica was quantitatively transferred into a polypropylene tube and washed once with desorption buffer (1 *M* NaCl in 10 m*M* phosphate buffer, pH 7.0), then twice with adsorption buffer (10 m*M* phosphate buffer, pH 7.0). The sorbents were saturated for 30 min. with 4 ml of 5% (w/v) lysozyme solution in 10 m*M* phosphate pH 7.0 buffer. The tubes were then vortex mixed gently every 10 min for 30 min. The silica was centrifuged to the bottom and the supernatant was discarded. To remove excess protein, the silica was washed thoroughly with (3×5 ml) adsorption buffer. Finally, the lysozyme was desorbed from the surface by washing the packing material with the desorption buffer (3×5 ml). This desorbed lysozyme was quantitated spectrophotometrically at 280 nm.

2.4.2. Ionic hemoglobin-binding capacity (Hbiec)

The same procedure was followed as given for lysozyme. However, the phosphate buffers of pH 5.5 were used throughout the procedure and the absorbance of the Hb desorbed was measured at 410 nm.

2.5. Covalent binding capacity assays

2.5.1. Activation of carboxylic acid groups of the stationary phase

Acrylate grafted silica (0.1 g) was transferred into a 20 ml polypropylene tube and 2 ml of 9.28 mg/ml solution (5 equiv.) of sulfo-N-hydroxy succinimide added. The mixture was degassed by purging with nitrogen for 10 min. Then 2 ml of dicyclohexyl carbodiimide (DCC) (8.85 mg/ml in dioxane, 5 equiv.) was added. Mixing was continued for 3 h, silica was centrifuged out, and the supernatant was discarded. The silica was then washed thoroughly with water and with dioxane to remove N,N′-dicyclohexyl urea and excess DCC. After a further dioxane wash, the silica was rinsed with water and dried.

2.5.2. Protein coupling to the activated surface

A 40 mg/ml solution of lysozyme in 0.1 *M* $NaHCO_3$, pH 7.5 was used. To the activated acrylate sorbent, 2 ml of the lysozyme solution was added and continuously agitated for 24 h at room temperature. After the silica was separated by centrifugation, it was washed according to the following sequence:

1. 0.1 *M* phosphate buffer pH 7.0 (1×5 ml).
2. 1.0 *M* NaCl in 0.1 *M* phosphate buffer pH 7.0 (1×5 ml).

3. 0.1 *M* phosphate buffer pH 7.0 (1×5 ml).
4. 10% ethylene glycol (1×3 ml).
5. 0.1 *M* phosphate buffer pH 7.0 (1× 5 ml).
6. Deionized water (1×5 ml).
7. Rinsed with THF and dried.

Elemental analyses (C, H, N, S) on silica-based stationary phases were performed by H.D. Lee, Purdue University, Department of Chemistry Microanalysis Laboratory.

2.6. Chromatographic evaluation

Portions of the polymer grafted silica were packed into 50×4.6 mm ID stainless steel columns. Conditions used for chromatography were: 10 min or 20 min gradient from 10 m*M* phosphate buffer, pH 7.0 to 1.0 *M* NaCl in 10 m*M* phosphate buffer, pH 7.0 at a flow-rate of 1.0 ml/min.

Protein samples were prepared in 10 m*M* phosphate buffer, pH 7.0 at a concentration of 4.0 mg/ml. Cation-exchange behavior and Z numbers were examined using a mixture of α-chymotrypsin A, cytochrome *c*, and lysozyme. Resolution of α-chymotrypsin A and lysozyme were examined at 0.5 ml/min., 1.0 ml/min., and 2.0 ml/min. Z numbers of acrylate grafted sorbents (see Section 3 were determined using retention times of proteins at different concentrations of NaCl in phosphate buffer, pH 7.0.

3. Results and discussion

3.1. Coating synthesis

Acrylates were chosen to generate the polymeric ion-exchange coatings used in these studies because they allow the introduction of a wide variety of stationary phase groups into the coatings. Two different approaches have been used to graft acrylates to a silica surface. One approach is the α,α'-azoisobutyronitrile (AIBN) initiated polymerization on a methacrylsilane or vinylsilane derivatized surface [7]. The other was by cerium catalyzed polymerization from a diol derivatized surface [3]. AIBN initiated polymerization occurred throughout the solution, in addition to grafting polymer to the surface. Cerium(IV), in contrast, has been reported to initiate polymerization predominantly at the surface [3]. Silica was chosen as the support matrix for these studies because elemental analysis of the polymer coating was much easier to interpret when there is little or no organic material in the support.

It was initially observed in 1956 [4] that both Ce(IV) nitrate and sulfate catalyze a one electron oxidation of organic reducing reagents such as alcohols, thiols, glycols, aldehydes, and amines to produce Ce(III) and a transient free radical species of the reducing agent that was used to initiate acrylate and vinyl monomer polymerization in the preparation of graft copolymers. Oxidization of γ-glycerylpropysilane derivatized silica with Ce(IV) in the preparation of ion-exchange tentacles is a special case of the more general oxidative cleavage of diols and alcohols reported in 1973 [8].

3.1.1. AMPS (2-Acrylamido-2-methyl-1-propanesulfonic acid) coating

Grafting acrylate polymers to the surface of diol silica by Ce(IV) catalysis was best achieved at acidic pH (data not shown). In the case of AMPS, the monomer is sufficiently acidic that it was not necessary to adjust the pH. However, low reactivity of the AMPS monomer necessitated heating the reaction mixture to 70 °C. Unfortunately, extended heating at this temperature leaches the γ-glycerylpropysilane by hydrolyzing siloxane bonds. This problem was circumvented by heating the reaction mixture to 70 °C for only 5 min every 3 h in a 14 h reaction. Following polymerization it was necessary to wash the sorbent exhaustively with 0.5 *M* H_2SO_4 to remove electrostatically adsorbed Ce ions. The percentage carbon in the polymeric coating increased in direct proportion to the AMPS monomer concentration in solution. Sorbents designated AM-3, AM-6, AM-9, and AM-12 had carbon contents of 1.11, 1.87, 2.6, and 3.52% respectively. (Carbon content of these coatings included the 0.51% carbon in the diol coating). Numbers in these designators refer to the percentage of AMPS monomer in the coating solution.

3.1.2. Polyacrylic acid coating

Acrylic acid is more reactive than AMPS. Reaction at 50 °C for 7 h was sufficient to obtain

maximum coating. Acidity of the acrylic acid monomer solution was adequate to bring reaction pH to the requisite pH 3–4. Sorbents designated AC-1, AC-2, AC-3, AC-6, AC-8, and AC-10 had carboxyl group densities of 2.72, 4.63, 9.54, 11.67, 11.74 and 12.5 mmol/m^2, respectively. This is at least six times the carboxyl group density achievable with a simple organosilane coating [9]. Numbers in these designators refer to the percentage of acrylate monomer in the coating solution in all cases except AC-1 which is oxidized diol and AC-2 where acrylate monomer concentration was 1.5%. Between 3% and 6% monomer, incorporation of acrylate monomer into the polymeric ion-exchange layer decreased rapidly. Above 6% acrylic acid there was little increase in the percentage of carbon in the stationary phase. It should also be noted that sorbents derived from 8% and 10% monomer produced columns of significantly higher operating pressure.

3.2. Oxidative cleavage

Previous publications on the use of cerium based catalysis for the preparation of polymeric coatings from acrylate monomers suggest that the coating polymer in these studies would be a linear tentacle anchored to the surface at one end through organosilane coupling [3]. The objective in this phase of characterization was to determine the molecular weight distribution of the grafted polyacrylates by cleavage of the Si–C bond and matrix assisted laser desorption ionization (MALDI) mass spectrometry of the released polymer. It has been reported that 3-chloroperoxybenzoic acid with three equivalents of KF may be used to cleave Si-C bonds of organoalkoxysilane in 92% yield with production of the respective alkyl alcohol [6].

When this oxidative cleavage procedure with certain modifications was applied to the AMPS coated sorbent a viscous, sticky white product was isolated after evaporating the dimethylformamide solvent under high vacuum. After thorough washing with acetone and water to remove KF and dimethylformamide the product was dried. Elemental analysis of the silica product revealed that approximately 64% of the coating had been removed and the C/H/N ratio of the residual coating was equivalent to that of the original. The relatively poor cleavage efficiency can be interpreted in two ways; either (i) the reaction proceeds more slowly in the case of a coated polymer surface or (ii) the residual polymer is sufficiently crosslinked that it is insoluble.

MALDI-time of flight mass spectrometry was relatively unsuccessful. The spectrum was of low intensity and showed a series of ions between mass 1000 and 1370 varying by 74 amu. Although these ions appear to be the result of a polymeric ladder, there is no apparent relationship between their mass and any structural features of a linear polymer of AMPS. This data may be explained in several ways. One would be that the MALDI matrix used for ionization was not suitable for a polymer of AMPS. The validity of this prospect can only be established by an exhaustive study of MALDI matrices for polymers of AMPS. Such a study was beyond the scope of this project. The second possibility is that the polymer could be crosslinked and of sufficiently high molecular mass that it either can not be ionized or fragments during flight. In regard to the prospects of crosslinking, it is known that Ce(IV) catalyzed polymerization can yield crosslinked products [10,11]. The poor water solubility of this very hydrophilic polymer implies that it is crosslinked. There is also precedent in the case of DNA and RNA oligomers ranging up to 50 bases in length that highly charged, hydrophilic polymers can be transported into the gas phase by MALDI [12]. It is reasonable to expect that polymers of AMPS up to 5000 amu should be ionizable by MALDI.

Taken together, (i) the poor yield of oxidative cleavage product, (ii) poor water solubility of the AMPS polymer, and (iii) the difficulty of obtaining MALDI mass spectra of the cleaved polymer would imply that there is some degree of crosslinking in the AMPS polymer. Although it is possible that this crosslinking could have been catalyzed by hydrogen peroxide during the cleavage reaction, it is more likely that if there is crosslinking, it occurred during polymerization as reported in the literature [10,11].

3.3. Static loading capacity

The static loading capacity of both the AMPS and acrylate polymer media are shown in Table 1. Based on the dimensions of lysozyme (LYS), it may be calculated that a single molecule in a monolayer

Table 1
Lysozyme (LYS) and Hemoglobin (Hb) binding capacities of polymeric stationary phases

Stationary phase	Ce(IV) g/g silica	Binding capacity (μmol/m^2)	
		LYS	Hb
AM-3	0.66	0.26	0.02
AM-6	0.5	0.659	0.05
AM-9	0.375	0.702	0.06
AM-12	0.28	0.823	0.08
AC-1	0.0	0.143	0.011
AC-2	0.5	0.26	0.026
AC-3	0.5	0.62	0.063
AC-6	0.5	0.98	0.12
AC-8	0.5	1.0	0.125
AC-10	0.5	1.0	0.124
AC-3a	0.25	0.57	0.055
AC-6b	0.125	1.22	0.16
AC-8c	0.094	1.276	0.163
AC-10d	0.075	1.485	0.185

would occupy approximately 1000 Å^2 of surface. This would mean that the theoretical loading capacity of a sorbent saturated with a monolayer of lysozyme would be 0.16 μmol/m^2. In similar fashion, it is expected that hemoglobin (Hb) would occupy approximately 5000 Å^2 of surface area and have a loading capacity of 0.022 μmol/m^2 . At the lowest levels of stationary phase loading it is seen in Table 2 that the loading of both lysozyme and hemoglobin approaches these values of a theoretical monolayer. It is also seen that loading capacity is independent of the type of stationary phase at low stationary phase density. Specific loading capacity for proteins, i.e., μmol/m^2, increases roughly in proportion to stationary phase density, generally reaching a maximum of four times that of the monolayer value. By lowering the concentration of Ce(IV) catalyst used in the synthesis of the polyacrylate coating, it was possible to increase the static capacity to six times the monolayer value with a concomitant increase in stationary phase loading.

Table 2
Description of the stationary phases used in this study

Abbreviation	Ce(IV) g/g of silica	Monomer % (w/v)	
		AMPS	Acrylic acid
AM-3	0.66	3.0	–
AM-6	0.5	6.0	–
AM-9	0.375	9.0	–
AM-12	0.28	12.0	–
AC-2	0.5	–	1.5
AC-3	0.5	–	3.0
AC-6	0.5	–	6.0
AC-8	0.5	–	8.0
AC-10	0.5	–	10.0
AC-3a	0.25	–	3.0
AC-6b	0.125	–	6.0
AC-8c	0.094	–	8.0
AC-10d	0.075	–	10.0
AC-1[a]	0.0	–	–

[a] Diol coating on LiChrospher 1000 Diol silica (10 μm, 1000 Å) was oxidized to COOH groups.

As a general rule, it may be concluded that static loading capacity of these ion-exchange sorbents increases in proportion to stationary phase charge density. Similar results were reported in the case of crosslinked polyethyleneimine (PEI) sorbents. The PEI sorbents are very different to the polyacrylates in that they are prepared by adsorbing 600 molecular mass PEI onto the surface of silica and exhaustively crosslinking it with multifunctional oxiranes.

3.4. Covalent binding capacity

It is frequently the case in molecular adsorption at surfaces that multiple layers are formed. In view of the fact that electrostatic interactions are long range phenomena, it was reasoned that by comparing specific ion-exchange capacity to the specific covalent binding capacity it would be possible to discriminate between monolayer and multilayer adsorption.

Covalent immobilization of lysozyme was achieved by activating polyacrylic acid stationary phases with 3-sulfo-N-hydrosuccinimide (S-NHS) using dicyclohexylcarbodimide as a water scavenger. Elemental analysis indicated that 90% derivatization of carboxyl groups was achieved. The relationship between ion-exchange vs. covalent loading capacities for a variety of sorbents varying up to 10 fold in loading capacity is seen in Table 3. Covalent loading capacity ranged from 43% to 62% that of the ion-exchange capacity, but generally converged on 50%. It was shown in Table 2 that the AC-1 sorbent had an ion-exchange capacity equivalent to the theoretical value calculated for a monolayer of lysozyme. Data in Table 3 indicate that the covalent binding capacity of this material is only 62% that of the theoretical monolayer. Several explanations may be

Table 3
Comparison of lysozyme binding capacities of polyacrylate sorbents

Stationary Phase	LYS covalent loading capacity (cov) (μmol/m^2)	LYS ion-exchange capacity (iec) (μmol/m^2)	iec/cov ratio
AC-1	0.085	0.143	1.7
AC-2	0.16	0.26	1.62
AC-3	0.31	0.62	2.0
AC-6	0.42	0.98	2.33
AC-8	0.44	1.00	2.27
AC-10	0.45	1.00	2.23
AC-3a	0.26	0.57	2.19
AC-6b	0.60	1.22	2.03
AC-8c	0.62	1.276	2.06
AC-10d	0.74	1.485	2.00

given as to why the covalent binding capacity would be 50% of the theoretical monolayer capacity in the cases of AC-1. One is that S-NHS activating agent spontaneously hydrolyzes during the protein coupling reaction and there is insufficient activated surface remaining for full loading. Preliminary data from another study [13] in which lysozyme was covalently immobilized with S-NHS, digested with trypsin, and the tryptic fragments analyzed by HPLC indicates that lysozyme is attached to the surface through multiple carboxyl groups. This precludes the possibility that hydrolysis of the S-NHS derivatized sorbent during immobilization reduces the concentration of S-NHS groups to the point that there are too few for monolayer surface coverage. Another explanation would be that in covalent immobilization, binding is random and molecules can not diffuse along the surface as in the case of electrostatic adsorption. Random binding and the absence of diffusion would not produce a close packed surface. It is to be expected that packing would be higher in the case of electrostatic adsorption where surface diffusion would allow closer packing.

Based on the fact that the organosilane ion-exchanger does not even saturate the surface in the case of AC-1 and protein loading of this material approaches the theoretical value for adsorption of a monolayer, it is concluded that there is no evidence for multilayer adsorption in the case of the AC-1 sorbent. It is further concluded that the prospect of multilayer electrostatic binding in the higher capacity AC-6, AC-8, AC-10, AC-6b, AC-8c, and AC-10d is unlikely. The fact that the ratio of electrostatic to covalent binding capacity is relatively constant over a 10 fold range of loading capacity is a strong indicator that the nature of the binding mechanism is very similar in all the sorbents examined. This means that the enhanced loading capacity of these polymeric stationary phases is due to increases in sorbent surface area as opposed to increases in surface potential causing the accumulation of multiple layers of protein at the sorbent surface.

3.5. Chromatographic characterization

3.5.1. Resolution

One of the concerns when polymeric bonded phases are introduced in the pore matrix of a support is that intraparticle diffusive mass transfer will be compromised. The intraparticle mass transfer properties of the sorbents prepared in these studies were evaluated by the resolution of a mixture of α-chymotrypsinogen A and lysozyme at increasing mobile phase velocity (Table 4). First, it should be noted that for this mixture the strong cation-exchange (AMPS) sorbents were of inherently higher resolving power than the weak cation-exchange (AC) materials. For example, AM-6 and AC-3 are of approximately the same loading capacity but the strong cation-exchangers had almost twice the resolution. This is because the strong cation-exchanger has greater selectivity for this mixture. It is also seen in Table 4 that the intermediate loading capacity AC-3 and AM-3 supports had both the highest resolution in their respective families and the greatest percent loss in resolution at high mobile phase velocity. The decrease in resolution for AC-3 and AM-3 in going from 0.5 to 2.0 ml/min flow-rate was 31% and 30%,

Table 4
The dependence of $R_{s_{\alpha-\text{CHYM-LYS}}}$ on stationary phase thickness and the ligand density

Column	Thickness (nm)	Ligand density (μmol/m^2)	$R_{s_{\alpha-\text{CHYM-LYS}}}$		
			0.5 ml/min.	1.0 ml/min.	2.0 ml/min.
AC-2	0.68	4.63	2.09	2.012	1.809
AC-3	1.088	9.54	3.579	2.876	2.359
AC-6	1.256	11.67	3.12	2.60	2.263
AC-8	1.288	11.75	2.526	2.418	2.144
AM-3	0.937	2.38	6.62	5.35	4.591
AM-6	1.611	5.39	6.511	5.37	3.766

respectively. The AM-6 support showed a 41% decrease in resolution over this same range of flow-rate. When compared to the AC-2 support which loses 14% of its resolution at elevated mobile phase velocity, there is no question that the higher capacity of the polymeric stationary phases is achieved at the expense of introducing some mass transfer limitations. Even so, the AC-3 material has almost twice the resolution of AC-2 at high mobile phase velocity with substantially higher capacity.

3.5.2. Z number

Reversible electrostatic binding of a protein to a sorbent is expressed by the equilibrium;

$$P_o + ZS_b \Leftrightarrow P_b + ZS_o$$

$$K_b = [P_b][S_o]^Z/[P_o][S_b]^Z \quad (1)$$

where $[P_o]$ is the protein concentration in solution, $[S_b]$ is the concentration of bound displacing ion, $[P_b]$ is bound protein, $[S_o]$ is the concentration of the displacing ion in solution, Z is the number of ions displaced when a protein binds to the sorbent, and K_b is the equilibrium constant [14]. $[S_b]$ is equal to the density of ion-exchange groups on the surface of the sorbent. It is seen in Eq. 2 that the bound to free ratio

$$K_d = [P_b]/[P_o] = k'/\phi \quad (2)$$

of protein, i.e., $[P_b]/[P_o]$, is equal to the chromatographic distribution coefficient (K_d) and the ratio of chromatographic capacity factor (k') to the phase ratio (ϕ). Substituting in Eq. 1 it is possible to relate chromatographic behavior, i.e., k', to the magnitude of a cluster of constants, i.e., $K_b\ \phi[S_b]^z$, and salt concentration in the mobile phase ($[S_o]^z$).

$$k' = \{K_b\phi[S_b]^Z\}\{1/[S_o]^Z\} \quad (3)$$

It is now well established that Z is a measure of the number of sites with which a protein interacts with a stationary phase [14–16].

It is seen in Table 5 that Z numbers on the non-polymeric surface of AC-1, are generally equal to or larger than those of the polymeric materials. The single exception is the case of lysozyme with AC-6 and AC-8 where the Z numbers were 10–20% higher. This is considered to be within the experimental error of Z number measurements. It would be expected that if the polymeric bonded phase engulfs the protein analytes, as has been proposed for tentacle sorbents, that the contact surface area with the protein would increase and Z numbers for proteins on the polymeric stationary phases would be higher than for non-polymeric stationary phases. Based on the Z number measurements there is little evidence for increased contact area with the polymeric phases. Molecular engulfment of protein analytes does not seem to occur. The polymeric stationary phases in these studies behave in a manner very similar to the rigid, organosilane derivatized silica surface of AC-1. The major dif-

Table 5
Retention properties of α-CHYM A, CYT c, and LYS with poly(acrylate) stationary phases

Column	Z numbers		
	α-CHYM A	CYT c	LYS
AC-1	3.829	4.12	3.23
AC-2	4.77	3.59	3.0
AC-3	3.478	4.08	3.9
AC-6	3.05	4.2	3.55
AC-8	2.51	3.74	3.43

ferentiating feature of the polymeric bonded phases appears to be that the polymeric phases are simply of higher surface area.

4. Conclusions

Data presented in this paper allow several conclusions to be made relative to the nature of polyacrylate ion-exchange phases prepared by Ce(IV) initiated polymerization and the mechanism by which these sorbents bind proteins. Based on the poor yield of oxidative cleavage product and limited water solubility of the AMPS polymer released from sorbent particles by oxidative cleavage it is concluded that there can be some degree of crosslinking in acrylate polymer bonded phases prepared by Ce(IV) initiated polymerization. It is possible that not all the acrylate polymer chains in tentacle type bonded phases are in fact tentacles.

It may be further concluded that electrostatic adsorption of protein at the surface of an inorganic support derivatized with 2.72 μmol/m^2 of ion exchanging stationary phase approaches a monolayer and that covalent immobilization at this surface approaches 50% of electrostatic adsorption for reasons of steric limitations. Although, electrostatic adsorption increases with stationary phase ligand density, the ratio of electrostatic to covalent bond protein is relatively constant over a ten fold range of ion-exchange capacity. This leads to the conclusion that (i) the adsorption mechanism in all the sorbents examined is similar, (ii) multilayer electrostatic binding is highly unlikely in any of the sorbents examined and (iii) polymeric acrylate phases do not seem to be unique in terms of their electrostatic to covalent binding capacity ratio. Furthermore, data from Z number measurements lead to the conclusion that contact surface area between proteins and the stationary phase does not increase in proportion to the polymeric character of the stationary phase. There was no evidence of the tentacle effect in which tentacles of polymer engulf or wrap around proteins. The mechanism by which polymeric stationary phases increase the ion-exchange loading capacity of sorbents seems to be simply that of providing greater surface area. This increase in surface area appears to relate directly to the amount of polymer applied to the surface until polymer begins to restrict entry to the sorbent pore matrix.

Acknowledgments

The authors thank E. Merck for their generous gifts of Diol silica and Lisa R. Hahn for her contribution in this project. This work was supported by NIH grant GM25431.

References

[1] W. Kopaciewicz and F.E. Regnier, J. Chromatogr., 358 (1986) 107.
[2] M.A. Rounds, W. Kopaciewicz and F.E. Regnier, J.Chromatogr., 362 (1986) 187.
[3] W. Muller, J. Chromatogr., 510 (1990) 133.
[4] G. Mino and S. Kaizerman, S.J. Polymer Sci., 31 (1958) 242.
[5] H.J. Carlsen, T. Katsuki, V.S. Martin and K.B. Sharpless, J. Org. Chem., 46 (1981) 3936.
[6] K. Tamao, T. Kakui, M. Akita, T. Iwahara, R. Kanatani, J. Yoshida and M. Kumada, Tetrahedron, 39 (1983) 983.
[7] H. Engelhardt, H. Low, W. Eberhardt and M. Maub, Chromatographia, 27 (1989) 535.
[8] W.S. Trahanovsky, L.H. Yong and M.H. Bierman, J. Org. Chem., 34 (1969) 868.
[9] F.E. Regnier and R. Noel, J. Chromatogr., 14 (1976) 316.
[10] E. Bacioechi and R. Ruzziconi, J. Org. Chem., 51 (1986) 1645.
[11] L.M. Weinstock, E. Corley, N.L. Abramson, A.O. King and S. Karady, Heterocycles, 27 (1988) 11.
[12] N.P. Christian, S.M. Colby, L. Giver, C.T. Houston, R.J. Arnold, A.D. Ellington and J.P. Reily, Rapid Communications in Mass Spectrometry, 9 (1995) 1061.
[13] H. Zhou, D. Patterson and F.E. Regnier, Manuscript in preparation, Purdue University, West Lafayette, IN.
[14] W. Kopaciewicz, M.A. Rounds, J. Fausnaugh and F.E. Regnier, J. Chromatogr., 266 (1983) 3.
[15] X.D. Geng and F.E. Regnier, Chromatographia, 38(3/4) (1994) 159.
[16] R.R. Drager and F.E. Regnier, J. Chromatogr., 359 (1986) 147.