ELSEVIER

Journal of Chromatography A, 673 (1994) 159–165

JOURNAL OF CHROMATOGRAPHY A

Adsorption and separation of proteins on composite anion exchangers with poly(N-diethylaminoethylacrylamide) bonded phases☆

Alexander E. Ivanov*, Vitali P. Zubov

Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Miklukho-Maklaya 16/10, 117871 Moscow V-437, Russian Federation

(First received May 9th, 1993; revised manuscript received February 23rd, 1994)

Abstract

Composite anion exchangers for chromatography of proteins were prepared by chemical adsorption of poly(*p*-nitrophenyl acrylate) on γ-aminopropylsilicas followed by coupling of the esters to 2-diethylaminoethylamine. Separation of standard proteins (bovine serum albumin and ovalbumin) on these anion exchangers established their enhanced selectivity and milder desorption conditions as compared with polyethyleneiminesilicas and DEAE-Toyopearl 650M. Frontal analysis was used to evaluate the maximum binding capacity for ovalbumin adsorption on the new packing, which was found to be 13 mg/ml and so nearly half those observed with polyethyleneiminesilicas of comparable pore diameter. The possible role of the excluded volume of the attached polymer is discussed with respect to the adsorptivity of the composite ion exchangers.

1. Introduction

Since polyethyleneimine-coated silicas were developed by Regnier and co-workers [1,2] for use as packings in the anion-exchange chromatography of proteins, attempts have been made to design other polymer-coated silicas for the same purpose. Apart from variations with polyethyleneimine coatings [3,4] and use of DEAE-dextran as the bonded phase [5], the synthetic approach of Müller [6] seems to be most interesting because it ensures the formation of end-grafted polyelectrolyte chains with maximum motional freedom and, therefore, the best adaptivity towards the absorbing proteins. Better separation properties of such "tentacle-like" composite sorbents as compared with the traditional ion exchangers based on cross-linked polymers were reported [6]. However, under overloading conditions the tentacle-type anion exchangers gradually lose their protein adsorption capacity [7]. This might be related to the peculiar behaviour of end-grafted chains or their leakage during the desorption stages.

The stability of the coating and the reproducibility of the loading capacities may be improved, in principle, by the preparation of bonded phases composed of multivalently attached macromolecules. On the other hand, the multivalently attached polymeric chains will have

* Corresponding author.

☆ Presented at the *17th International Symposium on Column Liquid Chromatography, Hamburg, May 9–14, 1993.* The proceedings of this symposium were published in *J. Chromatogr. A*, Vols. 660 + 661 (1994).

0021-9673/94/$07.00
SSDI 0021-9673(94)00205-N

less mobility and thus may give a diminished effect of tentacles.

In previous papers [8,9], we described the chemical adsorption of poly(*p*-nitrophenyl acrylate) (PNPA) as a method for the preparation of multivalently attached polymeric phases. Ester functions of the chemically adsorbed PNPA can be coupled to various primary amines and yield bonded phases composed of N-substituted polyacrylamides. If 2-diethylaminoethylamine is used for such a synthesis, an adsorbent with DEAE functions is formed with a chemical structure almost identical with that explored by Müller [6]. However, the arrangement of the grafted chains is different in our case and may lead to a different behaviour of separated solutes.

In this work we examined the separation properties of a composite anion-exchanger prepared by the PNPA method and compared them with those of polyethyleneiminesilica (PEI-silica) and DEAE-Toyopearl 650M, a wide-pore cross-linked polymer. We also considered some features of the chemical adsorption of PNPA on γ-aminopropylsilicas as they can help to elucidate the structure of the new bonded phases. To characterize its interaction with proteins, the adsorption isotherm was obtained and the maximum capacity was evaluated for ovalbumin binding.

2. Experimental

2.1. Materials

MPS-2000 VGKh wide-pore glass (WPG) with particle diameter 50–150 μm and mean pore diameter 2100 Å, manufactured by GOZ VNII NP (Nizhny Novgorod, Russian Federation) was washed with 6 *M* hydrochloric acid and distilled water and fractionated by sedimentation. Ultimately, the fraction with particle diameter 50–100 μm was collected and dried. Silica gel XWP-1500 with particle diameter 16–23 μm and mean pore diameter 1500 Å was a gift from Diagen (Hilden, Germany). Monospher (MS) non-porous silica beads with particle diameter 1.4 ± 0.1 μm were a gift from Professor K.K. Unger (Johannes Gutenberg-Universitat, Mainz, Germany). DEAE-Toyopearl 650M was obtained from Tosoh (Akasaka, Tokyo, Japan).

Poly(*p*-nitrophenyl acrylate) (PNPA) $\bar{M}_w$ = 42 000, $\bar{M}_w/\bar{M}_n$ = 3.3 ($\bar{M}_w$ = mass-average molecular mass; $\bar{M}_n$ = number-average molecular mass), was prepared by free-radical polymerization of the monomer in dry benzene with azobisisobutyronitrile as initiator at 70°C with nitrogen bubbling for 20 h. γ-Aminopropyltriethoxysilane was purchased from Fluka (Buchs, Switzerland). Dimethyl sulphoxide (DMSO) was supplied by Sigma (St. Louis, MO, USA). Tris-hydroxymethylaminomethane (Tris) was obtained from Gerbu (Gaiberg, Germany). 2-Diethylaminoethylamine was purchased from Fluka. Other chemicals (solvents and salts) of analytical-reagent grade were supplied by Reakhim (Moscow, Russian Federation) benzene and toluene being distilled.

Bovine serum albumin (BSA), fraction 5, was obtained from Boehringer-Mannheim (Vienna, Austria) and ovalbumin from Serva (Heidelberg, Germany).

2.2. Chemical modification of silicas

All silicas were chemically modified with γ-aminopropyltriethoxysilane and analysed as described previously [8]. The amino group contents were 38, 210 and 150 μmol g^{-1} for MS-NH_2, WPG-NH_2 and XWP-NH_2, respectively. Aminopropylsilicas were treated with PNPA solution as described previously [8], washed with DMSO and suspended in a 2% solution of 2-diethylaminoethylamine in DMSO. After 2 days of periodic shaking at room temperature, the deposits of silicas were washed with distilled water and incubated in 0.5 *M* ammonium acetate (pH 9.0) for 1 week. Ultimately, the prepared sorbents were washed with distilled water and stored as wet cakes at room temperature in 0.02% sodium azide solution.

2.3. PNPA isotherm measurements

Isotherms of the polymer adsorption were obtained by means of a probe isolation from the

reaction media. From 0.05 to 0.1 g of aminopropylsilica was incubated in 1–10 ml of 0.05–3% (w/v) PNPA solution in DMSO for 2 days with moderate shaking at 25°C, the molar ratio of ester to aminopropyl groups being not less than 3. Samples of 5 mg of the PNPA-silica were isolated from the reaction medium, washed with DMSO and acetone, dried under vacuum, weighed and treated with concentrated aqueous ammonia solution (25°C, 24 h). The *p*-nitrophenolate concentration in the supernatant was determined by UV spectrophotometry (λ_{max} = 405 nm, ϵ_{max} = 15 300 l mol^{-1} cm^{-1}) and the specific *p*-nitrophenyl ester group content in the PNPA-silicas was then calculated.

2.4. Ovalbumin isotherm measurements

The adsorption isotherm of ovalbumin was obtained by recording the breakthrough curves of ovalbumin (A_{280}) applied in a frontal mode to the thermostated column (25°C, 1.5 cm × 1 cm I.D.) packed with anion exchanger based on XWP-1500 silica (DEAE-PA-silica) at various concentrations of the protein dissolved in 0.02 *M* Tris–HCl buffer (pH 7.8). The saturation of the column was judged at the point where 10% of the feedstock absorbance was detected in the effluent. The amount of adsorbed protein was then calculated by multiplying the volume pumped by the concentration. The flow-rate in frontal experiments was 0.5 ml min^{-1}.

2.5. Analytical chromatography of proteins

Ovalbumin and bovine serum albumin [0.5 mg each in 0.5 ml of 0.02 *M* Tris–HCl buffer (pH 7.8)] was applied to a glass MS-PC 10/20 Whatman column (3.9 cm × 1 cm I.D.) equipped with a thermostated water jacket (25°C) and packed with the anion exchanger. For sample application a V-7 valve (Pharmacia, Uppsala, Sweden) connected to a 0.5-ml polyethylene tubing loop was used. A linear gradient of sodium chloride was formed by an Ultrograd 11300 gradient mixer (LKB, Bromma, Sweden) and an NP-1 peristaltic pump (Analytical Apparatus SKB, Kiev, Ukraine). A Holochrome HMD HPLC detector (Gilson, Villiers-le-Bel, France) connected to a Mettler (Zurich, Switzerland) GA17 recorder was used to detect absorbing fractions during chromatographic procedures.

The resolution (R_s) was calculated as $2(t_2 - t_1)/(\Delta t_1 + \Delta t_2)$, where t_1 and t_2 are the retention times and Δt_1 and Δt_2 are the peak widths at the baseline in minutes.

3. Results and discussion

3.1. Bonded-phase synthesis: chemical adsorption of PNPA to aminopropylsilicas

Chemical adsorption of PNPA to aminopropylsilicas proceeds via formation of amide bonds between the polymer and moieties of the immobilized silane [8]. Many ester functions, however, remain unreacted and held by segments of the fixed macromolecular coils. These segments, loops and tails form a diffuse interface which is dense enough to prevent adsorption of proteins on the surface of silica [8]. To characterize the structure of the polymeric bonded phases, the adsorption isotherms were obtained for PNPA binding to non-porous aminopropyl Monospher silica gel (MS-NH_2) and wide-pore aminopropyl-glass (PG-NH_2) from solution in DMSO (Fig. 1a).

The plateau value of PNPA adsorption to PG-NH_2 exceeds that for MS-NH_2 by a factor of 8. This may be ascribed to the larger surface area and higher aminopropyl group content in the porous glass carrier as compared with non-porous silica. Indeed, the positions and shapes of the two isotherms differ only slightly from each other if the amount of polymer adsorbed is expressed as the *p*-nitrophenyl ester group content divided by the initial content of aminopropyls (Fig. 1b). Apparently, the pore structure of glass does not seriously hinder the transport of reactive PNPA molecules into the depth of particles, so that the adsorption mechanisms are closely related on the two carriers. Theoretically, this phenomenon may be explained by the high ratio of the mean pore diameter of glass (2000 Å) to the hydrodynamic radius of polymer coil, the latter being evaluated as 80 Å [10]. One can

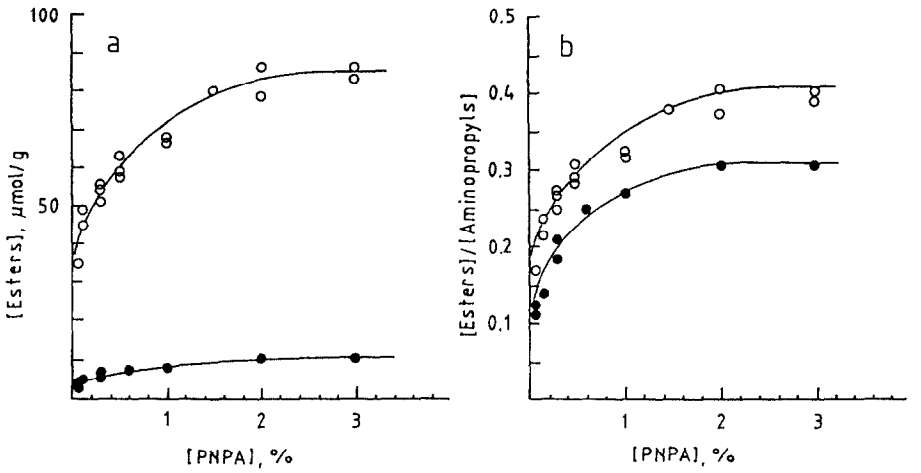

Fig. 1. Isotherms for poly(*p*-nitrophenyl acrylate) chemical adsorption on (○) aminopropylsilylated wide-pore glass and (●) non-porous silica.

expect, therefore, almost free diffusion of the polymer through the pores, concurrently with its chemical adsorption on the aminopropyl-glass surface.

The ester group content in the resultant composites increases with increasing PNPA concentration from 0 to 10 mg ml^{-1} and levels off at higher concentrations. The shape of the isotherms indicates the permeability of the adsorbed PNPA layer, which is assumed to be diffuse with flexible loops and tails. These findings correspond well with the conclusions of theories developed by Barford and co-workers [11,12] which predict more extended polymer profiles, as a function of adsorbate concentration, for a non-equilibrium mechanism of polymer adsorption rather than for the equilibrium mechanism.

Further, according to the above-mentioned theories, the density of segments plotted against the distance from the surface exhibits a slower decrease for neutral polymers than polyelectrolytes. One can expect, therefore, longer loops and tails in the PNPA adsorbed layers rather than in the adsorbed layers of PEI. Hence the effect of tentacles on protein separations seems to be more probable with PNPA- than with PEI-coated sorbents.

3.2. Analytical chromatography of proteins

For testing proteins by anion-exchange chromatography, composite sorbents with DEAE functions (DEAE-PA-silica and -glass) were prepared by the PNPA method from 20-μm particle size XWP-silica (average pore diameter 1500 Å) and from 80-μm particle size wide-pore glass (average pore diameter 2000 Å). The particles used were large enough to be packed into the Whatman standard glass column, which allows the use of low-pressure peristaltic pumping. The separation profiles obtained for commercial samples of ovalbumin and bovine serum albumin are shown in Fig. 2b and c. A typical separation performed with commercial DEAE-Toyopearl 650M, a cross-linked vinyl polymer [13], is given for comparison in Fig. 2a.

The better separation and sharper peaks observed for the DEAE-PA-silica are possibly due to its smaller particles and their narrow size distribution as compared with the Toyopearl material. However, the separation carried out with DEAE-PA-glass having larger particles (50–100 μm) is also better, as demonstrated in Fig. 2c.

The differences in the retention behaviour of the proteins indicates some other mechanism of

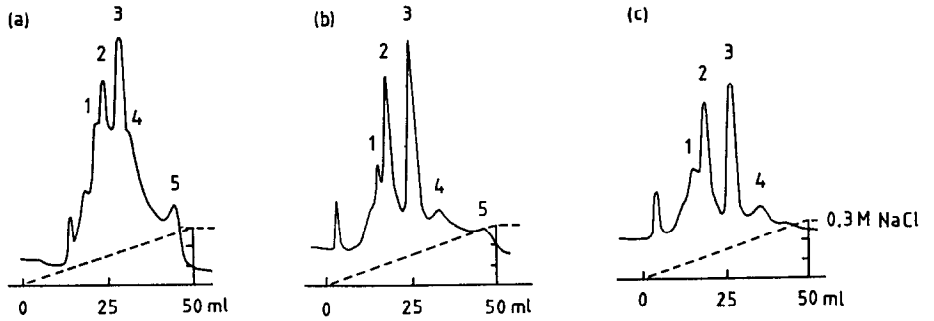

Fig. 2. Analytical separations of ovalbumin (peaks 1 and 2) and bovine serum albumin (peaks 3, 4 and 5) on 3.9 cm × 1 cm I.D. columns packed with (a) DEAE-Toyopearl 650M, (b) DEAE-PA-silica and (c) DEAE-PA-glass. Starting buffer, 0.02 *M* Tris–HCl (pH 7.8); sample, 0.5 mg of each protein in 0.5 ml of the starting buffer; elution, gradient from 0 to 0.3 *M* NaCl in the starting buffer; flow-rate, 1.1 ml min^{-1}; gradient time, 45 min. Proteins were detected by UV absorbance at 280 nm.

their adsorption, which contributes to the improved protein separation. Indeed, the main peak of ovalbumin elutes at 0.11 *M* NaCl concentration and the albumin peak at 0.16 *M* NaCl, the resolution factor being 1.4.

With a use of DEAE-Toyopearl 650M as a packing, ovalbumin elutes at 0.14 *M* NaCl and albumin at 0.19 *M* NaCl with $R_s < 0.8$, so that the better resolution of proteins on DEAE-PA sorbents is accompanied by milder desorption conditions.

The described effects are similar to those reported by Müller [6] for tentacle-like anion exchangers (DMA type). We believe that the diffuse structure of the polymer coating displaying many tails ensures an analogous improved function of DEAE-PA sorbents.

When compared with PEI-silicas, DEAE-PA-silica again shows a capability for better resolution. Vanecek and Regnier [3] studied the separation of albumin and ovalbumin on a series of PEI-silicas and obtained an optimum resolution factor of 1.40 on a 5 cm × 0.41 cm I.D. stainless-steel column with 10-μm PEI-LiChrospher Si 4000 with a 40-min gradient of sodium acetate.

DEAE-PA-silica of 20-μm particle size packed into a 3.9 cm × 1 cm I.D. column by the conventional low-pressure technique ensures comparable resolution factors for the same pair of proteins as listed in Table 1. It is noteworthy that PEI-silicas release the proteins at higher ionic strength than do DEAE-PA-silicas. Desorption of ovalbumin and albumin from PEI-silica requires 0.30 and 0.38 *M* sodium acetate, respectively, in 0.02 *M* Tris–CH_3COOH buffer (pH 8.0) [2].

The significantly milder desorption conditions observed for DEAE-PA-silicas prompted us to study the adsorption isotherm of ovalbumin in order to detect the possible deviations from the literature data for PEI-silicas and to evaluate the maximum loading capacity of the sorbent.

3.3. Adsorption isotherm

To obtain the adsorption isotherm, the dynamic load capacities (DLC) of DEAE-PA-silica were measured by frontal analysis at various concentrations of ovalbumin (Fig. 3). It was reported by Janzen *et al.* [7] that DLC may

Table 1
Resolution of bovine serum albumin and ovalbumin on DEAE-PA-silica at various flow-rates and gradient times

Flow-rate (ml min^{-1})	Gradient time (min)	R_s
0.6	90	2.0
0.7	45	1.4
1.2	45	1.4
1.4	22	1.2

R_s = resolution; for calculation, see Experimental.
Column size and chromatographic conditions as in Fig. 2.

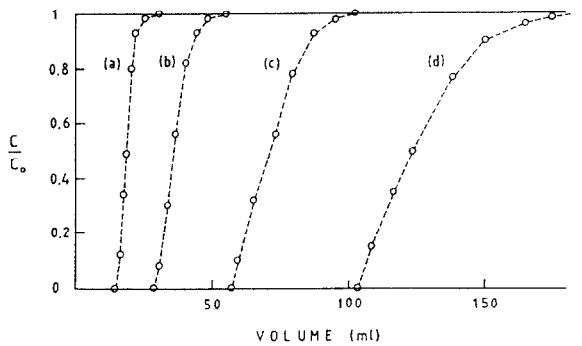

Fig. 3. Breakthrough curves of ovalbumin adsorption obtained on a 1.5 cm × 1 cm I.D. column packed with DEAE-PA-silica at various concentrations of the protein: (a) 1; (b) 0.4; (c) 0.2; (d) 0.1 mg ml^{-1}.

depend on the number of repetitive loadings on the same column. In this study, after six consecutive steps of loading and desorption, DLC was found to change by only 4% at a concentration of ovalbumin of 1 mg ml^{-1} and thus allowed the same packing to be used at least five times before it was replaced with a fresh portion of the sorbent. Moreover, virtually quantitative release of the protein (95–100%) was observed with DEAE-PA-silica and DEAE-PA-glass packings during the frontal experiments followed by a desorption step [0.5 *M* NaCl in 0.02 *M* Tris–HCl buffer (pH 7.8)].

The isotherm is illustrated in Fig. 4. Non-linearity of the Scatchard plot shows that the adsorption of ovalbumin strongly deviates from the course of the Langmuir isotherm, the same phenomenon having been observed earlier for adsorption of albumin on some PEI-silicas [7]. The maximum adsorption capacity was evaluated by extrapolation from a double reciprocal plot of the isotherm and equals 13 mg ml^{-1} of the sorbent. This amount is half that reported for ovalbumin adsorbed on 1000 Å pore diameter PEI-silicas (26 mg ml^{-1} for thin PEI coatings) [3].

Judging by the moderate protein capacity and milder desorption conditions, one may conclude that the synthesis based on PNPA chemical adsorption leads to an anion-exchange bonded phase exhibiting a sort of repellency towards proteins.

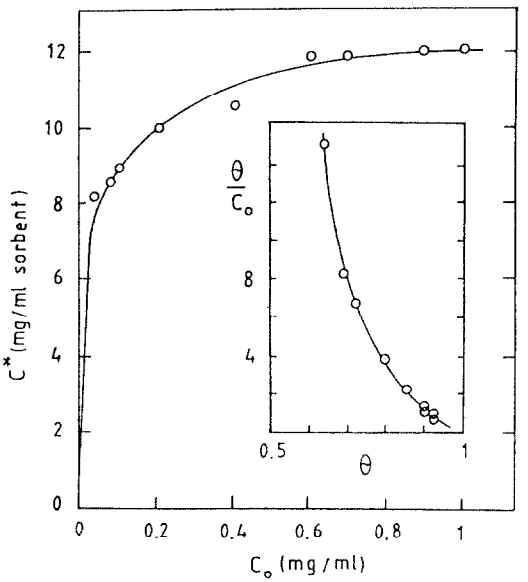

Fig. 4. Adsorption isotherm of ovalbumin on DEAE-PA-silica. The inset shows the Scatchard plot of the isotherm, where θ is the degree of sorbent saturation by the protein and c_0 is the amount of the protein adsorbed.

As was mentioned above, neutral reactive PNPA macromolecules are not as tightly adsorbed on silica as polycations of PEI. This is the reason why the resultant polymeric phase has a larger excluded volume and may produce an entropic repulsion towards the approaching particles [14]. Although the electrostatic attraction overwhelms this effect by far, something like a "springing" mechanism of protein adsorption may be suggested for polymeric ion exchangers with flexible loops and tails. For PEI-silicas with heavily cross-linked oligomeric coatings, no such "springing" may be expected. The same holds true for cross-linked vinyl polymers such as Toyopearl. The retention and adsorption of proteins are stronger on these materials.

4. Conclusions

Chemically bonded phases composed of multivalently adsorbed poly(N-diethylaminoethylacrylamide) exhibit an improved selectivity of protein separation and milder desorption conditions as compared with cross-linked anion

exchangers, an effect observed previously with end-grafted polymers [6]. The maximum loading capacities of the novel sorbents are, however, lower than those in the literature for analogous sorbents of a comparable pore size. On the other hand, the diffuse structure of the multivalently adsorbed polymer ensures highly reproducible protein adsorption capacities and an apparent absence of irreversible protein binding. These characteristics seem to be valuable for both the analytical and preparative chromatography of proteins.

Acknowledgements

We express our sincere gratitude to Professor K.K. Unger (Johannes Gutenberg-Universitat, Mainz, Germany) for presenting a sample of Monospher silica and to Dr. K. Henco (Diagen, Hilden, Germany) for the sample of XWP-1500 silica.

References

[1] A.J. Alpert and F.E. Regnier, *J. Chromatogr.*, 185 (1979) 375.
[2] G. Vanecek and F.E. Regnier, *Anal. Biochem.*, 109 (1980) 345.
[3] G. Vanecek and F.E. Regnier, *Anal. Biochem.*, 121 (1982) 156.
[4] M. Flashner, H. Ramsden and L.J. Crane, *Anal. Biochem.*, 135 (1983) 340.
[5] X. Santarelli, D. Muller and J. Jozefonvicz, *J. Chromatogr.*, 443 (1988) 55.
[6] W. Müller, *J. Chromatogr.*, 510 (1990) 133.
[7] R. Janzen, K.K. Unger, W. Muller and M.T.W. Hearn, *J. Chromatogr.*, 522 (1990) 77.
[8] A.E. Ivanov, L.V. Kozlov, B.B. Shojbonov, V.P. Zubov and V.K. Antonov, *Biomed. Chromatogr.*, 6 (1991) 90.
[9] V.P. Zubov, A.E. Ivanov and V.V. Saburov, *Adv. Polym. Sci.*, 104 (1992) 135.
[10] A.E. Ivanov, S.V. Belov and V.P. Zubov, *Vysokomol. Soed., Ser. A*, 35 (1993) 1320.
[11] W. Barford, R.C. Ball and C.C.M. Nex, *J. Chem. Soc., Faraday Trans. 1*, 82 (1986) 3233.
[12] W. Barford and R.C. Ball, *J. Chem. Soc., Faraday Trans. 1*, 83 (1987) 2515.
[13] *Toyopearl —The Packings for Medium Performance Liquid Chromatography*, Tosoh, Tokyo.
[14] J. Hermans, *J. Chem. Phys.*, 77 (1982) 2193.