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COATED SILICA SUPPORTS FOR HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY OF PROTEINS

F. L. ZHOU, D. MULLER*, X. SANTARELLI and J. JOZEFONVICZ
L.R.M. CNRS UA502, University Paris-Nord, Avenue J. B. Clément, 93430 Villetaneuse (France)

SUMMARY

Polymer-coated silica supports are potentially good stationary phases for high-performance affinity chromatographic separations of proteins. Silica beads have been coated with a polysaccharide (dextran or agarose), substituted by a calculated amount of positively charged diethylaminoethyl functions in order to neutralize the negatively charged silanol groups of silica and to facilitate the formation of a hydrophilic polymeric layer on the inorganic surface. The silica-based supports were prepared in two steps. First, the silica was impregnated with a solution of diethylaminoethylated polymer, and then the coating polymer was crosslinked in order to avoid leakage of the polymeric layer. The supports present minimal non-specific interactions with proteins, as tested by high-performance size-exclusion chromatography. These coated silica supports were coupled with active ligands, such as protein A, concanavalin A and heparin, by conventional coupling methods. The resulting affinity stationary phases were tested by the elution of proteins in order to study their performance in high-performance affinity chromatography.

INTRODUCTION

Porous silica beads have excellent mechanical proporties and can be prepared easily[1]. However, to be used as support in high-performance liquid chromatography (HPLC) of proteins, they have to be modified to avoid non-specific adsorption by the negatively charged silanol groups[1–6]. Silanol groups on the surface act as weak ion-exchanging groups and are responsible for this interfering adsorption phenomenon. Polysaccharide-based supports, such as Sepharose have excellent chromatographic properties in low-pressure liquid chromatography but, unlike silica, they have bad mechanical properties, leading to slow elution flow-rates[7]. It is interesting to combine the mechanical rigidity of silica with the hydrodynamic behaviour of polysaccharides[8,9]. We have prepared new stationary phases by coating silica beads with dextran or agarose substituted by a calculated amount of diethylaminoethyl (DEAE) functions. These DEAE units neutralize the ion-exchange capacity of native silica and facilitate the formation of a polymeric layer of the hydrophilic polysaccharides on the silica surface. The influence of several characteristics of these silica-based supports (*e.g.*, porosity of the starting silica beads, molecular weight of the

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polymer used, percentage of DEAE units, amount of polymeric coverage) was investigated by determining their performance in high-performance size-exclusion chromatography (HPSEC). These supports were activated by the classical methods used for hydroxyl-rich supports[10]. Ligands (protein A, concanavalin A and heparin) have been immobilized on these activated supports in a good yield. Tests were carried out by eluting samples of human IgG, ovalbumin, human antithrombin III (AT-III) and human α-thrombin from the affinity sorbents in order to investigate their performance in high-performance affinity chromatography (HPAC).

EXPERIMENTAL

The HPLC apparatus consisted of a Merck-Hitachi 655 A-12 gradient system from Labs Merck-Clevenot (LMC, Nogent sur Marne, France) with a Rheodyne 7126 injection valve, connected to an LMC variable-wavelength monitor and to a D2000 integrator. All solutions and buffers were prepared with doubly distilled water, which was degassed and then filtered through a 0.22-μm HA membrane (Millipore, Velizy, France). Molecular weight calibration curves for proteins were obtained as previously described[8]. The standard proteins were purchased from Sigma-Chimie (La Verpilliere, France). All chemical reagents were of analytical grade.

Preparation of silica-based supports

The silica-based supports were prepared in two stages. First, the silica beads (40–100 μm) were impregnated with a concentrated solution of DEAE polymer. Then, the polymer coating the beads was crosslinked in order to avoid leakage of the polymeric layer. The preparation of the dextran-coated support has been described previously[8].

Agarose (Indubiose A37 HAA and A37 NA), kindly provided by IBF Biotechnics (Villeneuve La Garenne, France), were modified by the following procedure. Agarose (10 g) was added to 210 ml of water at 60°C and stirred for 120 min. Then, 20 g of sodium hydroxide in 40 ml of water were added and the mixture was stirred at 55°C for 5 min. Next, 11.5 g of 2-diethylaminoethyl chloride hydrochloride (Janssen Chemica, Pantin, France) was added in several portions and the mixture was stirred at 55°C for 10–30 min. After the reaction had stopped, DEAE-agarose was precipitated with methanol–HCl (49:1, v/v) and the suspension was filtered under gentle suction in a 4-in. coarse sintered funnel. Finally, the filter cake was washed with ethanol, which was drawn off through the filter. The dried product was ground to a powder, and the remaining alcohol was removed under vacuum at 40°C. The characteristics of the DEAE-agarose were determined by acid–base titration of DEAE functions and by elementary analyse.

A batch method, followed by a cross-linking reaction, was used to coat the silica beads (silica X015 and silica X075, from IBF Biotechnics) with the DEAE-agarose. The starting silicas X075 and X015 have mean pore sizes of 300 and 1250 Å and specific surfaces of 100 and 25 m^2/g, respectively. An amount of 10 g of silica was impregnated with 0.46 g of DEAE-agarose in 23 ml of water (adjusted to pH 11.5) for 30 min at 80°C. The material was dried for 15 h at 80°C and the resulting powder was sieved. The impregnated silica was then added to a solution of 71 mg (0.55 mmol) of 1,4-butanediol diglycidyl ether (BDGE) (cross-linking agent) in 20 ml of diethyl ether and the mixture

was stirred for 30 min at 40°C. After evaporation of the solvent, the silica powder was dried for 15 h at 80°C, and the product was sieved. The amount of polymeric coverage, expressed as the weight percentage of impregnating polymer (g of polymer/100 g of support), was determined by elementary analysis for carbon.

Immobilization of active ligands

The supports were activated under the usual conditions for activation of hydroxyl-rich supports with either 1,1'-carbonyl-diimidazole (CDI) or BDGE[10]. Ligands (protein A, concanavalin A and heparin) were immobilized on the activated supports. Protein A and concanavalin A were supplied by IBF Biotechnics, and heparin (101 I.U./mg) was provided by Institut Choay (Paris, France). Immobilization of the ligands was performed by using 2.5 g of activated support suspended with the ligand in 12.5 ml of buffer (100 m*M* sodium carbonate, pH 8.7) at 20°C. The amounts of protein A and concanavalin A immobilized were determined by the Lowry method[11], and the amount of heparin immobilized was determined by the elementary analysis of sulphur on the dry stationary phase.

Performance of the affinity sorbents

The different biospecific affinity sorbents prepared from silica-based supports were used in chromatographic experiments in order to study their HPAC performance. Human IgG (IBF Biotechnics), ovalbumin (Sigma), human antithrombin III (37 I.U./ml, Centre Regional de Transfusion Sanguine, Lille, France) and human thrombin (1000 I.U./mg, Centre National de Transfusion Sanguine, Paris, France) were eluted from these stationary phases under salt gradient conditions. The eluted proteins were detected at 280 nm, and the chromatographic fractions were collected in order to determine the biological activity of the purified proteins and the recovery in the eluates.

RESULTS AND DISCUSSION

The basic principle of preparation of the silica-based supports is to coat inorganic stationary phases with hydrophilic polymer, substituted by a calculated amount of positively charged functional (DEAE) groups. These DEAE units neutralize the ion-exchange capacity of native silica and facilitate the formation of a polymeric layer of the hydrophilic polysaccharides on the silica surface. The hydrophilic layer will improve the chromatographic properties under aqueous eluting conditions. Moreover, these polymers can easily be activated by conventional activation methods, and ligands can be immobilized on the coated silica supports. It was demonstrated previously that the optimal substitution (expressed by the percentage of units bearing DEAE groups) for satisfactory coating is 4–13%[8]. The characteristics of the substituted polysaccharides used for preparation of the silica-based supports are given in Table I.

The amount of polymer covering the silica surface is the most important characteristic of a good coating. It is determined by elementary analysis of carbon, and is expressed as the weight percentage of impregnating polymer (g of polymer/100 g of support).

The effects of several characteristics of these silica-based supports (*e.g.*, the

TABLE I

CHARACTERISTICS OF SUBSTITUTED POLYSACCHARIDES

Modified polysaccharide	*Starting polysaccharide*	*Mw (g/mol)*	*Percentage of DEAE units*
DDT403	Dextran T40	35 600	4.3
DDT705	Dextran T70	68 000	4.5
DDT50011	Dextran T500	488 000	7.2
DHAA1	Indubiose HAA	–	10.0
DNA1	Indubiose NA	–	10.0

porosity of the starting silica beads, percentage of DEAE units and amount of polymeric coverage) have been investigated by studying the performance of the supports in HPSEC. Calibration curves for the standard proteins on the agarose-coated silica[8]. The results demonstrated that a minimum of 4% of polymer units bearing DEAE groups on the hydrophilic polymers covering the silica surface is necessary to minimize the ion-exchange capacity of the starting silica. If the percentage of polymer units bearing DEAE groups exceeds 13%, the cation-exchange capacity of the supports interferes with the steric-exclusion mechanism. A study of calibration curves indicated that silica coated with different polysaccharides exhibited similar chromatographic properties in HPSEC. The 1250-Å silica beads, coated with agarose or dextran, gave better resolution of proteins than the 300-Å silica beads (Fig. 1). Consequently this silica was selected as starting material for the preparation of HPAC supports. The characteristics of the supports used for HPAC are given in Table II.

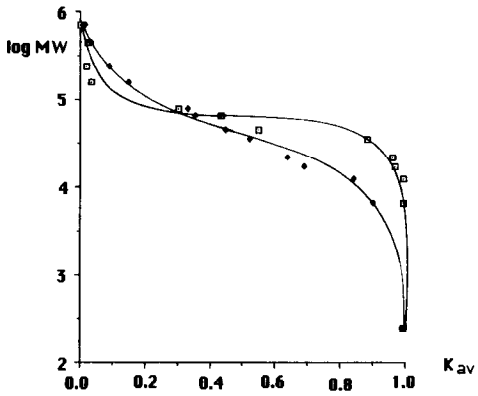

Fig. 1. Molecular weight calibration curves of standard proteins on different agarose-coated silica supports. Column, 25 × 0.7 cm I.D.; eluting buffer, 0.02 *M* Tris–HCl–0.15 *M* NaCl, pH 7.4; flow-rate, 1 ml/min.

Support	*Porosity (Å)*	*DEAE (%)*	*Impregnation (%)*[a]
SIA075HAA1 (□)	300	10.0	4.5
SIA015HAA1 (◆)	1250	10.0	4.7

[a] g of polymer/100 g of support.

TABLE II

CHARACTERISTICS OF SUPPORTS USED FOR HPAC

Support	*Silica porosity (Å)*	*Coating polymer*		*Impregnation (%)*[a]
		Name	*MW (g/mol)*	
SID403	1250	DDT403	35 600	8.1
SID704	1250	DDT704	68 000	10.4
SID5004	1250	DDT5004	488 000	10.1
SID50011	1250	DDT50011	488 000	10.1
SIANA1	1250	DNA1	–	4.5
SIAHAA1	1250	DHAA1	–	4.7

[a] g of polymer/100 g of support.

In order to confirm the passivation of silica, several plasma proteins [human albumin, human immunoglobulin (IgG), human AT-III and cytochrome] were injected into the different coated silica columns. No retention was observed, even at a 0.1 *M* ionic strength of sodium chloride, indicating that non-specific interactions had been minimized.

The immobilization of ligands (protein A, concanavalin A and heparin) on the coated silica supports was performed with CDI and BDGE as activating agents. The amount of immobilized ligand depended on the amount of activating agent used (Fig. 2). Similar coupling yields were obtained on DEAE-dextran- or DEAE-agarose-coated silica supports (Table III). The coupling yields of the coated silica supports were similar to those obtained with commercial polysaccharide-based supports.

In order to study their performance in HPAC, human IgG, ovalbumin, AT-III and thrombin were eluted from these coated silica supports grafted with different ligands. The results of elution experiments are presented in Table IV.

In the initial buffer, the proteins were strongly adsorbed on the coated silica supports grafted with the active ligands. A significant amount of protein was eluted by

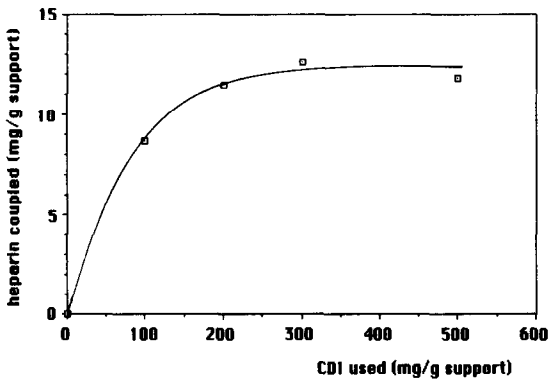

Fig. 2. Variation of the quantity of heparin coupled on SID50011 support with the quantity of 1,1-carbonyldiimidazole (CDI) used (20 mg of heparin/g support).

TABLE III

COUPLING CONDITIONS OF COATED SILICA SUPPORTS

Ql = quantity of ligand used (mg/g support). *Qa* = quantity of CDI used (mmol/g support). Con-A: concanavalin A.

Support	*Ligand*	*Ql*	*Qa*	*Yield (%)*
SID5004	Protein A	2.29	0.615	92
SIAHAA1	Protein A	2.29	0.615	89
SID5004	Con-A	20	1.23	71
SIAHAA1	Con-A	20	1.23	68
SID5004	Heparin	50	1.845	68
SIAHAA1	Heparin	50	1.845	68

a linear gradient. The conditions of desorption and recovery by elution from these affinity stationary phases were similar to those for the corresponding commercial polysaccharide-based affinity stationary phases. The nature of the polysaccharide (agarose or dextran) appears to have only a very slight influence on the chromatographic performance of the affinity stationary phases (conditions of desorption and recovery of eluted protein).

Results of a typical HPAC elution of human IgG on agarose-coated silica grafted with protein A are shown in Fig. 3. The protein was strongly adsorbed at pH 7.4 (0.02 *M* Tris–HCl, 0.15 *M* NaCl) and selectively desorbed by a decreasing pH gradient.

The elution of ovalbumin was carried out on an agarose-coated silica support grafted with concanavalin A. The glycoprotein was strongly adsorbed from the initial buffer (0.02 *M* Tris–HCl, 0.002 *M* $MnCl_2$, 0.002 *M* $CaCl_2$, pH 7.4) and specifically desorbed by competitive elution with glucose (Fig. 4).

Finally, a study was made of the elution of human AT-III (Fig. 5) and human thrombin (Fig. 6) from an agarose-coated support grafted with heparin. Thrombin and AT-III were strongly adsorbed at low ionic strength (0.02 *M* Tris–HCl, 0.1 *M* NaCl, pH 7.4) and selectively desorbed by raising the salt concentration in the eluting buffer. The eluted fractions were collected and then dialyzed against 0.15

TABLE IV

ELUTION CONDITIONS FOR HPAC OF PROTEINS

AT-III = antithrombin III; Pro-A = protein A; Hep = heparin.

Stationary phase	*Protein eluted*	*Conditions of desorption*	*Recovery (%)*	*Figure*
Pro-A SID5004	IgG	pH 3.40	84.5	
Pro-A SIAHAA1	IgG	pH 3.43	84.7	3
Con-A SID5004	Ovalbumin	0.082 *M* glucose	–	
Con-A SIAHAA1	Ovalbumin	0.082 *M* glucose	–	4
Hep-SID5004	AT-III	1.21 *M* NaCl	80	
Hep-SIAHAA1	AT-III	1.22 *M* NaCl	78	5
Hep-SIAHAA1	Thrombin	0.98 *M* NaCl	85	6

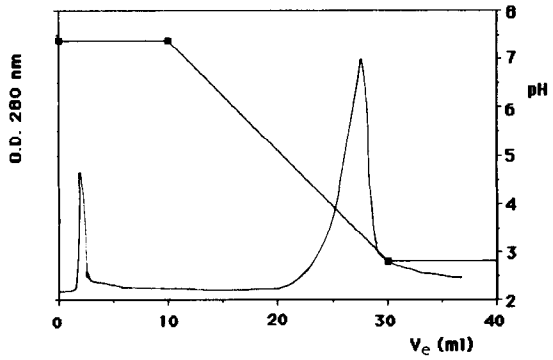

Fig. 3. Elution of human IgG from protein A–SIAHAA1 (gradient from pH 7.4 to 2.8). Column, 5 × 0.7 cm I.D.; sample, 100 μl human IgG (12.5 mg/ml); buffer A, 0.02 *M* Tris–HCl–0.15 *M* NaCl (pH 7.4); buffer B, 0.02 *M* glycine–HCl–0.15 *M* NaCl (pH 2.8); flow-rate, 1 ml/min.

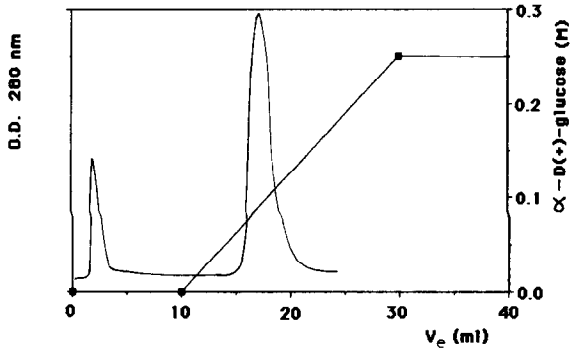

Fig. 4. Elution of ovalbumin from Con A–SIAHAA1 (gradient from 0 to 0.25 *M* α-D(+)-glucose). Column, 5 × 0.7 cm I.D.; sample, 100 μl ovalbumin (2.0 mg/ml); eluent 0.02 *M* Tris–HCl–0.15 *M* NaCl–0.02 *M* $CaCl_2$–0.02 *M* $MnCl_2$ (pH 7.4); flow-rate, 1 ml/min.

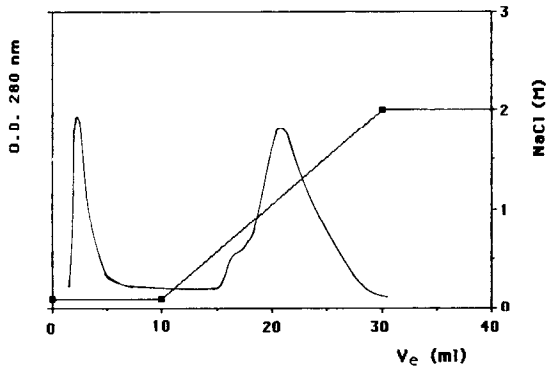

Fig. 5. Elution of human antithrombin III (AT-III) from Hep–SIAHAA1 (gradient from 0.1 to 2.0 *M* sodium chloride). Column, 5 × 0.7 cm I.D.; sample, 100 μl human AT-III (37 I.U./ml); eluent, 0.02 *M* Tris–HCl (pH 7.4); flow-rate 1 ml/min.

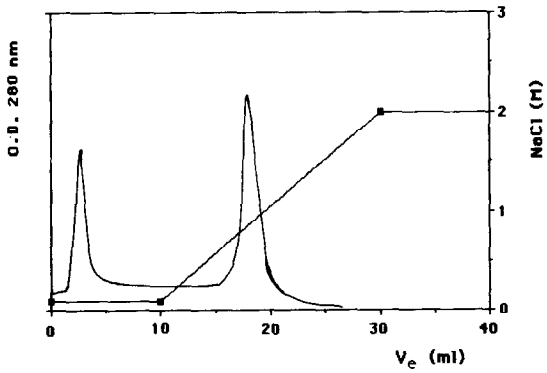

Fig. 6. Elution of human α-thrombin from Hep–SIAHAA1 (gradient from 0.1 to 2.0 *M* sodium chloride). Column, 5 × 0.7 cm I.D.; sample, 100 μl human thrombin (1000 U.NIH/ml); eluent, 0.02 *M* Tris–HCl (pH 7.4); flow-rate, 1 ml/min.

M NaCl aqueous solution for several hours at 4°C. The biological activity of AT-III was assayed for this suitability to inhibit thrombin in the presence of heparin by measuring the hydrolysis of Tos–Gly–Arg–pNa, *i.e.*, Chromo-Thrombin (from Diagnostica Stage, Asniéres-sur-Seine, France). The recovery of the eluted protein was determined by comparing the AT-III activity of human platelet-poor plasma with that of these fractions and of the starting material[12]. The thrombin time of these fractions was measured and compared with that of the starting sample. The recovery of the eluted protein, expressed as a percentage, could be calculated from a calibration curve[13]. Good recovery (>80%) was obtained in these elutions although α-thrombin was slightly inactivated during the separation at 22°C. For all of these separations, similar chromatograms were obtained with a dextran-coated support grafted with the same active ligand[9].

Twenty 100-μl portions of α-thrombin solution were injected successively into an affinity sorbent (Hep-SIAHAA1) under the same elution conditions. Similar chromatograms were obtained, indicating that there was no apparent change in the elution recovery during these elutions. This result demonstrated the excellent chemical stability of the coated silica supports.

CONCLUSION

In order to be used as chromatographic supports for proteins, silica beads must be passivated. This passivation can be carried out by a preliminary impregnation with a hydrophilic polymer having a relatively low percentage of units bearing positively charged DEAE groups. These DEAE units neutralize the ion-exchange capacity of native silica and facilitate the formation of a polymeric layer on the silica surface, so that non-specific adsorption of proteins on the coated silica is minimal. Because these coated silica beads possess the mechanical properties of the starting material and the hydrophilicity of the coating polymers, they can easily be grafted and used in HPAC. The active ligands can be immobilized in good yield by the conventional coupling methods used for polysaccharide-based supports. The chromatographic separations observed with these phases are similar to those obtained with commercial supports,

but can be obtained at high flow-rates. Moreover, because of the mechanical rigidity of the coated silica beads, the separation of proteins can easily be scaled up.

REFERENCES

1 K. K. Unger, *Porous Silica*, Elsevier, New York, 1979.
2 K. K. Unger, *Trends Anal. Chem.*, 2 (1983) 271.
3 F. E. Regnier, *Anal. Chem.*, 55 (1983) 1298A.
4 D. Muller, V. Baudin-Chinch and H. Wacjman, *Biosciences*, 5 (1987) 216.
5 L. Hagel and T. Anderson, presented at the *3rd Int. Symp. HPLC Proteins, Peptides and Polynucleotides, Monte Carlo, 1983.*
6 A. J. Alpert and F. R. Regnier, *J. Chromatogr.*, 185 (1979) 375.
7 J. L. Tayot, M. Tardy, P. Gattel, R. Plan and M. Roumiantzeff, in R. Epton (Editor), *Chromatography of Synthetic and Biological Polymers*, Vol. 2, Ellis Horwood, Chichester, 1978.
8 X. Santarelli, D. Muller and J. Jozefonvicz, *J. Chromatogr.*, 443 (1988) 55.
9 X. Santarelli, F. L. Zhou, D. Muller and J. Jozefonvicz, presented at the *Int. Symp. Biotech. Plasma Proteins, Nancy, 1988.*
10 *Practical Guide for Use in Affinity Chromatography and Related Techniques*, Technical note, IBF Biotechnics, Villeneuve la Garenne, 1983.
11 D. H. Lowry, N. J. Rosenbrough, A. I. Farr and R. J. Randall, *J. Biol. Chem.*, 265 (1951) 193.
12 O. R. Odegard, M. Lie and U. Abildgaard, *Thromb. Res.*, 6 (1975) 287.
13 C. Boisson, D. Gulino, J. Jozefonvicz, A. M. Fischer and J. Tapon-Bretaudiere, *Thromb. Res.*, 34 (1984) 269.