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Comparative affinity chromatographic studies using novel grafted polyamide and poly(vinyl alcohol) media

D. MÜLLER-SCHULTE*

Institut für Organische Chemie der RWTH Aachen, Prof-Pirlet-Strasse 1, W-5100 Aachen (Germany)
and
S. MANJINI and M. A. VIJAYALAKSHMI

Université de Technologie de Compiègne, Departement Genie Biologique, B.P. 649, 60206 Compiègne Cedex (France)

ABSTRACT

Radiation-grafted polyamide in microparticulate form was used for the affinity chromatographic separation of fibronectin, human immunoglobulin IgG1 and human serum albumin using gelatin, histidine and Cibacron Blue F3G-A as affinity ligands. The chromatographic properties, in particular the protein adsorption capacities of the grafted carriers, were tested in comparison with several commercial media (Sepharose, Spherosil, Eupergit, Fractogel, VA-Epoxy). As a result of grafting, long molecular spacers are introduced into the polymer carrier, leading to high ligand binding and protein loading capacities well above those achieved with commercial media. A novel support based on poly(vinyl alcohol) carrying a blood group-specific trisaccharide ligand for the removal of blood group antibodies from human serum is described. The poly(vinyl alcohol) carrier exhibits excellent antibody binding capacities when compared with supports carrying protein G and protein A ligands. New perspectives opened up by the new support with regard to *in vivo* haemoperfusion are discussed.

INTRODUCTION

A number of carrier media based on silica gels, polysaccharides or synthetic organic materials have been described for diverse chromatographic applications [1]. The increasing importance of separation technology in different areas has led to improved chromatographic media which are mainly used for the production of biologically active substances. Prominent examples include the purification of antibodies, blood components, growth factors, interferon, insulin and fibronectin. If one considers technical applications, affinity chromatography offers enormous advantages over other separation methods.

Depending on the degree of biorecognition of the ligand, the separation of biomolecules can be obtained in a single step, thereby simplifying the commonly used separation procedures which generally require several purification steps. Affinity chromatography can therefore offer a significant reduction in cost, time and technical effort.

Despite the enormous effort invested in the field of affinity chromatography,

a few aspects still remain critical, including insufficient ligand binding capacity of the support, the cost, the physical and chemical stability and the lack of biospecificity of the affinity ligand [2]. Another essential point is the different coupling method, the drawbacks of which have been critically discussed [1].

We have demonstrated recently that radiation-grafted polyamide-6 in microparticulate form and newly developed poly(vinyl alcohol) carriers exhibit distinctly higher protein and ligand binding capacities than those achieved with well established commercial media [2,3].

In this work, the performances of grafted polyamide-6 (Biograft) and poly(vinyl alcohol) (PVA) gels as carriers were tested in comparison with several commercial media.

Gelatin, Cibacron Blue F3G-A and *l*-histidine were used as affinity ligands for the separation of human fibronectin (Fn), human serum albumin (HSA) and human immunoglobulin IgG1. Likewise, a trisaccharide hapten specific against blood group A antibodies was coupled to different supports, the aim being to assess the feasibility of these carriers for *in vivo* haemoperfusion.

EXPERIMENTAL

Materials

The materials were purchased from the following sources: Sepharose and Superose media, Pharmacia–LKB (Freiburg, Germany); Fractogel TSK HW-75F, Merck (Darmstadt, Germany); Eupergit C, Röhm Pharma (Darmstadt, Germany); VA-Epoxy and VA-Hydroxy, Riedel-de Haën (Selze, Germany); Synsorb A and blood group A trisaccharide, Chembiomed (Edmonton, Canada); Spherosil, Rhône Poulenc (Paris France); IgG1 protein solution from human placenta, Institut Merieux (Lyon, France); and PVA, Hoechst (Frankfurt/M, Germany). All other chemicals were supplied by Fluka (Freiburg, Germany) and Merck._

Preparation of grafted polyamide and poly(vinyl alcohol) carriers

The grafting of polyamide-6 and the synthesis of the PVA carriers were carried out according to previously described procedures [4,5]. For grafting, 2-hydroxyethyl methacrylate together with N-vinylpyrrolidone and acrylamide were used, resulting in a graft uptake of 74% (w/w). Activation was carried out with epichlorohydrin in 1 *M* sodium hydroxide solution [4].

PVA was synthesized as follows [5]: PVA was dissoved with heating in ethylene glycol to obtain a 5% solution. The hot solution was dispersed in vegatable oil with stirring at 400–1000 rpm. The suspension was cooled and the beads formed were separated from the oil by several extractions with acetone. The diameter of the beads depends on the stirring speed, and ranges from 50 to 500 μm. Cross-linking was performed using epichlorohydrin in 3 *M* sodium hydroxide solution. Epoxy-activation with epichlorohydrin was performed as for the grafted polyamide.

Preparation of affinity supports

Gelatin support. Ten millilitres of a 1% gelatin (porcine skin, Fluka) solution in 1 *M* potassium phosphate (pH 7.5) were added to 3 ml of epoxy-activated support (Biograft, Eupergit C or VA-Epoxy) and the mixture was left for 30 h at 30°C with

gentle stirring. The excess of protein was removed by washing with 0.5 M potassium phosphate. The remaining oxirane groups were blocked by incubation for 2 h with 1 M ethanolamine solution (pH 8.0). The support was stored in 0.05 M Tris–HCl buffer (pH 7.5) containing 0.02% sodium azide.

Fractogel TSK HW-75F was activated for coupling with gelatin using 2-fluoro-1-methylpyridinium toluenesulphonate according to the method described by Ngo [6].

Cibacron Blue support. Coupling of Cibacron Blue F3G-A to 3 ml of Biograft and PVA was effected in a solution containing 20% sodium chloride and 1 M sodium carbonate for 2 days at 40°C [7]. In order to ensure maximum binding, a high dye concentration (100 mg/ml resin) was used. Extensive washing with water and 4 M sodium chloride solution containing 40% (v/v) ethylene glycol followed until all unbound dye had been removed. Before use, the matrices were equilibrated in 0.01 M potassium phosphate (pH 7.5).

Histidine support. Histidine was covalently coupled to epoxy-activated Biograft as described elsewhere [8]. Two coupling agents were used, epichlorohydrin and 1,4-butanediol diglycidyl ether.

Blood group A immunoadsorbent. For the preparation of the immunoadsorbents, 0.5 ml of epoxy carrier (Biograft or Eupergit C) was incubated with 1 ml of borate buffer (pH 9.5) containing 0.5 mg of the blood group hapten. After reaction for 20 h at room temperature, the adsorbent was washed several times with 1 M sodium chloride solution. Fractogel TSK HW-75F was activated with 2-fluoro-1-methylpyridinium toluenesulphonate as described above.

The activation of PVA using 1,6-hexamethylene diisocyanate dissolved in dimethyl sulphoxide and the subsequent coupling of the blood group hapten were carried out as described previously [2]. After the reaction, the matrix was washed with water and dimethyl sulphoxide. Before use, all supports were equilibrated with physiological sodium chloride solution.

Determination of immobilized Cibacron Blue

The amount of Cibacron Blue F3G-A coupled to the support was determined spectrophotometrically by measuring the differences in absorbance at 610 nm between added and unbound dye [7].

Chromatographic procedures

Gelatin support. Gelatin affinity support (3 ml) was packed into a chromatographic column (5 × 0.9 cm I.D.). After equilibration with 50 ml of phosphate-buffered saline (pH 7.5) containing 0.01 M citric acid and 0.02% sodium azide (buffer A), 20 ml of freshly prepared pooled citric human plasma (obtained by centrifugation for 10 min at 3000 g) were loaded on the column at a flow-rate of 50 cm/h. After the major unretained peak had eluted and the absorbance monitored at 279 nm had reached zero, the Fn was eluted with 3 M urea in buffer A. The Fn content of the eluted fraction was determined by a standard turbidimetric immunoassay test [9]. Briefly, antiserum to human Fn is added to the plasma sample and the absorbance is measured with a Behring laser nephelometer. The Fn content is automatically evaluated from the corresponding protein concentration of a standard dilution [9].

Cibacron Blue support. Cibacron Blue F3G-A affinity resin (3 ml) was packed into the column described above and equilibrated in 0.05 M sodium phosphate buffer

(pH 7.0) at a flow-rate of 25 cm/h. A 10-ml volume of sample solution containing 30 mg of HSA was loaded on the column. Unadsorbed protein was washed off the column with 20 ml of buffer solution. The bound HSA was subsequently eluted with 10 ml of 4 M sodium chloride and the fractions were collected at a flow-rate of 25 cm/h. The protein content was determined spectrophotometrically at 280 nm.

Histidine support. A 20 × 1 cm I.D. column was packed with 1 ml of support. Adsorption and washing were performed in 0.025 M Tris–HCl buffer (pH 7.4). The same buffer containing 0.2 M sodium chloride was used for desorption. The experiments were conducted at 4°C, except for Spherosil columns, for which the optimum retention was obtained at room temperature. The concentration of the eluted IgG1 was determined spectrophotometrically at 280 nm.

Blood group immunoadsorbent. Pooled human serum type O (1 ml) was added for 10 min at room temperature to a given amount of support (batch procedure; for details see Table IV). The content of antibody adsorbed was determined by the agglutination test as described previously [2].

RESULTS AND DISCUSSION

The performance of Biograft and PVA carriers was tested in comparison with seven different commercial supports: Sepharose, Superose, Fractogel, Eupergit C, VA-Epoxy, Synsorb A and Spherosil. As the commercial matrices chosen here are frequently used in practice, the results obtained should provide important data with regard to the performance of Biograft and PVA.

All supports were coupled under identical conditions, with the exception of gelatin Sepharose, protein A/protein G Sepharose and Synsorb A, which were used as supplied by the manufacturer.

As shown in Table I, the Fn adsorption capacity of gelatin Biograft is about 80% higher than that of Gelatin Sepharose. VA-Epoxy shows the best performance among the commercial media, which is in line with recent findings regarding the performance of this matrix in immunoaffinity chromatography [2]. Although the study of the different supports under identical test conditions is the main topic, it should be stressed that the performance of the separation can still be optimized by modifying certain test

TABLE I

ADSORPTION CAPACITY OF FIBRONECTIN ON DIFFERENT GELATIN AFFINITY RESINS

Human plasma (20 ml) was applied to 3 ml of adsorbent at a linear flow-rate of 50 cm/h at room temperature. The Fn concentration in the elution fraction was determined using an immunoturbidimetric standard test method [9]

Support	Approximate bead size (μm)	Capacity (mg Fn/ml gel)
Biograft	150	2.8
Gelatin Sepharose 4B	70	0.6
Fractogel TSK HW-75F	40	0.9
Eupergit C	150	1.4
VA-Epoxy	110	2.1

TABLE II

ADSORPTION CAPACITY OF HSA ON DIFFERENT CIBACRON BLUE F3G-A GELS

Flow-rate: 25 cm/h.

Support matrix	Approximate bead size (μm)	Ligand bound (mg/ml gel)	HSA adsorbed (mg/ml gel)
Biograft	150	0.5	0.59
PVA	80	1.4	0.81
Sepharose CL-6B	70	0.2	0.19
Fractogel TSK HW-75F	40	0.3	0.65
VA-Hydroxy	110	0.3	0.21

parameters, *e.g.*, amount of ligand bound, flow-rate, temperature and amount of protein loaded onto the column, as recently demonstrated by Regnault *et al.* [10] for the separation of Fn. This leaves room for further improvements when using grafted polyamide carriers.

HSA adsorption capacities on different Cibacron Blue resins are given in Table II. Biograft binds about three times as much HSA as VA-Hydroxy and Sepharose CL-6B. This correlates directly with the ligand uptake of these supports: 0.5 mg/ml (Biograft), 0.3 mg/ml (VA-Hydroxy) and 0.2 mg/ml Cibacron Blue (Sepharose) under the chosen test conditions. PVA shows the highest protein adsorption with 0.81 mg/ml, yet this amount is relatively low considering the large amount of the ligand bound (1.4 mg/ml). The performance of Fractogel TSK HW-75F is not in accordance with the other results of this test series, insofar as it adsorbs relatively large amounts of HSA (0.65 mg/ml), which is in contrast to its low Cibacron Blue uptake. An explanation of this could be the non-specific adsorptions due to the partly non-polar nature of the

TABLE III

COMPARISON OF IgGl ADSORPTION CAPACITIES OF DIFFERENT HISTIDINE-COUPLED AFFINITY RESINS

For details see Experimental.

Support	Approximate bead size (μm)	Capacity (mg IgCl/ml gel)
Biograft	150	0.69
Sepharose 4B		
Without spacer[a]	70	0.05
With spacer[b]		0.23
Superose 12 with spacer[b]	30	0.29
Spherosil		
Without spacer[c]	80	0.15
With spacer[d]		0.28

[a] Activated with epichlorohydrin.
[b] Activated with 1,4-butanediol diglycidyl ether.
[c] Activated with γ-glycidyloxypropyltrimethoxysilane.
[d] Activated with γ-glycidyloxypropyltrimethoxysilane and subsequently reacted with aminocaproic acid; coupling of histidine was conducted using carbodiimide [8].

Fractogel structure. A reason for the relatively low protein adsorption achieved with PVA regarding the very high ligand binding density could be that a high ligand density, owing to steric effects, prevents an appropriate interaction of the non-polar moiety of the ligand with the non-polar region of HSA [11].

A comparison of the IgG1 binding capacities on different histidine affinity supports is shown in Table III. Grafted polyamide adsorbs about three times more IgG1 than Sepharose 4B, Superose 12 and Spherosil. The influence of a spacer molecule on the protein binding efficacy is demonstrated by comparing the results obtained with 1,4-butanediol diglycidyl ether, which functions as a spacer, and epichlorohydrin. The differences between these two coupling modes are obvious, especially with Sepharose 4B: 0.05 mg IgG1/ml bound without spacer and 0.23 mg IgG1/ml bound with spacer. In contrast, the grafted support does not require an additional spacer-like coupling agent, which would also contribute to the overall high costs of affinity media.

The above-described ligands have one common feature, namely that they are all pseudo-specific. However, as they are inexpensive and are readily available, they are frequently used in the laboratory. This applies especially to the use of some textile dyes such as Cibacron Blue, Remazol Yellow and Procion Red [12]. Although these ligands interact with a number of proteins [7], Cibacron Blue F3G-A has been applied successfully to the separation of HSA, enzymes, growth and coagulation factors and interferon [12]. HSA is technically produced by a chromatographic process using Cibacron Blue columns [13]. A comparison of the performances of different Cibacron Blue supports is hence of fundamental interest with regard to technical applications.

By adopting defined adsorption or desorption modes, a resolution and degree of purification comparable to those of immunoadsorption can be obtained with pseudo-specific ligands. This applies especially to histidine, which interacts through its carboxyl, amino and imidazole groups with several proteins at or around their isoelectric point and has shown particular efficacy in separating IgG subclasses [14]. This mode of chromatography also has some potential if one considers its technical application [15].

TABLE IV

REMOVAL OF BLOOD GROUP A ANTIBODIES FROM HUMAN SERUM

Pooled human serum type O (1 ml) was incubated for 10 min at room temperature (batch procedure). The antibody content was determined by agglutination test [3].

Support	Sample volume (ml)	Approximate bead size (μm)	Antibody bound (%)
PVA	1.0	500	> 90
Biograft	0.5	150	> 90
Eupergit C	0.5	150	> 90
Fractogel TSK HW-75F	0.5	40	< 30
Synsorb A	1.0	500	< 50
Protein A			
Sepharose CL-4B[a]	0.3	70	< 10
Protein G			
Sepharose 4[a]	0.3	70	< 10

[a] Used as supplied by the manufacturer without blood group hapten.

The reason for the overall superior performance of the grafted supports in comparison with well established media lies in their special molecular structure. Grafting leads to long tentacle-like molecular chains which function automatically as spacer arms [4]. These grafted chains obviously possess a high accessibility for ligands and proteins, as shown in recent enzyme and antibody immobilization tests [3,16].

Affinity chromatography has also been successfully used in the medical field. In bone marrow transplantation, ABO incompatibilities can be circumvened by anti-A and anti-B antibody removal from blood and serum. For this purpose, a novel PVA carrier was developed to which a specific blood group oligosaccharide was attached
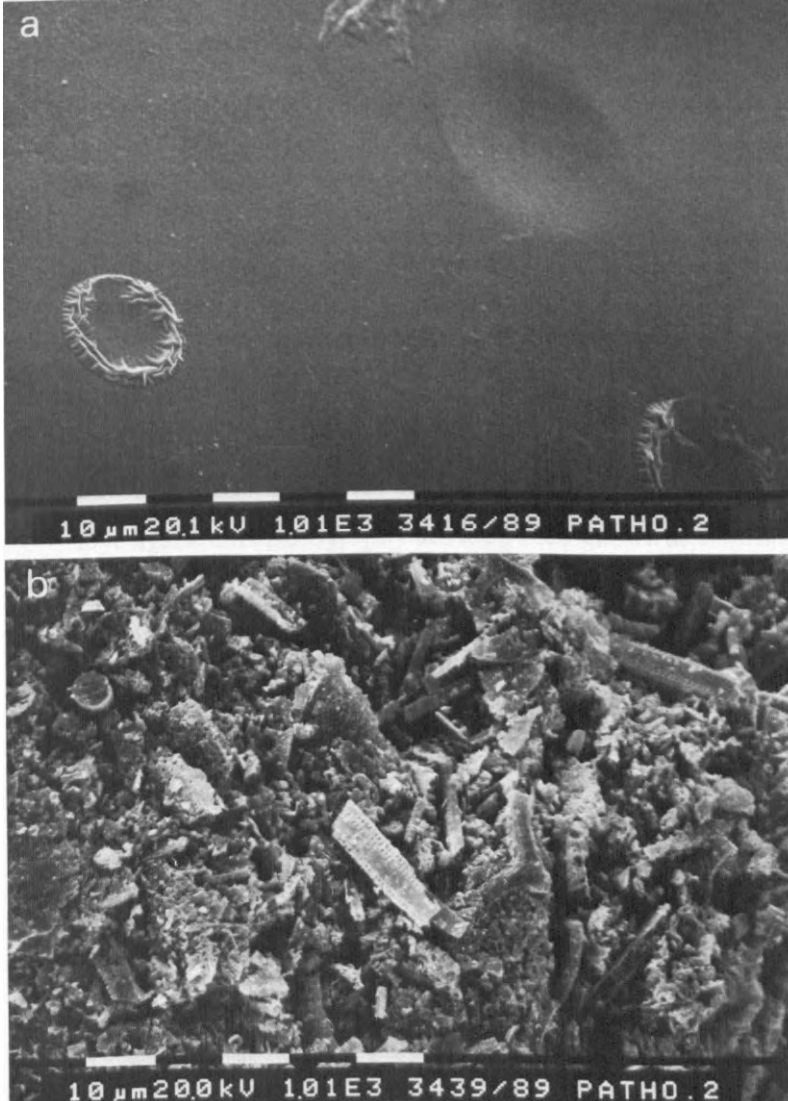

Fig. 1. Scanning electron micrographs of (a) PVA and (b) Synsorb A immunosorbent carriers, showing the smoothness of the surface morphology of PVA beads in comparison with Synsorb.

[2,17]. A comparative study of the blood group antibody adsorption capacities on different immunosorbents is shown in Table IV.

PVA, Biograft and Eupergit C bind over 90% of the antibody, whereas Fractogel and Protein A and Protein G Sepharose adsorb relatively low protein contents (<30% and <10%, respectively). The PVA protein binding ability is outstanding if one considers the large bead size of this gel (*ca.* 500 μm). This underlines both the efficacy of the isocyanate coupling technique and the suitability of PVA as an affinity support. Further, it becomes obvious that the blood group trisaccharide ligand exhibits distinctly better specificity and hence binding properties than protein A and protein G, ligands frequently used for IgG separations [18].

Although Biograft and Eupergit C also show good protein adsorptions, these carriers are only suitable for *in vitro* usage owing to their small bead sizes. Synsorb A, a commercial immunosorbent based on silica gel [19], also has a bead size of *ca.* 500 μm and shows reasonable adsorption properties. However, this support might not be useful for *in vivo* application as it consists of very small single building blocks (Fig. 1) which produce a rough surface morphology. The surface morphology, however, is vital for biocompatibility as it directly influences the mode of blood flow. Rough surfaces can cause micro turbulences and thus promote thrombus formation [20]. Another detrimental reaction is the activation of the complement system [21]. Hence PVA remains the only choice for practical haemoperfusion because of its excellent adsorption capacity, smooth surface morphology (Fig. 1) and good blood compatibility, which is superior to that of common carrier types [21].

Apart from the biochemical performance, the newly developed media possess excellent chemical stability. Treatments in 1–6 M sodium hydroxide are possible without detectable destruction [5]. This is a unique feature among all common chromatographic supports and it seems that these media are useful alternatives to currently used supports.

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