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Novel media for chromatography and immobilization using a radiation grafting technique

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ABSTRACT

Radiation grafting onto polyamide and poly(vinyl alcohol) was performed using different vinyl monomers. This grafting technique permits the synthesis of carrier media with a wide range of physical and chemical properties. A number of immobilization tests with antibodies and enzymes *e.g.*, penicillin acylase, glucose isomerase and formate dehydrogenase, are described, exhibiting binding capacities which are distinctly higher than those achieved with commercial media. The epoxy- and isocyanate-activated grafted copolymers were used for the affinity chromatographic separation of insulin, factor VIII and human serum albumin using antibodies as affinity ligands. The radiation-modified media allow a high antibody coupling, thus overcoming drawbacks of currently available commercial media. The separation of blood group antibodies can be performed by using novel antibody-specific oligosaccharide ligands, which permit a much more specific separation than protein G coupled media.

INTRODUCTION

The interest in and demand for biospecific molecules (enzymes, antibodies and glycoproteins) in biotechnology, biochemistry and medicine have contributed to an increased exploitation of affinity chromatography. As affinity chromatography represents by far the most powerful separation method, great efforts have been made in the whole field of chromatography to extend beyond the laboratory scale and to adapt this technology to large-scale industrial production. Essential requirements for transfer to a technical level are physical and chemical stability, hydrophilicity, appropriate ligand-binding capacity and suitable activation and coupling procedures. If one takes into account the technical usage, price and reusability must then also be considered.

Despite tremendous efforts, none of the currently available commercial media fulfil all of these criteria. Ineffective and laborious coupling techniques, low binding capacities and high costs are the main drawbacks among current supports[1]. In this respect large-scale affinity chromatography must be regarded as being in a preliminary stage.

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In this present study, radiation-grafted polyamide-6 (Biograft) and poly(vinyl alcohol) (PVA) as media for affinity chromatography (AC), enzyme immobilization and haemoperfusion are described. As a result of grafting, a molecular structure is formed which enables high ligand–protein binding densities well above those achieved using established commercial matrices. Simple coupling techniques via epoxy- (Biograft) and isocyanateactivated carriers (PVA) contribute to a high binding and separation performance.

EXPERIMENTAL

Materials

The materials were purched as follows: Affi-Gel Hz, Bio-Rad Labs. (Munich, F.R.G.); Sepharose media, Pharmacia–LKB (Freiburg, F.R.G.); Fractogel media, Merck (Darmstadt, F.R.G.); Eupergit C, Röhm Pharma (Darmstadt, F.R.G.); VA-Epoxy, Riedel-de Haën (Seelze, F.R.G.); Synsorb A, blood group A oligosaccharide, Chembiomed (Edmonton, Canada); polygalacturonase, formate dehydrogenase, penicillin acylase, Braunschweiger Biotechnologie (Braunschweig, F.R.G.); horseradish peroxidase, Sigma (Deisenhofen, F.R.G.); glucose isomerase, CPC/Europe (Vilvoorde, Belgium); anti-human serum albumin (anti-HSA), anti-human growth hormone (anti-HGH), anti-insulin, Sorin (Saluggia, Italy); and insulin, HSA, Behring Werke (Berlin, F.R.G.). All other chemicals were obtained from Fluka (Neu-Ulm, F.R.G.).

Preparation of affinity and immobilization carriers

Radiation grafting of polyamide-6 and PVA with 2-hydroxyethyl methacrylate (HEMA), N-vinylpyrrolidone (NVP), acrylamide (AA) and 2-dimethylaminoethyl methacrylate (DAEM) was conducted using a simultaneous grafting technique as described elsewhere[2–4]. Such a grafting procedure is performed as follows: 5 ml of HEMA, 0.5 ml of NVP, 3 ml of AA (20% aqueous solution), 1 ml of DAEM, 1 ml of formamide, 4 ml of tetrahydrofuran, 7 ml of methanol and 2 ml of water were added to 5 g of polyamide powder with a bead size of *ca.* 150 μm. The mixture was exposed to a cobalt γ-radiation source for 1 h, receiving a total dose of 0.18 Mrad. After washing with methanol, the graft uptake (weight per cent increase of the starting polyamide powder) was 62%. A 1-g amount of the grafted and dried resin was activated with 3 ml of epichlorohydrin with addition of 10 ml of 0.5 *M* NaOH for 2 h at 55°C. After intensive washing with water, the oxirane content of the dried carrier was determined according to Sundberg and Porath[5], amounting to 800–1000 μmol/g resin. For a detailed description of the protein coupling procedures to the epoxy-activated carriers, see the legends of the figures and tables. Isocyanate activation per gram of PVA was carried out with addition of 10 ml of dimethyl sulphoxide containing 2 ml of 1,6-hexamethylene diisocyanate at 40°C for 60 min, followed by thorough washing with acetone.

Activation of Fractogel TSK HW-75F with 2-fluoro-1-methylpyridinium toluene sulphonate (FMP) and coupling of ligands to this carrier were conducted following the method described by Ngo[6]. Antibodies were coupled to cyanogen bromide (CNBr)–Sepharose ad Affi-Gel Hz according to the manufacturer's instructions[7,8]. For the coupling of the blood-group hapten to isocyanate-activated PVA,

2 mg of hapten were dissolved in 5 ml of dimethyl sulphoxide and incubated with 1 g of carrier for 5 h at ambient temperature.

Procedures for affinity chromatography

All affinity chromatographic tests were carried out as a batch process. The affinity media coupled with the ligand were placed in a 2-ml plastic syringe equipped with a 0.2-μm pore size sterile filter unit. After the protein solution to be separated had been incubated for 12 min, the solution was pressed through the filter and the remaining protein content in the filtrate was analysed according to Lowry *et al.*[9]. For further details, see the figure and table legends.

Analytical methods

The amount of protein immobilized was calculated from the difference between the initial amount of protein applied, determined by Lowry *et al.*'s method[9], and the amount in the supernatant after coupling. The blood-group A antibody was determined using an agglutination test, each with 20 μl of undiluted serum and freshly prepared erythrocyte suspension. Using increasing serum dilutions (1:1, 1:2, 1:5 and 1:10), the agglutination times were measured and served as calibration values for the quantitative detection of antibodies. The enzymatic tests were carried out following the Merck[10] and Boehringer Biochemical[11] standard test methods.

RESULTS AND DISCUSSION

Some carrier manufacturers offer affinity ligands that include an attached affinity ligand, *e.g.*, Cibacon Blue-, heparin-, protein A-, concanavalin A- and benzamidine-coupled media. Despite the frequent use of these affinity media, they only express group specificity and do not show specificity towards a single compound. Owing to the lack of specific ligands, an increased usage of antibodies and in particular monoclonal antibodies as affinity ligands has been observed in the last few years. This trend has been enhanced by hybridoma technology.

In this study, a number of antibody immobilization tests on different carriers were conducted in order to assess the performance of the grafted media in comparison with established matrices.

Fig. 1 shows a plain anti-HGH coupling on four epoxy-activated carriers. The

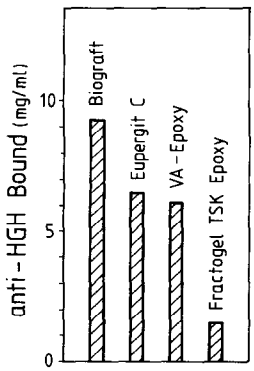
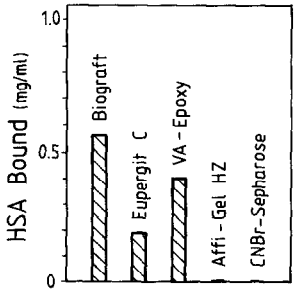


Fig. 1. Immobilization of anti-HGH on different epoxy-activated carriers. Coupling conditions: 3 mg of protein per 100 mg of carrier in 1 *M* potassium phosphate (pH 8.0) for 24 h at room temperature.

coupling of the grafted sample (Biograft) lies well above that of the commercial media. With the introduction of high-molecular-weight affinity ligands, the drawback regarding accessibility increases, as is evident on comparing the HSA and insulin recoveries on different anti-HSA and anti-insulin affinity supports (see Figs. 2 and 3). The coupling differences between Biograft and Affi-Gel Hz (a hydrazide-activated gel) and CNBr-activated Sepharose respectively are large. This is in accordance with recent practical experience, namely that CNBr is an ineffective coupling agent[12]. These media were therefore not considered suitable for further tests. In contrast, coupling via epoxy groups clearly shows a better performance, as can be demonstrated by the results obtained using Eupergit and VA-Epoxy, both oxirane-containing carriers.

Based on the present and recent studies, it could be shown that coupling via epoxy and isocyanate groups is superior, from the methodological and kinetic point of view, to currently used methods[2,13]; this also applies to the excellent stability of the bond formed. Recently described methods recommending tresyl chloride[14], 1,1'-carbonyldiimidazole[15] and FMP[6] are too expensive and the techniques too laborious for technical application. Further, these agents split off organic moieties during the coupling procedure, which could be detrimental to the biological activity of a bound biomolecule (see enzyme immobilization tests in Table I); interference with UV detection is another crucial aspect of these methods.

Coupling, however, is only one aspect contributing to the overall performance, as was found when correlating the anti-insulin loading degree and insulin recoveries (Fig. 3). From these results, one can conclude that steric aspects play an important role regarding the accessibility of the affinity ligands when their immobilization density is increased. This trend applies to both VA-Epoxy and Biograft carriers; however, the protein recovery obtained with Biograft remains a factor 2 higher than that obtained with VA-Epoxy. The reason for this difference lies in the unique molecular structure of the grafted sample. Long tentacle-like spacer arms are introduced into the matrix



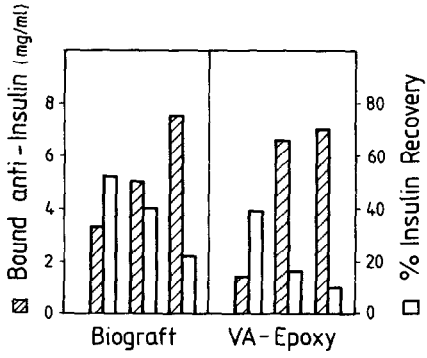


Fig. 2. HSA recoveries on different anti-HSA carriers. Coupling conditions: 3.6 mg of antiserum per 100 mg of carrier in 1 *M* potassium phosphate buffer (pH 7.5) for 24 h at room temperature; amount of HSA applied, 0.5 mg in 25 m*M* phosphate buffer (pH 7.5); incubation period, 12 min; eluent, 8 *M* aqueous urea.

Fig. 3. Immobilization of anti-insulin and insulin recoveries on Biograft and VA-Epoxy resins. Amount antiserum applied, 4 mg per 100 mg of carrier in 1 *M* potassium phosphate buffer (pH 7.5), for 24 h at room temperature; amount of insulin applied, 1.5 mg in 25 m*M* phosphate buffer (pH 7.5); incubation and elution as in Fig. 2.

TABLE I

IMMOBILIZATION OF ENZYMES AND HEPARIN TO DIFFERENT SUPPORTS (% ACTIVITY RETENTION)

Initial enzyme concentration for coupling; 0.5–3 mg per 100 mg of dry carrier.

Immobilized biomolecule	*Support*						
	Biograft	*Eupergit*	*Sepharose 6B Epoxy*	*Sepharose 4B Tresyl*	*VA-Epoxy*	*Fractogel TSK Epoxy*	*FMP-Fractogel TSK HW-75F*
HR Peroxidase	77	36	7	nd[a]	32	8	nd
Penicillin acylase	69	39	8	12	34	10	15
Polygalacturonase	49	<5	<5	nd	<5	<5	nd
Glucose isomerase	74	50	10	16	nd	nd	18
Formate dehydrogenase	78	37	nd	nd	41	8	11
Heparin (mg/g)	26	14	<1	nd	10	<1	<1
Bead size (m)	150	150	70	70	110	50	50

[a] nd = Not determined.

during grafting, thus rendering the ligands more accessible to high-molecular-weight components. There is further evidence that the enlarged surface structure rather than the overall porosity plays a decisive role in the separation performance among grafted carriers[2]. The immunopurification of factor VIII on different supports which are depicted in Table II confirms the above test results.

Affinity chromatographic data obtained with the grafted matrices in comparison with commercial media were substantiated by a number of enzyme immobilizations (Table I). The activity retentions are expressed in per cent; his represents the residual enzymatic activity after immobilization. The activity of the enzyme in solution is set at 100% (blind test). With the same amount of enzyme used for the blind test, the immobilization with a defined amount of carrier is performed. This correlation permits a more realistic evaluation of the immobilized enzyme than relating the activity to the actual bound enzyme, as is frequently done[16].

Based on these results and the excellent physico-chemical stability of Biograft (treatments in 4 *M* NaOH and also magnetic stirring are possible without detectable destruction) a pilot study is currently being done to evaluate the feasibility of the technical production of matrix-bound penicillin acylase. Apart from applications in chromatography, efforts have been made in the last few years to extend the principle of affinity chromatography to the medical field. One promising approach is the development of media for hemoperfusion. The main aim is the removal of blood-group antibodies or toxins from blood using appropriate affinity media[17].

Using specific oligosaccharde blood-group determinants, novel media on the basis of PVA have been developed[18,19]. The optimum matrix for hemoperfusion is PVA, as it exhibits the highest blood compatibilities among all common matrices including dextrans and agarose[20]. In the tests described here, the oligosaccharide α-GalNAc(1–3)-β-Gal-α-Fuc(1–2) specific to blood-group A antibodies was bound to different matrices to assess the antibody clearance performance (Table III).

The efficient antibody-binding properties of isocyanate-activated PVA and

TABLE II

IMMUNOAFFINITY PURIFICATION OF FACTOR VIII ON DIFFERENT ANTI-FACTOR VIII IMMUNOGLOBULIN G (IgG) (MOUSE) COUPLED CARRIER MEDIA

Test results were kindly supplied by Dr. E. Rauenbusch, Bayer, Wuppertal, F.R.G. Coupling conditions: 10 ml of 1 *M* potassium phosphate buffer (pH 7.5) containing 3 mg of IgG per 0.5 g of carrier ; remaining oxirane groups were blocked by incubation with 2 ml of 1 *M* ethanolamine (pH 8.0) for 24 h at room temperatures; sample applied, 1 ml of crude protein solution containing *ca.* 100 μg of protein.

Carrier	*IgG immobilization yield (%)*	*Factor VIII recovery (%)*
Biograft A[a]	99	21
Biograft B[a]	78	39
Biograft C[a]	100	22
Controlled-pore glass	90	17
Eupergit	100	31
VA-Epoxy	95	5

[a] Biograft A, 45% graft uptake; Biograft B, 88% graft uptake; Biograft C, 62% graft uptake.

TABLE III

REMOVAL OF ANTI-A ANTIBODIES FROM HUMAN SERUM TYPE 0

Concentration of hapten in coupling solution, 2 mg per gram of carrier; 400 μl of pooled human serum were incubated for 10 min at room temperature; the antibody content was determined by the agglutination test (for details, see text).

Carrier	*Sample taken (mg)*	*Bead size (μm)*	*Antibody bound (%)*
PVA	50	200–500	>90
Synsorb A	50	200–500	>90
Eupergit	50	150	>90
Fractogel TSK HW-75F[a]	30	50	<50
Protein G Sepharose 4[b]	30	70	<30

[a] Activated with FMP.
[b] Used as supplied by the manufacturer without blood-group hapten.

Synsorb A are excellent, considering the large bead sizes. The high specificity of the oligosaccharide hapten is notable in comparison with the antibody binding performance of protein G, which is recommended as an affinity ligand for immunoglobulins[21].

The described affinity chromatographic and immobilization tests have demonstrated the benefits of grafted polymers in chromatography. The high performance of these carriers in conjunction with their high chemical and physical stability indicate that these supports may be useful alternatives to the currently used media for laboratory applications and provide new prospects with regard to technical usage.

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