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USE OF AVIDIN-COATED GLASS BEADS AS A SUPPORT FOR HIGH-PERFORMANCE IMMUNOAFFINITY CHROMATOGRAPHY

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SUMMARY

Immunoaffinity chromatography, using immobilized antibodies, is a useful technique for the specific isolation and purification of biological materials. We have developed an avidin-coated glass bead which can easily immobilize and retain under adverse conditions, such as acid elution, any antibody which is covalently labelled with biotin. The physical strength of the bead, together with the expanding repertoire of commercially available biotinylated monoclonal antibodies makes this support ideal for both medium- and high-performance immunoaffinity chromatography.

INTRODUCTION

Affinity chromatography using immobilized antibodies is a rapid and specific technique for the isolation of active biological materials from a variety of different materials[1–4]. This technique can become even more useful when the controlled conditions of high-performance liquid chromatography are applied. However, the major disadvantage of this latter technique is the lack of suitable support packings which can provide both the ability to withstand high flow-rates and also possess suitable side-chains to which antibodies may readily be attached.

Avidin, a protein derived from the chicken egg white has the ability to bind up to four molecules of biotin per avidin molecule[5]. Biotin, a small vitamin, can easily be attached to proteins and carbohydrates by a series of commercially available reactive biotin derivatives. It has been shown that the binding forces exhibited between avidin and biotin are strong enough to survive extremely harsh conditions such as those encountered during the acid elution phase of immunoaffinity chromatography[6]. This binding is strong enough so that the immobilized antibody does not require further modification, such as chemical cross-linking in order that it will remain bound to the avidin during repeated elution and regeneration cycles.

We have used the streptavidin form of avidin to coat glass beads which are able to bind and retain any biotinylated antibody and provide a suitable packing medium for both medium- and high-performance immunoaffinity chromatography.

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THEORETICAL

Antibody–antigen reactions

The basis of immunoaffinity chromatograaohy is the specificity of an immobilized antibody to recognize and capture its reactive antigen. The generalized reaction is presented below:

immobilized antibody + antigen → antibody–antigen complex

In this way, the antibody (in this work the antibody was a mouse-derived monoclonal antibody directed against the B27 human leukocyte antigen component of human leukocyte membranes) retains the appropriate antigen (B27 antigen) while the unreactive material is washed through the column (Fig. 1A). The antibody–antigen complex (Fig. 1B) is held together by weak bonds, which can easily be broken by a variety of different agents, the most common of which are either acid or chaotropic ion buffers. By changing the running buffer from a low concentration neutral salt to the elution agent, the bonding between the antibody and the antigen is broken and the released antigen is washed through the column by the mobile phase. The eluted antigen appears as a secondary peak (Fig. 1C) which is measured by the column detector and collected, in an active form, for further studies.

EXPERIMENTAL

Materials

Glass beads (diameter 1 mm) were obtained from Kontes Scientific Glassware (Vineland, NJ, U.S.A.). Purified streptavidin was obtained as a lyophilized, pure product (Bethesda Research Labs., Gaitherburg, MD, U.S.A.) and reconstituted in 0.01 *M* phosphate buffer, pH 7.0. Mouse monoclonal antibodies (MAbs) directed

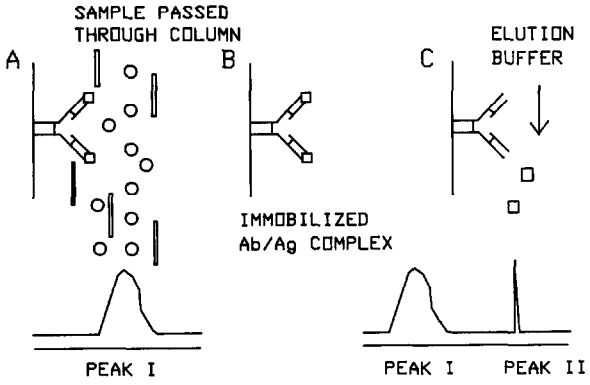

Fig. 1. Diagrammatic representation of immunoaffinity chromatography. (A) The immobilized antibody selects its antigen from the material passing through the column. (B) This material is retained as an antibody–antigen complex while the unreacted material passes through the column (peak I). (C) The bound material is recovered by passing an elution buffer through the column, which breaks the antibody–antigen bonds and the released antigen is eluted as peak II.

against human B27 antigen were obtained as a purified biotinylated and unbiotinylated IgG fraction from Chemicon International (El Segundo, CA, U.S.A.). The biotin hydrazine and the carbodiimide, N,N'-dicyclohexylcarbodiimide were obtained from Pierce (Rockford, IL, U.S.A.). All column fittings were obtained from BioRad Labs. (Rockville Centre, NY, U.S.A.). B27 positive human lymphocytes were obtained by consent from volunteers.

Derivatization of the glass beads

The glass beads were washed in double distilled water before preparing them for silanilization and derivatization[7]. Briefly, 10 g of the washed beads were added to 50 ml of 1 *M* hydrochloric acid and sonicated, for 25 min. The beads were then washed by sedimentation in 200-ml volumes of 1 *M* hydrochloric acid until the supernatant became clear. The clean beads were placed in a round bottomed flask, equipped with an overhead stirrer and refluxed for 30 min in 200 ml of 1 *M* nitric acid. Following recovery and drying of the beads, they were resuspended in 50 ml of 10% 3-aminopropyltriethoxysilane in toluene and refluxed for 16 h, with constant stirring. The beads were removed and washed in 95% methanol before being refluxed for 20 min in 95% methanol, to remove the unreacted silanization reagent. Following this, the beads were washed in distilled water and dried prior to adding them to 10 ml of 10% succinyl chloride dissolved in dry chloroform. A volume of 10 ml of 10% triethylamine dissolved in chloroform was added to the mixture, which was then refluxed for 30 min. The beads were collected and washed repeatedly in chloroform before attaching the succinimide side-chains.

The beads were dried at 100°C and then re-suspended in 20 ml of anhydrous dioxane, containing 0.1 *M* N-hydroxysuccinimide and 0.1 *M* N,N',dicyclohexylcarbodiimide. The mixture was stirred for 2 h at room temperature, with constant slow stirring. The beads were collected and washed in dioxane, followed by methanol, to remove the accumulated dicyclohexylurea. Finally the beads were washed in dioxane and stored, in enough dioxane to cover the bead surface, in light-tight containers for up to four months.

Biotinylation of MAbs

Although we used commercially available biotinylated MAbs in our experiments, we also biotinylated MAbs using the reagent biotin hydrazine. This reagent couples the biotin to the carbohydrate moieties of the antibodies and does not attach to other parts of the molecule[8]. The carbohydrate portion of most antibodies is present in the Fc or tail region and attachment of the biotin to this area would ensure correct orientation of the antibody on the bead surface.

Briefly, the MAb was suspended in 1 ml of sodium acetate, pH 5.0 and cooled to 4°C. A volume of 1 ml of 10 m*M* sodium metaperiodate was mixed with the MAb solution and incubated for 20 min at 4°C in the dark. The reaction was stopped by adding 20 ml of 5% ethylene glycol and dialysing the mixture for 10 h against 0.01 *M* phosphate buffer at pH 7.0. The MAb solution was removed from the dialysis sac and placed in a glass, capped tube. A volume of 1 ml of phosphate buffer containing 1 mg/ml sodium cyanoborate and 1 mg/ml biotin hydrazine was added to the MAb solution and incubated for 1 h at toom temperature. The reaction was stopped by dialysis against 0.01 *M* phosphate buffer overnight at 4°C.

Column construction

The beads were washed three times in double distilled water prior to the addition of 10 g of the derivatized beads to 2 mg of the streptavidin, which had previously been dissolved in 10 ml of 50 m*M* carbonate buffer, pH 9.0. The beads–streptavidin mixture was placed into a 15-ml capped glass tube and mixed for 18 h at 4°C on an overhead mixer. Following this incubation, the beads were sedimented by gravity and washed ten times in phosphate buffered saline (PBS, pH 7.0) by sedimentation and decantation.

Biotinylated mouse MAb (250 μg) was attached to the streptavidin-coated beads by adding the antibody to a 2-g batch of the coated beads suspended in 5 ml of PBS. The mixture was placed into a 10-ml glass capped tube and mixed for 1 h at 4°C on the overhead mixer. Following this incubation, the beads were washed three times in PBS and the unreacted biotin receptors on the avidin molecule blocked by incubating the beads in 5 ml of a 1 mg/ml solution of biotin. This incubation was performed at 4°C for 30 min on the overhead mixer. The beads were washed five times in PBS by sedimentation and decantation before being slurry-packed into 10 cm × 4.6 mm I.D. stainless-steel columns at 250 p.s.i.

Chromatography

The bead-packed column was installed into a Beckman 340 isocratic high-performance chromatography system (Beckman, Palo Alto, CA, U.S.A.), equipped with a Model 112 pump, a Model 160 UV detector, set at 280 nm and a Shimadzu C-RIB peak integrator (Shimadzu Scientific Instruments, Columbia, MD, U.S.A.). The system was equipped with an Autochrom Model III OPG/S solvent selector/gradient controller (Autochrom, Milford, MA, U.S.A.), which automatically controlled the elution profile of the system. Samples were injected by means of an Altex 210 injection port with a 100-μl sample loop. The column was isocratically developed in 0.9% sodium chloride–0.1 *M* sodium acetate buffer at pH 6.5 for 10 min at flow-rates varying from 0.5 to 1.5 ml/min. The column was jacketed and maintained at 4°C with a recycling ice bath, connected to the column jacket. Following the initial 10 min, during which time the immobilized MAb reacted with and retained its specific antigen and the unreacted material had run through the column, an antigen elution phase was started. Either a pH gradient was automatically generated from pH 6.5

TABLE I

ELUTION BUFFERS FOR IMMUNOAFFINITY CHROMATOGRAPHY

Buffer	*Application*
Acid buffers	
0.33 *M* Citrate, pH 2.0	General protein and glycoprotein elution
0.1 *M* Glycine, pH 1.5	General protein, glycoprotein and polysaccharide elution
0.1 *M* Tris–HCl, pH 1.0	General purpose elution buffer for non-delicate materials
Chaotropic buffers	
2.5 *M* Sodiom thiocyanate	General purpose elution agent for delicate materials
2.5 *M* Polyvinylpyrrolidone–iodine	Delicate antibody elution
6–8 *M* Sodium chloride	Elution buffer for delicate antigens which bind strongly to the immobilized antibody

to 1.0 by the addition of 0.1 *M* hydrochloric acid to the initial running buffer, over a 10-min period, or the solvent selector changed the running buffer to a chaotropic ion solution and developed an ionic gradient from 0 to 2.5 *M* over a 10-min period of time. Table I gives a summary of the buffers used in high-performance immunoaffinity chromatographic (HPIAC) isolations.

Recovery of the antigen was achieved during the gradient phase, and the upper limit of the gradient was maintained for a further 10 min, before reverting back to the original running buffer to regenerate the column. Fractions of the immunoaffinity isolated antigen were collected in a modified ISCO fraction collector (ISCO, Lincoln, NB, U.S.A.). The purity of the peaks were analyzed by dot-blot assay, using the original monoclonal antibody. During all of the HPIAC runs, samples of the column effluent was collected and tested for the presence of the immobilized antibody.

RESULTS

At present we have produced over 20 batches of streptavidin-coated glass beads and have found that the derivatized beads are able to bind between 1.5 and 1.85 mg of streptavidin per 2-g batch of beads. Once coated with the streptavidin, the beads can bind between 195 and 245 μg of hydrazine biotinylated MAb and 160–200 μg of commercially available biotinylated MAb. The production of the coated beads can be performed in either small or large batches and stored at 4°C or lyophilized and stored dry in a sterile container.

Packed columns kept at room temperature are useable for 1–2 weeks, depending on the type of antibody incorporated into the column. Generally IgG MAbs last longer than the IgM type of MAb, which rapidly lose their specificity. Cooling the columns to 4°C greatly improved their effective working lives as did elutions with chaotropic ions. The running temperature of the column greatly effected the functional life of the immobilized antibody. Column packing which were stored at 4°C and run at the same temperature gave excellent performance but at higher temperatures the effective column life was greatly reduced, even when the packing material

TABLE II

WORKING CHARACTERISTICS OF THE AVIDIN-COATED BEADS

Parameter	*Effective bead life*
Elution parameters	
Acid elution	10–20 elutions and regenerations
Chaotropic elution	20–40 elutions and regenerations
Running temperatures	
Room temperature	1–2 weeks or 5–10 elutions and regenerations. Peaks diffuse
Cold (4°C)	4–6 months or 10–40 elutions and regenerations depending on elution agent. Well defined peaks
Warm (37°C)	1 week or 2–10 elutions and regenerations depending on elution agent. Poorly defined peaks
Storage	
4°C	Up to 6 months or longer
Room temperature	1–2 weeks

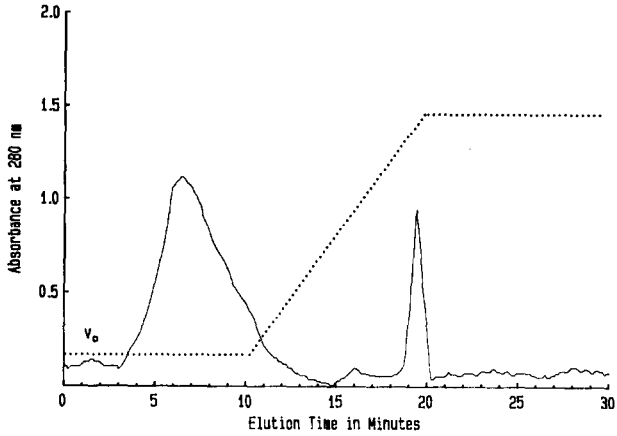

Fig. 2. Chromatogram of an HPIAC isolation of the B27 antigen isolated from detergent solubilized membranes. The chromatogram was developed using an acid pH gradient elution from pH 6.5 to 1.0. The trace was produced on a 10 cm × 4.6 mm I.D. HPIAC column run at 0.5 ml/min in 0.9% sodium chloride–0.1 *M* sodium acetate buffer (pH 6.5) and monitored at $O.D._{280\ nm}$ with the detector set at 0.005 a.u.f.s. The column was maintained at 4°C throughout the run. The dotted line indicates the pH gradient profile. V_0 indicates the void volume of the column.

was stored in the cold between runs. Table II summarizes our findings on the working life and efficiency of the streptavidin-coated beads.

Typical elution profiles, obtained with acid and chaotropic elution conditions are shown in Fig. 2 and 3. The elution peak (peak II) produced by the acid elution (Fig. 2) is broader than the same peak produced by chaotropic elution with sodium thiocyanate (Fig. 3).

The flow-rates of the column did not effect the elution profile over the range 0.5–1.5 ml/min (Fig. 4). The major difference seen in the comparison of the three different flow-rates is that there is a significant shift in the position of the second peak between that seen in Fig. 4A, which is run at 0.5 ml/min and the same material,

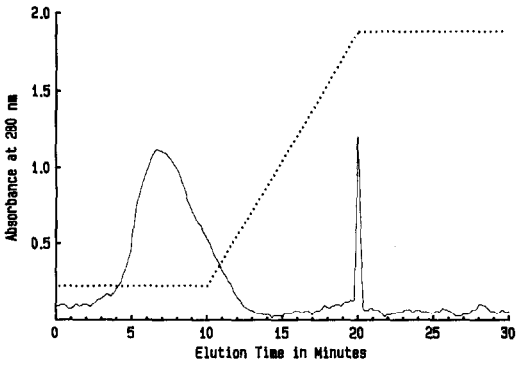

Fig. 3. Chromatogram of an HPIAC isolation of the B27 antigen using a chaotropic ion gradient elution. The running conditions are identical to those described in Fig. 2, except that the dotted line indicates the chaotropic ion gradient.

eluted at either 1 ml/min (Fig. 4B) or 1.5 ml/min (Fig. 4C). The shift in peak position, seen with increasing flow-rates, was due to the increase in speed with which the released antigen was washed through the column. The increase in column flow-rate also resulted in changes in the area under the second peak, which decreased as the antigen was eluted in a smaller volume, at a greater speed. Speeds greater than 1.5 ml/min resulted in an inefficient binding of the antigen to the immobilized antibody during the initial phase of the immunoaffinity procedure.

Dot-blot analysis of the immunoaffinity isolated antigen demonstrated that superior results are obtained with chaotropic elution at flow-rates of 1–1.5 ml/min. Although the efficiency of the immobilized antibody started to decline after ten column elutions and regenerations, we were unable to detect leakage of the immobilized antibody, even after sixty column recycles.

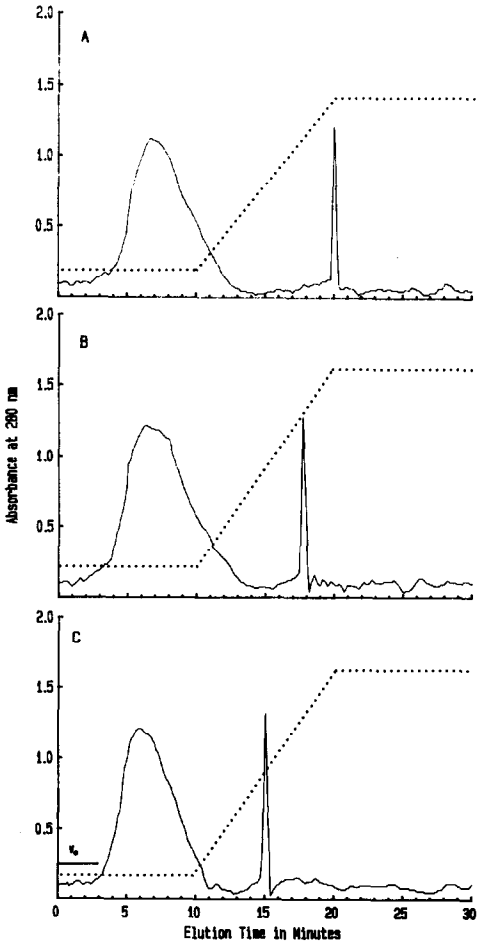

Fig. 4. Effects of flow-rate on the elution profiles of an HPIAC isolation of the B27 antigen using a chaotropic gradient elution buffer. The running conditions are as described in Fig. 2 except that the flow-rate was (A) 0.5 ml/min; (B) 1 ml/min and (C) 1.5 ml/min. The dotted line indicates the chaotropic ion gradient.

DISCUSSION

HPIAC is applicable to the rapid isolation and measurement of many different types of biological material. The major disadvantage of the technique is the availability of a suitable packing material which easily can be used to immobilize antibodies.

Previously we have reported the use of the bacterial coat protein, protein A, as a support medium for the immobilization of IgG molecules to glass beads[9,10]. However, this technology has some disadvantages in that only certain immunoglobulin classes are able to bind to protein A, which was restrictive in the use of this packing medium for the immobilization of certain classes of mouse and rat MAb[11].

This problem can be overcome by using the avidin–biotin system, especially as there is a growing repertoire of commercially available biotinylated polyclonal and monoclonal antibodies. Avidin or streptavidin, a more pure form of avidin, derived from *Streptomyces avidinii*, exhibit extremely high binding capacity for biotin. This capacity expresses a dissociation constant of 10^{-15} M, which means that the avidin–biotin conjugation cannot easily be disssociated even under extreme pH conditions[6]. This is a major advantage concidering the ionic changes which take place during the elution and regeneration phases of immunoaffinity chromatography.

Newer forms of biotin, such as hydrazine biotin, improve the basic biotin labelling of protein molecules such as antibodies. This derivatized biotin can be used to selectively label the carbohydrate portion of the antibody molecule, which is located in the Fc or tail region[8]. In this way, the advantages of the protein A-coated bead is still retained, but the selectiveness of the protein A for different subclasses of antibody is avoided[12]. Biotinylation can be performed on all classes of antibody, which makes the avidin-coated bead a more universal support medium than protein A[11].

We feel that the development of this support will provide an easy packing medium for all applications of immunoaffinity chromatography, where either antibodies or antigens are used as the immobilized ligand.

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