Agarose polyacrolein microsphere beads

New effective immumoabsorbents

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1. INTRODUCTION

The reaction of polysaccharide supports with cyanogen bromide [1,2] continues to be the most widely used method for binding amino ligands. However, the cyanogen bromide activation method suffers from several disadvantages, such as, instability of the isourea bond formed by the ligands and the polysaccharide supports, resulting in continous leakage of the ligands from the supports into the solution, high toxicity of cyanogen bromide and high pH needed for the activation procedure. Several new immunoabsorbents and activation procedures have been developed in the search for better supports and coupling methods [2–6]. In the present publication the synthesis and the application in affinity chromatography of new effective immunoabsorbents based on agarose polyacrolein microspheres beads (PAMB) is described [7]. Polyacrolein microspheres (PA, 4% wt/vol) were encapsulated with agarose (4% wt/vol) to form beads ranging from 50 μm to 150 μm diameter. Magnetic APAMB were formed by carrying out the encapsulation procedure in the presence of magnetic particles (Ferrofluid). Various ligands containing primary amino groups, such as proteins, antibodies, lectins, hormones and drugs were bound covalently in a single step under physiological pH to the beads through their aldehyde groups. The potential use of some of the conjugates: beads–proteins (immunobeads) in affinity chromatography was illustrated successfully. Leaching of proteins bound to the beads under physiological pH and eluting conditions was not detected.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Goat anti-mouse immunoglobulin (gxmIg) and monoclonal anti-mouse Thy1,2 were obtained from Z. Eshhar, Rehovot, Israel. All other chemicals were from standard commercial suppliers.

2.2. *Antisera*

Rabbit and goat antisera used in this work were obtained by immunizing the animals with an emulsion containing the appropriate antigen in Freunds complete adjuvant (1 mg/ml of antigen, final concentration). Each animal received 2 ml injected intradermally into at least 10 different sites. The animals were boosted 10 days after the first dose in the same manner.

2.3. *Determination of proteins*

Quantities of proteins bound to the beads were determined by measuring the unbound proteins with the Folin–Ciocalteau reagent using Lowry's method [8]. Quantities of antibodies bound and eluted from the immunobeads were determined by the quantitative precipitation reaction [9].

2.4. *Synthesis of PA microspheres*

PA microspheres were synthesized by polymerizing acrolein with a cobalt radiation, as described in a previous publication [10].

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2.5. *Synthesis of the APAMB [7]*

A solution containing 0.96 g agarose A in 16 ml distilled water was heated to 95°C until the gel was melted into a clear solution. The temperature was then decreased to 70°C and 8 ml of PA microspheres solution (12% wt/vol) heated to 70°C were added. The solution was stirred for another 30 minutes and was then poured into 100 ml of stirred (3000 rev./min) peanut oil at 70°C. Ten minutes later the solution was cooled with ice. The APAMB produced were purified from the oil with ether which was then removed by evaporation. Magnetic APAMB were formed by carrying out the same procedure in the presence of ferrofluid (5% wt/vol).

2.6. *Binding of the spacer polylysine–glutaraldehyde to the APAMB*

Twenty ml of the APAMB in 35 ml of distilled water were shaken for 24 h with 40 mg of polylysine. The polylysine conjugated beads produced were washed free of unbound polylysine by repeated decantation. The beads solution (20 ml in 35 ml H_2O) was then shaken for 12 h with 2 ml glutaraldehyde (50%) and the resultant polylysine–glutaraldehyde conjugated beads were filtered and then washed with large amounts of distilled water. The binding of polylysine–glutaraldehyde to the magnetic APAMB was carried out in a similar procedure. The derivatized magnetic beads were washed free of unbound spacer by repeated decantation in the presence of a magnetic field.

2.7. *Preparation of the immunoabsorbents*

Twenty ml of the APAMB with or without a bound spacer were shaken for 24 h at 4°C with 400 mg of an appropriate antigen in 200 ml PBS. Unbound antigen was then removed by repeated decantation with large amounts of phosphate buffer saline (PBS). The remaining aldehyde groups were then blocked by shaking the beads for 12 h with 1 ml ethanol amine solution, brought to pH 7.2 with HCl. The immunobeads were then washed successively with PBS, eluting medium (0.2 M glycine–HCl buffer at pH 2.4), and again with PBS. The preparation of the magnetic immunoabsorbents was similar. The removal of the unbound antigen and ethanol amine was achieved by repeated decantation with PBS in the presence of a magnetic field.

2.8. *Isolation of antibodies*

The immune serum was passed in a rate of 1 ml/min through a column containing the appropriate immunoabsorbent (5 to 10 ml serum for each 1 ml of the immunobeads). The immunobeads were then washed several times with PBS. Absorbed antibodies were then eluted with 0.2 M glycine–HCl buffer solution at pH 2.4, neutralized with NaOH, dialysed against PBS and then analysed by polyacrylamide gel electrophoresis [11]. The immunobeads, after the treatment with glycine–HCl buffer, were washed several times with PBS and stored at 4°C in presence of sodium azide (0.05%) until reused. The isolation of antibodies with the immunomagnetic beads was carried out batchwise. The removal of unbound antibodies and the washing steps was achieved by successive decantation with a magnetic field.

3. RESULTS AND DISCUSSION

Fig.1a represents a light microscopy picture of the APAMB. Cross-section photomicrographs of the PA microspheres and the magnetic particles encapsulated in the agarose are shown in fig.1b and c, respectively. Amino ligands, e.g., proteins, diffuse through the porous agarose and bind covalently in a single step under physiological pH to the PA microspheres to form the polyvalent Schiff base bonds. A further reduction of a Schiff base bond to a stable single C–N bond [6,12] may be required when the ligand bound to the beads is provided only with a single amino group.

The binding capacity of the APAMB towards various amino ligands, e.g., proteins (BSA, DNP-BSA, and RIgG), antibodies (gxmIg and anti-mouse Thy1,2), lectins (Con A and soybean agglutinin), hormones (bovine insulin) and drugs (Desferoxamine, an iron chelating agent) are illustrated in table 1. Steric requirements may explain the significant increase (3–6-fold) in the binding capacity of the APAMB bound to the spacer arm polylysine–gluteraldehyde. The beads preserved their physical and mechanical properties after the coupling of the ligands. Leakage of bound proteins from the beads to the supernatant was not detected by using the method of Lowry et al. [8] for protein determination. Furthermore, radioactivity in the supernatant of a solution containing beads bound

Table 1

Binding capacity of the APAMB to various amino ligands

Ligands	Spacer	Binding capacity (mg)	
		APAMB	Magnetic APAMB
BSA	–	2.2	2.2
BSA	Polylysine-glutaraldehyde	14.8	14.0
DNP-BSA	–	1.8	–
DNP-BSA	Polylysine-glutaraldehyde	9.0	–
RIgG	–	1.0	1.0
Soybean agglutinin	Polylysine-glutaraldehyde	5.9	–
RIgG	Polylysine-glutaraldehyde	10.0	8.0
Con A	–	2.4	–
Con A	Polylysine-glutaraldehyde	11.5	–
Bovine insulin	–	7.2	–
Bovine insulin	Polylysine-glutaraldehyde	22.0	–
gxmIg	–	2.4	–
gxmIg	Polylysine-glutaraldehyde	12.4	–
Anti-mouse Thy1,2	Polylysine-glutaraldehyde	9.8	–
Desferoxamine	Polylysine-glutaraldehyde	40.0	–

1 ml of the APAMB was shaken with excess quantities of the various ligands in 5 ml PBS for 12 h at room temperature

to radioactive BSA ^{131}I was not detected during the radioactive iodine life time.

The binding capacity of some of the immunobeads to the appropriate antibodies and the resultant eluting data are given in table 2. In all cases antibodies were not detected after the adsorption step, as was determined by the ring test with the antisera. Antibodies eluted from the column were submitted to analysis by polyacrylamide gel electrophoresis and were found to contain only IgG. Normal rabbit serum was passed through a column containing the immunobeads. The beads were then treated as described for the isolation of antibodies. Under these conditions proteins were not eluted from the beads, indicating that non-specific absorption of proteins onto the beads did not occur. The immunobeads have also used repeatly during a period of 6 months without any significant loss of their antibody binding capacity, as shown in table 3.

Table 2

Isolation of antibodies with the agarose polyacrolein immunoabsorbents (1 ml APAMB)

Beads	Spacer	Antigen	Antigen bound (mg)	Antiserum used	Antibody bound (mg)	Antibody eluted (mg)
APAMB	–	BSA	2.2	Rabbit	2.4	2.2
APAMB	Polylysine-glutaraldehyde	BSA	14.8	Rabbit	16.5	17.0
APAMB	Polylysine-glutaraldehyde	DNP-BSA	9.2	Rabbit	8.5	8.4
APAMB	Polylysine-glutaraldehyde	RIgG	10.0	Goat	12.0	12.0
Magnetic APAMB	–	BSA	2.2	Rabbit	3.0	4.0

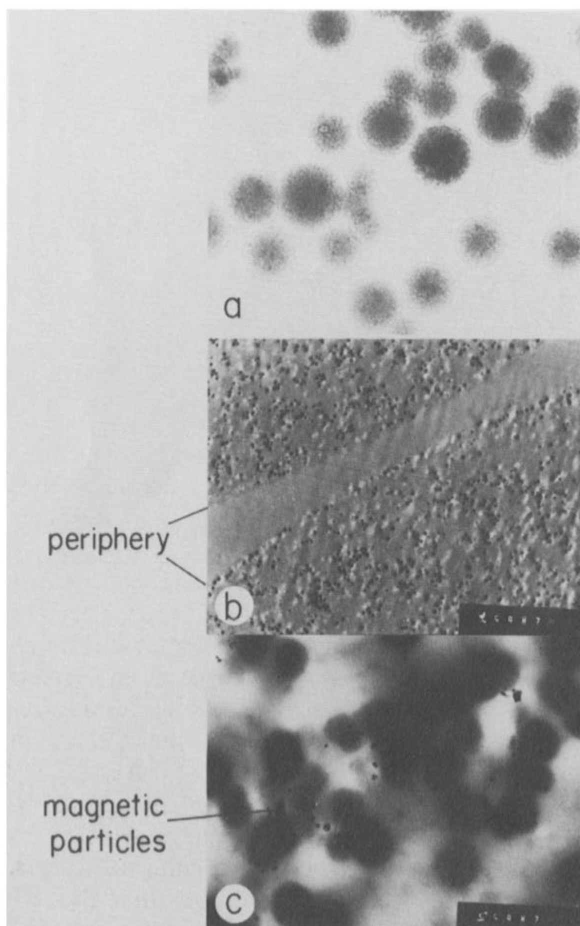

Fig.1. (a) A light microscopy photomicrograph of the APAMB (× 100). (b) A Transmission Electron Microscopy (TEM) picture describing a cross section of the APAMB (× 3300). (c) A TEM picture describing a cross section of the magnetic APAMB (× 55 000).

In conclusion, in a previous publication [10], the use of PA microspheres for cell labeling and cell separation was demonstrated. In the present paper the PA microspheres were encapsulated in agarose and the resultant APAMB were used successfully as new immunoabsorbents in affinity chromatography. Currently, research related to the potential use of the APAMB for other biological applications, e.g., diagnostic purposes, cell separation and hemoperfusion is on going in our group and will be published in the future.

Table 3

Quantity of rabbit anti BSA eluted from the APAMB bound to polylysine–glutaraldehyde during 3 succesive isolations in 6 months

Time (months)	Rabbit anti-BSA eluted (mg)
1	16.5
3	16.0
6	17.0

1 ml beads bound to 14.8 mg BSA

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