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AFFINITY SEPARATION WITH POLYALDEHYDE MICROSPHERE BEADS

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SUMMARY

Agarose polyaldehyde microsphere beads were prepared by encapsulating polyaldehyde microspheres of various diameters, *e.g.*, polyacrolein or polyglutaraldehyde microspheres, within agarose beads. Amino ligands such as proteins or drugs can be bound covalently to the beads in a single step at physiological pH. The binding capacity of the beads towards various amino ligands is inversely related to the diameter of the microspheres encapsulated in the agarose matrix. Different reagents, *e.g.*, bovine serum albumin, ethanolamine and hydroxylamine, were studied as blocking reagents of the free aldehyde groups. Blocking the remaining aldehyde groups after coupling the amino ligands to the beads is essential for increasing or retaining the reactivity of the ligands conjugated to the beads. Among the reagent studied, hydroxylamine was found to be the most suitable blocking reagent of the free aldehyde groups of beads conjugated with proteins. The extent of leakage of amino ligands bound to the agarose–polyaldehyde microsphere beads was studied as a function of the pH of aqueous solutions of the beads. At physiological pH the leakage was negligible. At acid pH, leakage of ligands containing several primary amine groups, *e.g.*, proteins, was insignificant. However, significant leakage was detected for ligands containing a single amino group. The leakage of proteins bound to the agarose–polyaldehyde microsphere beads was found to be much less than the leakage of the same proteins bound to agarose beads through the cyanogen bromide activation method.

INTRODUCTION

Affinity chromatograpy is a very useful technique in a variety of analytical and separation procedures. The development and wide use of this technique depends on the support materials and the facile methods for attaching ligands and proteins to them. In order for affinity chromatography to be effective, the ligands coupled to the supports should retain most of their reactivity and the bonds between the ligands and the support materials should be stable under absorbing and eluting conditions. A few recent articles have summarized the support materials and methods of attachment used for purification of biomolecules through the affinity chromatography technique[1–4].

Agarose is the most commonly used support for the affinity purification of

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biomolecules. Agarose contains hydroxy groups through which amino ligands, *e.g.*, proteins, are covalently bound. Few activity methods for coupling proteins to agarose (or other supports with hydroxy groups) have been developped[1]. These activation methods require the use of pure organic solvents and reagents, such as cyanogen bromide, tosyl chloride, tresyl chloride, N-hydroxysuccinimide, imidazoles, chloroformates or activated pyridines. Coupling of proteins to these activated supports usually requires basic conditions. In previous papers we described the synthesis and use of agarose–polyaldehyde microsphere beads (APAMB) for affinity separations[5,6]. These beads were prepared by encapsulating polyacrolein (PA) microspheres or polyglutaraldehyde microspheres of average diameter 0.15 μm in an agarose matrix. The beads formed contained on the surface of the embedded microspheres aldehyde groups through which primary amino ligands, *e.g.*, proteins or drugs, can be bound covalently in a single step at physiological (or other) pH. In further studies of the APAMB we found that their binding capacity towards amino ligands is inversely related to the diameter of the microspheres encapsulated in the agarose matrix. Different reagents, *e.g.*, bovine serum albumin (BSA), ethanolamine and hydroxylamine, were studied as blocking reagents of the free aldehyde groups. Blocking of the remaining aldehyde groups after coupling the amino ligands to the beads is essential for increasing or retaining the reactivity of the ligands conjugated to the APAMB. The extent of leakage of proteins bound to the agarose–polyaldehyde microsphere beads was also examined.

EXPERIMENTAL

Reagents

The following materials were purchased from commercial sources: polyethylene oxide, average mol. wt. 100000, from Polyscience; human serum albumin (HSA), bovine serum albumin (BSA, fraction V), digoxin, paraquat, chymotrypsin, protein A, biotin and alkaline phosphatase (type VII-NT) from Sigma; goat immunoglobulin (goat Ig), human Ig and concanavalin A from Bioyeda; avidin from Belova; agarose A and Sepharose 4B cross-linked (CL) from Pharmacia; hydroxylamine hydrochloride, ethanolamine, divinyl sulphone and acrolein from Aldrich; and [^{14}C]BSA, [^{14}C]-paraquat and [^{131}I]digoxin from Amersham. Acrolein was distilled at atmospheric pressure before use.

Synthesis of PA microspheres

PA microspheres were obtained as described in previous publications[7,8]. Briefly, microspheres of average diameter 0.15 μm were prepared by irradiation with a cobalt source (0.5 Mrad) of an air-free aqueous solution containing 7.5% (w/v) acrolein and 0.5% (w/v) polyethylene oxide. The microspheres were then washed by repeated centrifugation at 10000 rpm. The diameter of the microspheres obtained was controlled by changing the surfactant or monomer concentration, *e.g.*, microspheres of 0.08 μm were obtained in a similar procedure using an aqueous solution containing 1% (w/v) of surfactant and 5% (w/v) of monomer.

Synthesis of the APAMB

The APAMB were synthesized as previously described[5]. Briefly, an aqueous

solution at 80°C containing 3% PA microspheres with the desired diameter and 4% agarose was poured into stirred peanut oil at 70°C, then the solution was cooled with ice. The APAMB obtained were purified from the oil by several extractions with diethyl ether, which was then removed by evaporation. APAMB with diameters ranging from 100 to 200 μm were obtained by sieving the APAMB. The APAMB were cross-linked with divinyl sulphone according to a previously described procedure[9]. The APAMB were stored at 4°C with 0.05% (w/w) merthiolate.

Antiserum

Rabbit antiserum was obtained by immunizing rabbits with an emulsion containing the appropriate antigen in Freund's complete adjuvant[5,9–11].

Determination of proteins

The amounts of proteins bound to the APAMB were determined by measuring the unbound proteins with Folin–Ciocalteau reagent using Lowry *et al.*'s method[12]. The amounts of proteins bound to the immuno-APAMB were determined by measuring the unbound proteins by the quantitative precipitin reaction[13]. The amounts of eluted proteins were also determined by the method of Lowry *et al.*[12]. Avidin was determined by a sensitive enzyme assay as described by Bayer *et al.*[11]. Digoxin, Paraquat, rabbit antidigoxin and rabbit antiparaquat were determined by radioimmunoassay (RIA)[9,10].

Preparation of the immuno-APAMB

APAMB (1 g) in PBS (5 ml) were shaken for 12 h at room temperature with an appropriate amount of the desired protein and unbound protein was removed by repeated decantation with PBS. The remaining free aldehyde groups were then blocked by shaking 1 ml of the immuno-APAMB in 5 ml of PBS for 12 h at room temperature with one of the following reagents: BSA (10 mg), aqueous ethanolamine solution at pH 7.2 (1 ml) or aqueous hydroxylamine solution (100 mg/g) at pH 7.2 (1 ml). The blocked immuno-APAMB were washed free of unbound hydroxylamine by repeated decantation with PBS. Non-covalently adsorbed proteins or blocking reagents were removed by washing the beads with buffers at pH 4 (0.1 *M* acetate buffer containing 1 *M* sodium chloride) followed by a buffer solution at pH 8 (0.1 *M* hydrogencarbonate buffer containing 1 *M* sodium chloride). Then the beads were resuspended in PBS.

Affinity separations

The separation of proteins by affinity chromatography with the immuno-APAMB was performed as previously described[5]. Briefly, the immune serum was passed at a rate of 1 ml/min through a column containing the appropriate immunoabsorbent (5–10 ml of serum for each gram of the immunobeads). The immunobeads were washed several times with PBS. Absorbed antibodies were then eluted with 0.2 *M* glycine–hydrochloric acid buffer solution at pH 2.4, neutralized with sodium hydroxide, dialysed aganst PBS and then analysed by polyacrylamide gel electrophoresis[14]. The immunobeads, after the treatment with glycine–hydrochloric acid buffer, were washed several times with PBS and stored at 4°C in the presence of sodium azide (0.05%) until reused.

Binding capacity of PA towards amino ligands

PA microspheres of average diameter 0.15 μm (100 mg) in water (5 ml) were shaken at room temperature for 48 h with various amino ligands (500 mg of each ligand in 5 ml of aqueous solution at pH 7.0). Proteins, *e.g.*, BSA, were determined by measuring the unbound protein by Lowry *et al.*'s method[12]. Other amino ligands, *e.g.*, hydroxylamine or ethanolamine, were determined by removal of the ligand solution by at least six repeated centrifugation cycles through water at 12 000 rpm. The conjugated product was then vacuum dried at 60°C and submitted to nitrogen analysis.

Kinetic studies

APAMB (1 g) bound to rabbit antidigoxin or protein A were shaken at room temperature with PBS (50 ml) containing digoxin or human Ig, respectively. Samples were taken at intervals and checked for digoxin by RIA[9] or protein A by measuring the absorption at 280 nm.

Stability and safety

The release of microspheres or beads fragments into saline during perfusion was studied as previously described[10]. Briefly, saline was circulated through cross-linked APAMB (25 g, containing microspheres of size 0.15, 0.08 and .03 μm) for 2 days at a flow-rate of 150 ml/min. Samples of saline were taken periodically and measured for their turbidity (Hach 2100A turbidimeter). The limit of detection of the instrument was found to be 30 ng/ml. Release of monomeric acrolein was studied by measuring the absorbance of the samples at 210 nm. The limit of detection of acrolein by this method is 1 μg/ml

Stability of the covalent APAMB–amino ligand bond

Leakage of proteins bound to APAMB was studied by sampling the supernatant APAMB solution at intervals and determining the protein content. In some instances the leakage of proteins was also studied under perfusion conditions. Leakage of BSA and human Ig was determined by coupling to the APAMB 2 μCi [^{14}C]BSA and cold BSA, or 3 μCi [^{14}C]human Ig and cold human Ig, respectively. The conjugated APAMB (1 g) were stored in PBS (2 ml) at 4°C and sampled at intervals to measure the radioactivity in the supernatant. At each sampling period the supernatant was replaced with the same volume of PBS. Under similar conditions leakage of avidin was determined by an enzyme assay[11]. Leakage of avidin, rabbit antidigoxin and rabbit antiparaquat in plasma and PBS was also studied under the following perfusion conditions: plasma or saline (35 ml of each) was circulated (35–50 ml/min) at room temperature or 37°C through columns containing 10 g of the conjugated APAMB. Samples were taken after 4 h of perfusion and analysed. Antibodies were quantified by RIA, using ^{131}I for antidigoxin and ^{14}C for antiparaquat[9,10]. Avidin was determined as mentioned above by an enzyme assay[11].

Leakage of the blocking reagents, *e.g.*, hydroxlamine, was studied under physiological and acidic conditions. PA microspheres of average diameter 0.15 μm or APAMB coupled with hydroxylamine (100-mg microspheres or 1 g of APAMB) were added to PBS solution (20 ml) or to 0.2 *M* glycine–hydrochloric acid solution at pH 2.4 (20 ml). The supernatant of the beads was replaced each day after repeated centrifugation at 12 000 rpm or by decantation. After a week, all samples of the water-washed beads were vacuum dried at 60°C and submitted to nitrogen analysis.

RESULTS AND DISCUSSION

Photomicrographs

Fig. 1A shows a light microscopy photomicrograph of the APAMB. Fig. 1B is a transmission electron microscopy photomicrograph of the APAMB, showing the PA microspheres of average diameter 0.15 μm encapsulated within the agarose.

Effect of the diameter of the encapsulated microspheres on the binding capacity

Table I illustrates the inverse relationship between the binding capacity of the APAMB towards amino ligands and the diameter of the PA microspheres encapsulated in the agarose. For example, under the conditions described in Table I, APAMB containing PA microspheres of diameter 0.15 μm bind only 5 mg from an initial amount of 15 mg of goat Ig, whereas APAMB containing microspheres of 0.03 μm diameter completely bind the entire 15 mg of goat Ig. When the initial amount of goat Ig is 100 mg, 18 mg of the protein are bound to beads containing microspheres of diameter 0.15 μm, whereas 70 mg of the protein are bound to beads containing microspheres of diameter 0.03 μm. Similar differences were obtained with other proteins, *e.g.*, avidin, HSA and chymotrypsin.

The performance of the conjugated APAMB containing microspheres of smaller

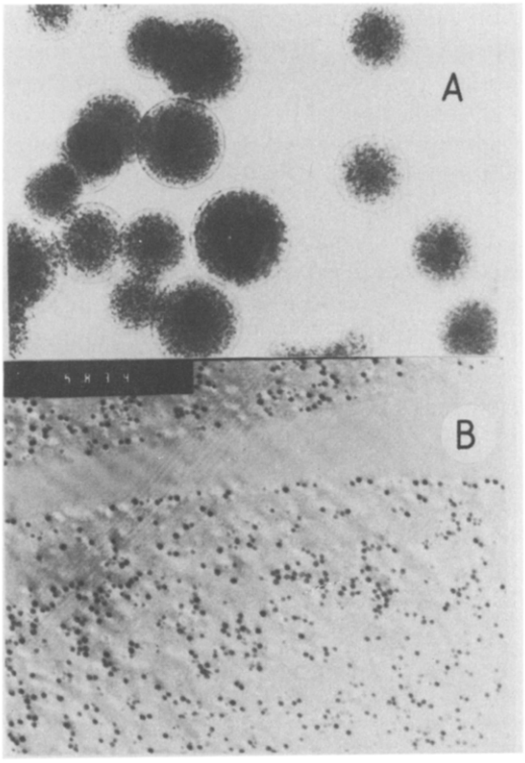

Fig. 1. (A) A light microscopy photomicrograph of the APAMB. (B) A transmission electron microscopy photomicrograph of a thin section of the APAMB showing PA microspheres of average diameter 0.15 μm within the agarose matrix. Peripheries of two beads are depicted.

TABLE I

EFFECT OF THE DIAMETER OF THE ENCAPSULATED MICROSPHERES ON THE BINDING CAPACITY OF THE APAMB

APAMB (1 g) in 5 ml of saline were shaken at room temperature with various amounts of proteins for 12 h.

Ligand	*Amount (mg)*	*Bound ligand (mg/g beads)*		
		0.15-μm microspheres	*0.08-μm microspheres*	*0.03-μm microspheres*
Goat Ig	15	6	13	15
	30	–	20	30
	100	18	30	70
HSA	15	3	4	6
	30	–	–	20
	100	14	–	40
Concanavalin A	100	54	–	72
Chymotrypsin	100	8	–	52
Avidin	15	13	–	15
Protein A	15	5	8	13

diameter in the affinity purification of proteins is also significantly improved, as shown in Table II. After three affinity separation cycles the amount of antiavidin eluted through avidin–APAMB containing microspheres of diameter 0.08 μm is 2.5 times higher than that of antiavidin eluted through conjugated beads containing microspheres of diameter 0.15 μm. Antiavidin antibodies eluted from the various kinds of avidin–APAMB shown in Table II were submitted to analysis by polyacrylamide gel electrophoresis and were found to contain only IgG.

Reagents for blocking remaining unreacted aldehyde groups

Table III illustrates the extent of reaction between PA microspheres and various amino ligands. The degree of reaction between the microspheres and the ligands did not change significantly on washing the microspheres with distilled water to remove

TABLE II

EFFECT OF THE DIAMETER OF THE ENCAPSULATED MICROSPHERES ON THE ISOLATION OF RABBIT ANTIAVIDIN

APAMB (1 g) in 5 ml of saline were shaken at room temperature with 20 mg of avidin for 12 h. Unbound avidin was then washed by repeated decantation. Immune serum was passed at a rate of 1 ml/min through a column containing the APAMB–avidin conjugate. Adsorbed antibodies were eluted with 0.2 *M* glycine–hydrochloric acid buffer solution (pH 2.4).

Encapsulated microspheres diameter (μm)	*First run*		*Second run*		*Third run*	
	Amount of bound antiavidin (mg)	*Amount of antiavidin eluted (mg)*	*Amount of bound antiavidin (mg)*	*Amount of antiavidin eluted (mg)*	*Amount of bound antiavidin (mg)*	*Amount of antiavidin eluted (mg)*
0.08	19	18	18	16	16	16
0.15	12	9	9	6.5	6.5	6.5

TABLE III

BINDING CAPACITIES FOR THE REACTION OF PA MICROSPHERES AND AMINO LIGANDS

Microspheres (diameter 0.15 μm) (100 mg) in 5 ml of water were shaken at room temperature for 48 h with 500 mg of the appropriate amino ligands in 5 ml of aqueous solution at pH 7.0. The microspheres were thoroughly washed with distilled water by repeated centrifugation to remove free ligands.

Ligand	*N (%)*	*Bound ligand (mmol/g microspheres)*
Hydroxylamine	14	10
Hexanediamine	1.8	1.3
Ethanolamine	2.0	1.4
Glycine	0.6	0.4
Phenylalanine	0.6	0.4
BSA	–	$4.2 \cdot 10^{-2}$

free ligands. On the other hand, a gradual decrease in the extent of reaction of the microspheres with the primary amino ligands was observed when washing of the microspheres was accomplished at acidic pH [*e.g.*, glycine–hydrochloric acid buffer, 0.2 *M* (pH 2.4)].

Hydroxylamine is a common reagent for the determination of aldehyde groups in insoluble polymers[15–17]. As expected, this reagent interacted completely with the aldehyde groups of the PA microspheres. On the other hand, other amino ligands reacted to a much smaller extent with the aldehyde groups of the PA. For example, ethanolamine reacted with 14% of the total aldehyde functionality, whereas glycine blocked only 4% of the aldehyde groups. Further evidence for the complete reaction of PA with hydroxylamine is illustrated in Fig 2. The aldehyde absorption of PA at 1720 cm^{-1} (Fig. 2A) decreased slightly after the interaction with ethanolamine (Fig. 2B) and almost disappeared on reaction with hydroxylamine (Fig. 2C).

The effect of blocking the remaining aldehyde groups after coupling proteins to the APAMB is illustrated in Fig. 3. The reactivity of antidigoxin–APAMB for the removal of digoxin is increased by approximately 40% by blocking the remaining aldehyde groups with hydroxylamine after coupling the rabbit antidigoxin. The reactivity of the blocked antidigoxin–APAMB was retained with time, *e.g.*, 3 months. On the other hand, the reactivity of the non-blocked antidigoxin–APAMB decreased by 20% after 3 months. However, by blocking these non-conjugated beads the reactivity towards digoxin increased to the original value obtained with the blocked conjugated APAMB. A similar effect was obtained when protein A was coupled to the APAMB. The reactivity of the bound protein A to human Ig was 30–50% higher when the remaining aldehyde groups were blocked with ethanolamine or hydroxylamine.

Stability and safety

The nephelometric experiments showed no detectable release of microspheres or agarose fragments, indicating that strong physical forces (*e.g.*, hydrogen bonds and Van der Waals forces) hold the microspheres within the agarose matrix. Spectrophotometric measurement showed no detectable release of acrolein from the APAMB.

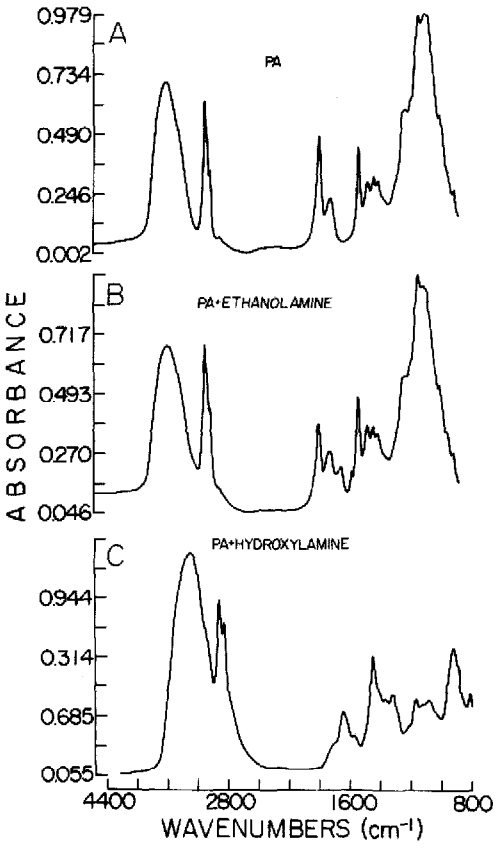

Fig. 2. (A) Infrared spectra of PA microspheres, (B) after reaction with ethanolamine and (C) after reaction with hydroxylamine. PA microspheres (average diameter 0.15 μm) (100 mg) in 5 ml of saline were shaken at room temperature for 48 h with 500 mg of ethanolamine or hydroxylamine in 5 ml of aqueous solution at pH 7.0.

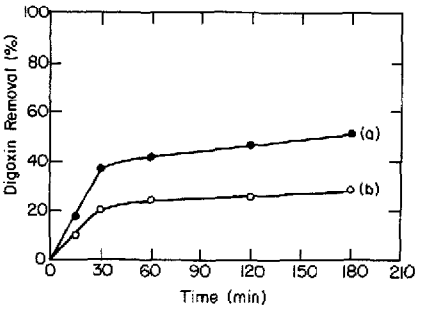

Fig. 3. Kinetics of removal of digoxin from PBS with antidigoxin–APAMB: (a) with hydroylamine as a blocking reagent; (b) without a blocking reagent. Antidigoxin–APAMB (4 mg of antidigoxin were bound to 1 g of APAMB containing PA microspheres of average diameter 0.15 μm) were shaken at room temperature in 50 ml of PBS containing 0.4 μg/ml of digoxin.

TABLE IV

LEAKAGE OF PROTEINS BOUND TO APAMB AND TO SEPHAROSE 4B CL

APAMB (1 g) (containing PA microspheres of average diameter 0.15 μm) and Sepharose 4B CL conjugated with proteins (BSA, 3 mg; human Ig and avidin, 10 mg each) were held in 2 ml of PBS at 4°C. For each period of time the supernatant was applied and reconstituted with the same volume of PBS.

Time (days)	*Amount released (%)*			
	*BSA–APAMB**	*Human IG–APAMB**	*Avidin–APAMB*	*Avidin–Sepharose 4B CL*
1	$1.5 \cdot 10^{-3}$	$4 \cdot 10^{-3}$	0**	0.1
7	$7 \cdot 10^{-3}$	$8.5 \cdot 10^{-3}$	0**	0.4
90	$7.5 \cdot 10^{-3}$	$38 \cdot 10^{-3}$	0**	0.4

* These experiments were carried out both under physiological conditions (PBS) and under aqueous acidic conditions [0.2 *M* glycine–hydrochloric acid buffer (pH 2.4)]. Similar results for the leakage were obtained.

** The detection limit for avidin using an enzyme assay[11] is 0.15 μg/ml or $3 \cdot 10^{-3}$% of released avidin.

Stability of the APAMB–amino ligands bond

Leakage of proteins bound to APAMB is low, as illustrated in Tables IV and V. BSA, human Ig and rabbit antidigoxin were detected in trace amounts in the supernatant of the conjugated APAMB. Avidin was detected in low levels in plasma and saline at 37°C. On the other hand, avidin in saline at room temperature and antiparaquat in plasma were not detected at all. The detection limit for avidin determination using the enzyme assay[11] is 0.15 μg/ml, or 0.3 μg/g of beads. Hence leakage of avidin bound to APAMB is at least 30 times lower than the leakage of avidin bound to Sepharose 4B via the cyanogenbromide activation method (Table V).

The leakage of ligands containing single primary amine group, *e.g.*, hydroxylamine, is illustrated in Table VI. Under physiological conditions the leakage, if any, is insignificant. However, under acidic conditions [0.2 *M* glycine–hydrochloric acid (pH 2.4)] which mimic the eluting conditions used to break the bond between antigen and antibody, a significant leakage of hydroxylamine was noted.

TABLE V

LEAKAGE OF PROTEINS BOUND TO APAMG DURING PERFUSION WITH PLASMA OR SALINE

Plasma or saline (35 ml) was circulated (at 30–50 ml/min) at room temperature and at 37°C through columns containing 10 g of APAMB (containing PA microspheres of average diameter 0.15 μm) conjugated with proteins (antidigoxin, 5 mg/g; antiparaquat, 18 mg/g; avidin, 10 mg/g). Samples were assayed after perfusion for 4 h.

Ligand	*Temperature (°C)*	*Medium*	*Ligand released (%)*
Antidigoxin	37	Plasma	$0.3 \cdot 10^{-3}$
Antiparaquat	37	Plasma	0
Avidin	37	Plasma	$9 \cdot 10^{-3}$
Avidin	Room	Plasma	$9 \cdot 10^{-3}$
Avidin	37	Saline	$2 \cdot 10^{-3}$
Avidin	Room	Saline	0

DISCUSSION

In previous papers we described the synthesis and use of APAMB for affinity chromatography[5,6,18]. During extensive studies carried out with the APAMB, several difficulties became apparent and were resolved.

Binding capacity

The binding capacity of APAMB containing microspheres of average diameter 0.15 μm toward amino ligands were described previously[1], *e.g.*, 1 g of APAMB bound 6 mg of goat Ig from a solution containing 15 mg of goat Ig. In order to increase the binding capacity of the APAMB, PA microspheres of smaller diameter were encapsulated in the agarose matrix. Microspheres with a smaller diameter have a much higher surface area and thereby their binding capacity is increased significantly (Table I). APAMB containing PA microspheres with diameters smaller than 0.1 μm possess additional useful characteristics and advantages compared with beads containing PA microspheres with diameters larger than 0.15 μm. They are more transparent, they have higher porosity and their performance in affinity separation is significantly improved (Table II).

Blocking the remaining aldehyde groups

In previous papers describing the use of polymeric beads containing aldehyde groups for affinity purification, the reagents examined and used for blocking remaining aldehyde groups, after coupling of the proteins, were glycine, BSA or ethanolamine[5,18–21]. However, glycine and BSA bind only 4% of the remaining aldehyde groups whereas ethanolamine blocks 14% of the remaining aldehyde groups (Table III). Therefore, it is expected that the reactivity of proteins bound to polyaldehyde beads blocked with the above reagents would not be optimal and may decrease with time because of the continued interaction of the lysine residue of the proteins bound to the APAMB with the free aldehyde groups on the beads. The hydroxylamine reagent interacts completely with all of the remaining aldehyde functionality of PA (Table III and Fig. 2). Therefore, this reagent seems to be a better choice for blocking the unreacted aldehyde groups.

Stability of the bond between APAMB and amino ligands

PA interacts reversibly with water to form various hydrated products[22,23]. Several of these hydrated forms are shown in Fig. 4. Primary amino ligands could bind to the aldehyde groups of PA through the free aldehyde form to give reversible Schiff base bonds and through the hydrated forms to give the irreversible cyclic products based on aminotetrahydropyran (Fig. 5). The lack of leakage obtained on reaction of proteins and PA may be explained by the resultant polyvalent bond, which is

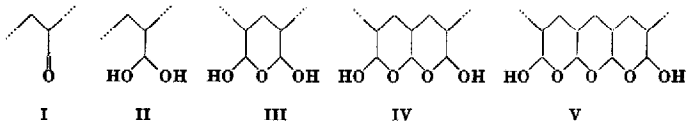

Fig. 4. Illustration of some products obtained by the reversible reaction of PA with water.

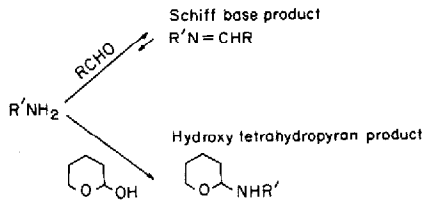

Fig. 5. Scheme of the reaction of primary amino ligands with the aldehyde groups of PA via the free aldehyde form and through the 2-hydroxytetrapyran form.

composed partially of Schiff base bonds and partially of the derivatized amino-tetrahydropyran forms. Amino ligands containing a single primary amino group, *e.g.*, hydroxylamine, may form two main types of products by their reaction with PA. The first is composed of a single Schiff base bond, which may lead eventually to leakage of the bound ligand from the APAMB into the solution. The second type of product may be based on the formation of a single derivatized aminotetrahydropyran form and should be stable.

Tables III and VI show that under physiological conditions the leakage of hydroxylamine bound to APAMB is insignificant. However, at acidic pH (2.4), which mimics the conditions employed to break the bond between antigen and antibody, a significant leakage of hydroxylamine bound to APAMB was obtained (Table VI). Therefore, it is suggested that when a low pH is used to break the antigen–antibody bond one should again block the remaining aldehyde groups after each five to eight affinity chromatography cycles. Another possibility for obviating the need for the reblocking step is to use conditions of high ionic strength at neutral pH, *e.g.*, 3.5 *M* aqueous sodium thiocyanate, to break the antigen–antibody bond.

Other possible reagents that can sometimes be used to stabilize the bond between the APAMB and ligands containing a single amino group are borohydride reducing reagents, *e.g.*, sodium borohydride or sodium cyanoborohydride[24]. Further, sodium borohydride may sometimes also be an efficient blocking reagent of the remaining

TABLE VI

LEAKAGE OF HYDROXYLAMINE BOUND TO PA MICROSPHERES AND TO APAMB

PA microspheres of average diameter 0.15 μm (100 mg) and 1 g of APAMB (containing PA microspheres of average diameter 0.15 μm) blocked with hydroxylamine were added to 20 ml of PBS solution or to 20 ml of 0.2 *M* glycine–hydrochloric acid solution at pH 2.4. The supernatant of the beads was replaced by the same volume each day. After 1 week, samples of washed beads were vacuum dried and submitted to nitrogen analysis.

Conditions	*PA microsperes*		*APAMB*	
	N (%)	*Hydroxylamine bound per 100 mg of PA (mmol)*	*N (%)*	*Hydroxylamide bound per gram of APAMB (mmol)*
Before treatment	14	1	7	0.5
pH 7.2	13.7	0.98	6.8	0.49
pH 2.4 once and then pH 7.2	11	0.78	6	0.43
pH 2.4	8.8	0.63	5	0.36

aldehyde groups (experiments in our laboratory at a variety of pHs, *e.g.*, pH 3, 4 and 5, showed that sodium cyanoborohydride in aqueous solution did not reduce the aldehyde groups of PA microspheres). However, our and other studies[24,25] indicated that in many instances the immunological activity of proteins, *e.g.*, antibodies, bound to various supports decreased significantly because of the use of the borohydride reducing reagents.

The search for new, effective immunoadsorbents is still continuing[26,27]. Most of the current advanced studies are carried out with polymeric beads containing hydroxy groups, *e.g.*, silica beads or Sepharose beads. The standard cyanogen bromide activation method that is used to bind amino ligands, to polymeric beads containing hydroxy groups suffers from several major disadvantages, *e.g.*, high toxicity of the cyanogen bromide reagent, a low yield of the reaction and instability of the isourea bond formed by the cyanogen bromide activation method towards hydrolysis and nucleophilic substitution reactions[26]. Recently, Wilchek and co-workers[28,29] elucidated the mechanism of the cyanogen bromide activation method. On the basis of their studies, they were able to increase the yield of the reaction between amino ligands and polysaccharide resins and thereby the amount of cyanogen bromide required for the activation could be decreased significantly. However, the instablity of the isourea bond still create a major difficulty in some systems. In order to eliminate the unstable bonds created by the cyanogen bromide activation method, Kohn *et al.*[30] developed alternative methods for the activation and immobilization of proteins to polymeric beads containing hydroxy groups based on reagents such as *p*-nitrophenyl chloroform at, N-hydroxysuccinimide chloroformate and trichlorophenyl chloroformate.

Several publications on the synthesis of beads derivatized with aldehyde groups suggest their use in affinity chromatography. An aldehyde-activated polyacrylamide support was prepared by Fiddler and Gray[31] from a commercially available aminoethyl polyacrylamide gel. Guesdon and Avrameas[20] prepared aldehyde-activated polyacrylamide agarose beads. Miron *et al.*[19] synthesized aldehyde-activated beads by reacting cyanogen bromide-activated agarose with varous bishydrazides to give hydrazidoagarose. Here, we have described the synthesis and studies performed with the improved APAMB. These beads are stable[10,18], they covalently bind amino ligands in a single step at physiological (or other) pH, they have a high binding capacity to amino ligands, the leakage of proteins bound to the APAMB is insignificant and they are highly biocompatible and blood compatible[18]. Very recent studies showed that the porosity of the APAMB (containing PA microspheres of average diameter 0.15 μm) is slightly lower than that of Sepharose 4B, and that the reactivity of a few proteins (*e.g.*, protein A) bound to similar APAMB, quenched with hydroxylamine, is similar to the reactivity of the same proteins bound to Sepharose 4B via the cyanogen bromide activation method. Further studies are in progress in our laboratory.

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