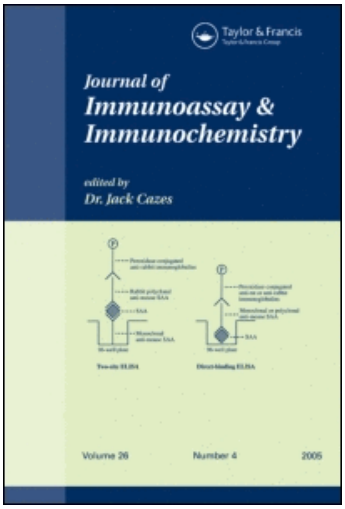

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POLYACROLEIN MICROSPHERES AS A NEW SOLID PHASE FOR RADIOIMMUNOASSAY

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ABSTRACT

Polyacrolein (PA) microspheres contain reactive aldehyde groups through which ligands containing primary amino groups such as proteins and drugs can be covalently bound in a single step at physiological pH. Antibodies against cyclic-AMP, digoxin and rabbit serum were thus coupled to PA microspheres. The immuno-microspheres were kept in suspension or freeze-dried, with insignificant decrease in their binding capacity. The conjugates were used in the respective radioimmunoassay (RIA) systems to facilitate the separation of the free and the antibody-bound ^{125}I ligands, in comparison with precipitation of Protein A of *Staphylococcus aureus*. Cyclic-AMP was assayed using PA microspheres coupled either with the primary antibody or with anti-rabbit serum as a secondary antibody, in a buffer system, in chick plasma, in urine and in media in which avian dispersed kidney cells had been stimulated by various agents. The results obtained using the immuno-microspheres and the bacterial separation methods were indistinguishable. Other ^{125}I-ligands, such as digoxin in buffer system or thyroxine and triiodothyronine in chick plasma, were assayed in the picogram range. Owing to the solubility of non crosslinked microspheres conjugates in toluene-based scintillation fluids, both the free and the bound fractions could be counted when using ^{3}H-ligands. Corticosterone was assayed using this technique.

(Key words: Polyacrolein Beads; Polyacrolein Microspheres; Radioimmunoassay; Solid Phase.)

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INTRODUCTION

Since its inception, radioimmunoassay (RIA) has been modified in numerous ways for the specific assay of a multitude of substrates (1-3), especially concerning the separation of the free from the antibody-bound fractions. Since the bound complexes usually do not precipitate spontaneously at the low concentrations employed, an appropriate technique is required to separate the bound and the free fractions. An ideal separation technique should fulfill the following criteria: 1) Permit complete separation of the bound and the free fractions without interference with the primary binding reaction. Any deviation from this requirement will impair the precision and sensitivity of the assay. 2) Not be affected by biological fluids such as plasma, serum and urine, such interactions necessitating a uniform concentration of these fluids in the standard tubes. 3) Provide a method which is rapid, simple and inexpensive.

Many separation techniques exist, based on different principles. These include differential migration caused mainly by a charge disparity (4,5), adsorption of the free fractions by coated charcoal or silicates, (6,7), adsorption of the bound fraction using anion exchange resins (8), passive adsorption on small chromatographic tubes (9,10), magnetic separation (11), etc. The most widely applicable systems are those depending on precipitation of the antigen-antibody complexes by secondary antibody directed against the specific antibody (double antibody technique) or by a suspension of *Staphylococcus aureus* containing the membrane-bound protein A which binds globulins without interfering with the active binding site of the antigen (12,13). The primary antibody, the secondary antibody or the purified protein A can be bound to a solid phase such as discs, tubes or various beads (14,15). Most of these methods require several steps and are based on non specific binding or hydrophobic interactions. Hereby, we propose polyacrolein microsphere as a new, widely applicable solid phase for RIA. These beads con-

tain reactive aldehyde groups through which ligands with primary amino groups, such as proteins, can be covalently bound, by a single step and at a physiological pH. Rabbit antibodies against digoxin or cyclic-AMP, as primary antibodies, and goat anti rabbit serum as secondary antibodies were bound to the PA microspheres and the conjugates were used in the respective RIA systems to facilitate the separation of the free and antibody-bound ligands. The immunomicrospheres can also be used in RIA systems including ^{3}H ligands, owing to their solubility in toluene-based scintillation fluids. This paper describes the characteristics of the assay using PA microspheres and discusses its advantages.

MATERIALS AND METHODS

Materials

Adensoine 3,5 cyclic phosphoric acid 2-0-succinyl-(^{125}I) iodotyrosine methyl ester (^{125}I cyclic-AMP) 600 Ci/mmole, L-(3,5 ^{125}I) thyroxine (T_4) 1.2 mCi/μg, L-(3-^{125}I) triiodothyronine (T_3) 1,2 mCi/μg and corticosterone-(1,2,6,7-^{3}H) 100 Ci/mmole, were obtained from the Radiochemical Centre, Amersham, England. Digoxin-^{125}I was obtained from Ames-Yissum, Jerusalem, Israel. Antibodies against cyclic-AMP, T_3, T_4 and corticosterone, raised in rabbits, and goat antirabbit serum were obtained from Bio-Yeda, Rehovot, Israel. Cholera toxin and DNase were from Sigma, St. Louis, MO, USA, and synthetic human parathyroid hormone 1-34 (hPTH 1-34) was a gift from Revlon Health Care Group, Tuckaho, NY, USA.

Preparation of polyclonal antidigoxin antibodies

Inbred white rabbits, weighing 3.5 to 4.5 kg were immunized with BSA-digoxin, prepared by the periodate oxidation method (16,17), in Freunds complete adjuvant. Weekly intradermal injections of 1 mg BSA-digoxin were given

into at least eight different sites. The pooled immune rabbit serum was passed through an affinity column as described previously (18). The antidigoxin antibodies were eluted with glycine HCl 200, mmol/L, pH 2.3. The eluates were neutralized to pH 7.0 and stored at -20°C until used. Purity of the antidigoxin antibody fraction was ascertained by polyacrylamide gel electrophoresis.

Synthesis of polyacrolein microspheres

The PA-microspheres were synthesized as described previously (19). Briefly, NaOH 200 mmol/L was added dropwise to an aqueous solution containing 300 ml/L acrolein and 5 g/L of the surfactant polyglutaraldehyde-sodium bisulfite adduct (19) until a pH of 11.0 was reached. The reaction was continued for 4 h, after which the mixture was dialyzed extensively against distilled water and then centrifuged four times at 500 g for 10 min. Monodispersed microspheres of 3.0 μ average diameter were collected. Crosslinked microspheres were obtained by cobalt irradiation (3 Mrad) of the microspheres.

Preparation of T_3 and T_4 free serum

Fifty ml of normal chick serum were mixed with 10 g of charcoal and kept overnight at 4°C. The charcoal was separated from the serum by centrifugation at 12,000 g and the supernatant was filtered through a Whatman no. 40 ashless filter paper. By this procedure, 99% of the T_3 and T_4 was removed with the charcoal.

Binding of the antibodies to the microspheres

Ammonium sulfate precipitation of the different immunoglobulins was performed as described by Nowotny (20). The equivalent of 1 ml serum was incubated with 200-300 mg of the PA microspheres in 15 ml phosphate-saline buffer, 10 mmol/L (PBS), pH 7.4, for 3 h at room temperature and then overnight at

4°C. The unbound proteins were separated from the conjugated microspheres by centrifugation (150 g, 5 min). The immuno-PA microspheres were resuspended in the appropriate buffer for the specific RIA.

Preparation of avian dispersed kidney cells and plasma, and urine collection were as described previously (21,22).

Calculation of assay results

After the appropriate correction for the background and non-specific binding, the ratios of the bound and free fractions were calculated for all assayed tubes. The standard curve was linearized by a log-logit transformation (23). The regression coefficient of the standard curve and the SD of the B/F ratio for each of the unknown samples were calculated according to Snedecor and Cochran (24).

RESULTS

Figure 1 represents a scanning electron microscope (SEM) photomicrograph of PA microspheres used for the RIA.

Use of Immuno-PA microspheres in cyclic-AMP assay

Standard curves for cyclic-AMP were obtained by incubating ^{125}I cyclic-AMP (4000 cpm) and standards in the range of 10-625 fmole/tube in sodium acetate buffer, 70 mmol/L, pH 6.1, containing 20 g/L BSA, by either of the following procedures.

1. Incubation with the antiserum solution (final dilution 1:6400, B/F=0.6) overnight at 4°C. The bound and free fractions were separated by adding either 1 mg of antirabbit serum bound to PA microspheres for an additional hour,

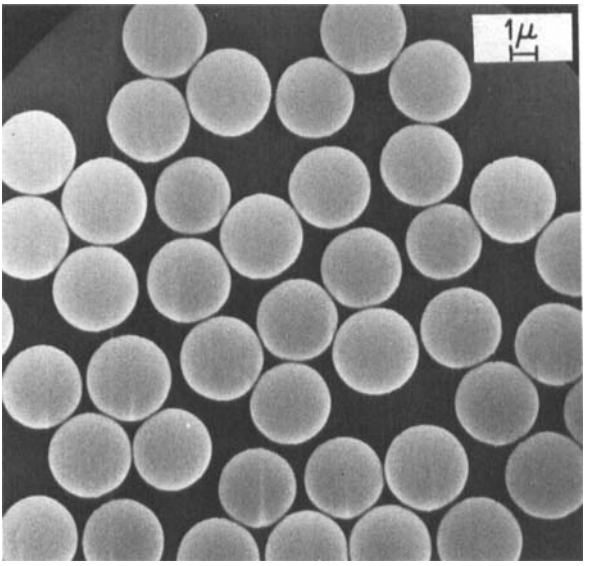

Figure 1. SEM photomicrograph of the PA microspheres.

or 500 μl of formaldehyde-treated *Staphylococcus aureus* suspension (13). The tubes were centrifuged at 250 g for 5 min or 1000 g for 20 min, respectively. The supernatant was decanted and both fractions were counted.

2. Incubation with 1 mg of cyclic-AMP antiserum bound to PA microspheres as the primary antibody for 1 h following centrifugation at 250 g for 5 min. Figure 2 shows the log-logit transformation of the standard curves. The sensitivity of the assay was the same for all procedures and the minimum detectable dose (concentration resulting in a response two SDs from the zero dose response) was 1-2 fmole/tube. Table I shows the cyclic-AMP concentration in avian plasma, urine and dispersed kideny cells *in vitro* as assayed by RIA, using immunomicrospheres or a bacterial suspension to separate the bound and free fractions. The two methods gave similar results.

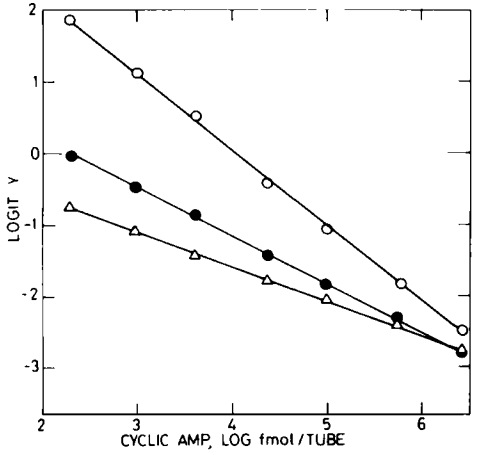

Figure 2. Ratios of bound to free fractions as a function of the cyclic-AMP concentrations, given as the log-logit transformation of the standard curves. Cyclic-AMP in the range of 10-625 fmole/tube was assayed with PA microspheres coupled with the primary antibody (o), with the secondary antibody (o) or with *Staphylococcus aureus* (Δ). The calculated regression equations were Y=1.26–0.63X, Y=4.76–1.17X and Y=0.28–0.46X, and the SD of the slopes was 0.02, 0.05 and 0.05, respectively.

Table I. Cyclic-AMP concentrations in avian plasma, uringe and dispersed kidney cells as assayed by immuno-PA microspheres and with *Staphylococcus aureus* suspension.[a]

Sample	PA Microspheres coupled with second antibody	*Staphylococcus aureus*
Plasma (pmole/ml)	3.1 ± 0.7	3.1 ± 0.8
Urine (μmole/GFR)	1.4 ± 0.3	1.4 ± 0.2
Kidney cells (pmole)	1.4 ± 0.2	1.4 ± 0.2
Kidney cells + 50 ng hPTH (pmole)	7.2 ± 0.5	6.9 ± 0.2
Kidney cells + 50 ng ch.toxin (pmole)	6.0 ± 0.7	6.3 ± 0.7

[a] Avian plasma and urine was collected and processed as described previously.[20] The kidney cells ($25x10^4$ vial) were incubated with hPTH for 15 min or with Cholera toxin (ch.toxin) for 3 h. The results are the mean ±SE of 5 different experiments.

Stability of the immuno-PA microspheres

Antirabbit serum coupled to PA microspheres was kept at room temperature for 70 days. At various intervals, RIA's for cyclic-AMP were performed and the initial ratios of bound to free fractions were calculated. Figure 3 shows that the microspheres retained their binding capacity during the entire period (2% decrease after 70 days). At the end of the experiment, the non-specific binding was less then 3%.

Freeze-dried microspheres kept for at least 6 months, and then reconstituted to their initial volume, retained 96% of their initial binding capacity.

Immuno-PA microspheres in thyroid hormone assays

The RIA was performed at 4°C for 12 h in a final volume of 250 μl containing ^{125}I T_3 or T_4 (4000 cpm) in buffer composed of sodium barbital,50 mmol/L, barbiton,10 mmol/L,and BSA,5 g/L. Standards (30-1000 pg/tube) were prepared in thyroid hormone-free serum and incubated with the specific antiserum (1:5000). The free and bound ligands were separated by adding 1 mg of the secondary antibody bound to PA microspheres in the same buffer for an additional 1 h followed by centrifugation at 250 g for 5 min. Both fractions were counted and the log-logit transformation was plotted (Fig. 4). The minimum detectable dose for both hormones was 4-5 pg/tube.

Assay for digoxin

Figure 5 shows the standard curves for digoxin assayed with PA microspheres coupled with the primary or secondary antibodies. The RIA was performed by incubating ^{125}I digoxin and the digoxin standards (30-240 pg/tube) either with microspheres conjugated with purified antidigoxin antibodies for 2 h in 300 μl of PBS, or with antidigoxin serum for 1 h and then for an additional hour with the microspheres conjugated with secondary antibody (1 mg). At the end

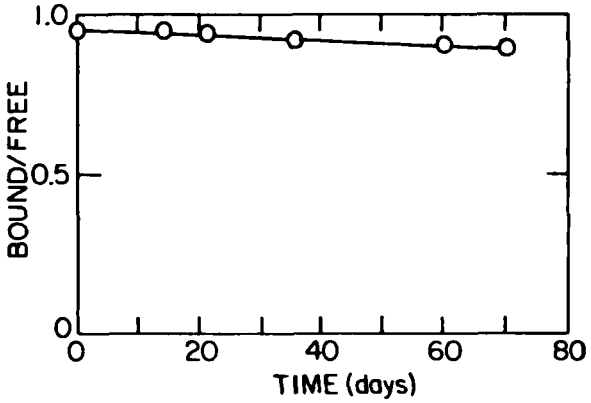

Figure 3. Stability of the immuno-PA microspheres. PA microspheres bound to the secondary antibody were stored at room temperature for 70 days. At intervals, samples were taken and their binding capacity in the cyclic-AMP RIA was measured.

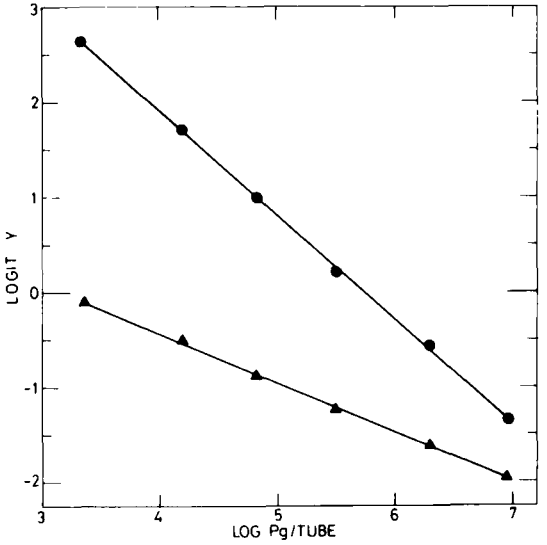

Figure 4. Ratios of the bound to free fractions as a function of T_3 and T_4 concentrations, given as log-logit transformation of the standard curves. T_3 (Δ) and T_4 (o) at concentrations of 30-1000 pg/tube in thyroid hormone free serum were assayed by RIA. The bound fractions were separated from the free by adding 1 mg of PA microspheres coupled with the second antibody following centrifugation. Both fractions were counted. The calculated regression equations were Y=1.55–0.5X and Y=6.2–1.1X, and the SD of the slopes was 0.098 and 0.038, respectively.

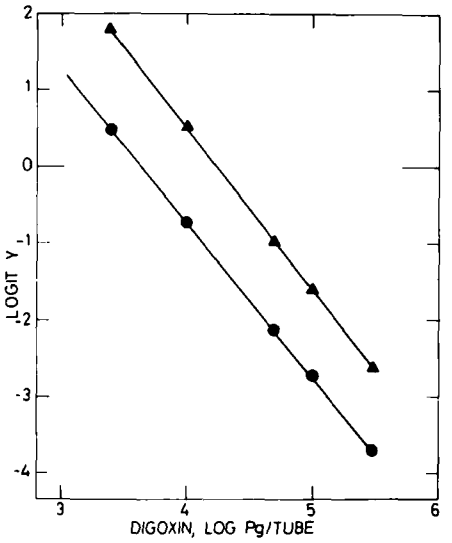

Figure 5. Digoxin standard curves. Digoxin (30-240 pg/tube) was assayed using PA microspheres coupled with the primary antibody (o) or with the secondary antibody (Δ). The calculated regression equations were Y=6.64-1.89X and Y=8.22-1.90X, respectively, and the SD of slope was 0.1 in both cases.

of the incubation, the tubes were centrifuged and both fractions were counted. The minimum detectable dose in the two procedures was in the range of 10-20 pg/tube.

Assay for ^{3}H ligand

^{3}H-corticosterone and standards were incubated with corticosterone antiserum in phosphate buffer,50 mmol/L,pH 7.4 containing BSA,5 g/L, at 4°C for 12 h. Thereafter, 1 mg of non-crosslinked immuno-microspheres coupled with the secondary antibody was added for another hour. The tubes were centrifuged for 5 min at 150 g and the bound and free fractions were separated. Because of the solubility of the non-crosslinked PA microspheres' conjugate in toluene-based

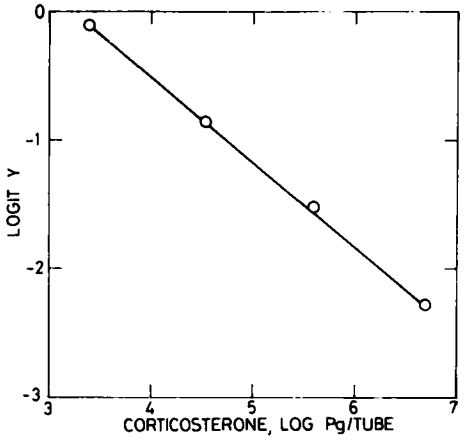

Figure 6. Corticosterone standard curve. Corticosterone (30-800 pg/tube) was assayed using ^{3}H-corticosterone as a tracer and PA microspheres coupled with the second antibody for separation. Both the free and the bound fractions were counted and the bound-to-free ratio was calculated for each tube. The regression equation was Y=2.66–0.65X and the SD of the slope was 0.03.

Table II. Corticosterone concentration in chicken adrenal cells as assayed by immuno-PA microspheres.[a]

Treatment	Corticosterone pg/10^4 cells
Control	50
+ ACTH (10 ng)	250

[a] Avian adrenal cells were prepared similarly to the method described for kidney cells (21), using only DNase for dispersing the cells.

scintillation fluids, both the bound and the free ligands could be counted (Fig. 6). To each fraction, 3 ml of the scintillation fluid was added (Insta-gel, Packard, IL, USA). Since there were differences in quenching between the bound and the free fractions, had to be corrected.

Assays of corticosterone secreted *in vitro* by ACTH-stimulated chicken adrenal cells (Table II) confirm the potential of this method for assaying tritiated ligands in biological systems. Table II shows the results from five experiments.

The adrenal cells were incubated at 37°C with or without ACTH for 1 h following centrifugation at 1000 g. The supernatant was assayed for corticosterone by RIA using immuno-microspheres for separation the free and the antibody-bound ligands.

DISCUSSION

The adaptation of PA microspheres as a solid phase for RIA offers several advantages; the antibodies are covalently bound to the microspheres through their aldehyde groups in a single step at physiological pH. Special equipment or techniques are not required. The immuno-PA microspheres are stable for a long period of time. As shown in this paper, different immuno-conjugates can be prepared, including both primary and secondary antibodies bound to PA microspheres. Proteins other than antibodies, such as receptors to specific ligands, can also be bound to microspheres. Binding second antibody to PA mcirospheres, or alternatively purified protein A, provides a universal solid phase for the RIA of numerous ligands differing in their chemical and physical properties (cyclic-AMP, T_3, T_4, digoxin, corticosterone, etc.). Binding the first antibody to the microspheres gives a solid phase specific to only one ligand, but saves time and labor by reducing the amount of pipetting needed, thereby reducing

the possibility of error, especially when a large number of assays is required. The immuno-microspheres can be used in buffer systems or in biological fluids, such as plasma, serum or urine, with the same efficiency as the *Staphylococcus aureus* suspension. Separation between the free and antibody-bound ligands is achieved by low-speed centrifugation for a short time, 250 g for 5 min as compared with 1000 g for 20 min with the bacteria.

Another advantage of the immuno-PA microspheres is that they permit counting of both the free and the bound fractions even when the tracers are β-labeled ligands. Although the γ labeled ligands have proved most suitable for RIA, ^{3}H is sometimes used, especially when iodination alters the binding between the antibody and the antigen or when few assays are needed over a long period of time. Using immuno-PA microspheres, the ratio of bound to free ligand can be calculated for each tube.

In conclusion, we present in this paper a simple, highly reliable solid phase for RIA, which can be applied to a wide range of test substances incorporating different isotopes.

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