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ISOLATION OF PLASMA MEMBRANE FROM EUKARYOTIC CELLS ON POLYLYSINE-COATED POLYACRYLAMIDE BEADS

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Summary

The conditions for covalently binding polylysine to polyacrylamide beads used for membrane isolation have been analyzed. Larger amounts of bead bound polylysine were required for maximizing plasma membrane purification from HeLa cells than from *Dictyostelium discoideum*. The least was needed for erythrocytes. The amount of polylysine bound to the bead was dependent on the carboxyl content of the bead and on the concentration of the polylysine used during the bead-polylysine coupling reaction.

A procedure was recently reported [1] for the rapid isolation and exposure of the cytoplasmic surface of erythrocyte plasma membrane on polylysine-coated beads. The method depends on an interaction between the negatively-charged outside surface of many cells with the positively-charged surface of a bead. After interaction, the unattached portions of the membrane and the cellular debris are washed away by cell lysis and a brief sonication, leaving behind beads covered by plasma membrane with its cytoplasmic face exposed to the bathing medium. Since the report with erythrocytes, it has become apparent that the manner in which the beads are modified and, in particular, the concentration of polylysine on the bead surface, is critical if the technique is to be consistently reproducible with various cell types.

Based upon the results presented here, the following procedures of bead preparation were found optimal for the isolation of plasma membranes from erythrocytes, HeLa cells and *Dictyostelium discoideum:* 10 g of polyacrylamide beads (Biogel P-2, 200—400 mesh, Bio-Rad Laboratories, Richmond,

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Calif.) were placed in a 250 ml flask and hydrated at 90°C for 3 h, then cleansed with a Heat Systems Sonifier set at 50 W for 1 min. The beads were allowed to settle and the fine material decanted. This was repeated three times. The next step was to hydrolyze the beads to form carboxyl groups to which polylysine could be subsequently linked by amide bonds. The hydrolysis procedure is a modification of that previously given [1]: 200 ml of 0.5 M Na_2CO_3 was stirred into the flask of hydrated beads; after the beads settled, the supernatant was aspirated and another 200 ml aliquot of Na_2CO_3 was added. The beads were stirred every 10—15 min during incubation at 60°C for 90—100 min. They were then washed 6—7 times with 6 vol. of distilled water. After the beads settled, the supernatant was removed and 10 ml of 1 M pyridine containing 400 mg of polylysine (90 000 to 300 000 daltons, Sigma Chemical Co., St. Louis, Mo.) was added. The mixture was agitated at room temperature on a rotary shaker set at a speed sufficient to keep the beads from settling. After 90 min, 6 ml of 5.2 M pyridine·HCl, pH 5.1 (as determined at room temperature), was added; while the beads were agitating, six 0.5-ml aliquots of freshly prepared 1 M 1-ethyl-3(3-dimethylaminopropyl)carbodiimide·HCl was added at 1—2-min intervals to activate and couple the carboxyl groups to polylysine. After 48 h, three 0.5-ml aliquots of fresh 1 M carbodiimide were similarly added followed by 2 h of incubation. The beads were allowed to settle and the polylysine solution was removed. The negative charge of unreacted carboxyl groups, such as those in the pores of the polyacrylamide not penetrated by the polylysine, was 65—75% blocked by adding 40 ml of 2 M pyridine·HCl (pH 5.0) to the beads, followed by sufficient solid NH_4Cl to make the solution 6 M. This was followed by addition of 5 ml of 2 M carbodiimide and 2 h of agitation. The beads were washed 3 times in water, 6 times in 0.15 M Tris·HCl (pH 7.4) and then stored at 4°C in the same buffer containing 0.02% NaN_3.

Firm ionic attachment of intact cells to the polylysine-coated bead surface is essential for plasma membrane isolation. The ionic interaction must be so tenacious that when sonication breaks up the cells, it leaves behind the part of the plasma membrane initially in contact with the bead. We have determined the conditions which maximize the positive charge on the bead surface so as to optimize cell attachment and subsequent membrane isolation.

Carboxyl groups to be coupled to the ϵ-amino groups of polylysine were formed by incubating the beads in 0.5 M Na_2CO_3. The mol fraction of carboxyl groups increased as a function of the hydrolysis time [2]. For the Biogel P-2 we used the mol fraction = $4.5 \cdot 10^{-4}$ (min of hydrolysis) + $1.8 \cdot 10^{-2}$ for up to 240 min (coefficient of determination was 0.96). As the mol fraction increased, the degree of bead swelling also increased. Swelling was most likely due to the formation of negatively-charged groups that repelled each other and to the reduction of cross links between neighboring polyacrylamide chains. Since the porosity of the beads was dependent upon the degree of cross-linking, the more they were hydrolyzed, the greater became their porosity. The degree of swelling was measured as a percent increase in volume of hydrolyzed beads compared to an equal weight of unhydrolyzed ones. The percent increase equaled 282 × (mol fraction of

carboxyl group) — 0.72 (coefficient of determination was 0.95) at pH 7.5.

The amount of polylysine covalently bound the beads was proportional to the concentration of polylysine used in the reaction medium (Fig. 1) and did not saturate the system even at 100 mg/g bead. Use of much higher concentrations were complicated by the extreme viscosity of the solution. Up to a certain mol fraction of carboxyl groups, the amount of polylysine bound increased as the mol fraction of carboxyl groups increased (Fig. 2).

At first thought, it might appear that beads extensively hydrolyzed and incubated in high concentrations of polylysine would maximize membrane binding. However, it was reasoned that since the bead porosity increased as the time of hydrolysis increased, much of the bound polylysine might be inside the bead instead of on its surface, where it must be to promote cell and membrane adherence. In fact, when polylysine-coated beads with a range of carboxyl contents were tested for their ability to bind erythrocytes, membrane protein attachment to the beads decreased as the mol fraction of carboxyl groups increased beyond about 0.04 (Fig. 3). The effect was less pronounced with higher molecular weight polylysine which would not so easily penetrate into the bead pores. Thus, for membrane isolation it is desirable that the polyacrylamide beads not be overly hydrolyzed, especially when low molecular weight polylysine is used. In the case report-

TABLE I

PURIFICATION OF PLASMA MEMBRANES COATED WITH DIFFERENT AMOUNTS OF POLYLYSINE

All values represent an average of three determinations with a standard deviation of less than 10%. Erythrocytes were attached to beads in 220 mosM sucrose containing 90 mosM phosphate buffer, pH 7.4 [1]. *D. discoideum*, grown to 8—10·10^6 cells/ml [3] were washed three times at room temperature in buffer A (150 mM KCl plus 20 mM phosphate buffer, pH 6.0). The cells were then left as a 50% suspension and diluted with 5 vol. of ice-cold buffer B (150 mM sucrose plus 50 mM acetate buffer, pH 5.0). Cells were washed twice in buffer B and left as a 50% suspension. As the cell suspension was gently agitated, an equal volume of beads previously washed in buffer B and brought to a 40—50% suspension was added drop-wise and then left on ice for 10 min. Excess cells were removed by diluting the beads with 10 vol. of buffer B and after the beads with attached cells settled, the unattached cells in suspension were aspirated. This was repeated three times. Attached cells were ruptured by adding 10 vol. of 10 mM Tris·HCl, pH 7.4, while vigorously vortexing the mixture. The beads were washed twice in the Tris buffer, left as a 33% suspension, then sonicated for 10 s with the sonifier set at 20 W. The cellular debris was removed by three washes in Tris buffer. HeLa cells grown to late log were prepared and attached to beads indicated above except buffer A was 280 mosM NaCl plus 30 mosM phosphate buffer, pH 7.4; buffer B was 220 mosM sucrose and 90 mosM Tris·HCl, pH 7.4. All assays were done with 0.3 ml of membrane-covered beads. Protein and phospholipid were determined by previously published procedures [1] as was alkaline phosphatase [4] and ouabain sensitive (Na^++K^+)-ATPase [5]. The beads were added to the cell homogenate enzyme reaction mixture to account for effects they might have had on the activity.

Polylysine		Plasma membrane purification			
Added	Bound	Erythrocytes		*Dictyostelium*	HeLa cells
		Protein[a]	Phospholipid[a]	Alkaline phosphatase purification[b]	(Na^++K^+)-ATPase purification
10	5.2	1.27	0.63	6.0	5.9
20	8.5	1.32	0.64	7.5	10.8
50	15.0	1.40	0.63	7.6	12.0

[a] $mg \cdot g^{-1}$ bead.
[b] Homogenate specific activity was 8 nmol PO_4 released·min^{-1}·mg^{-1} protein and for (Na^++K^+)-ATPase, 2.6.

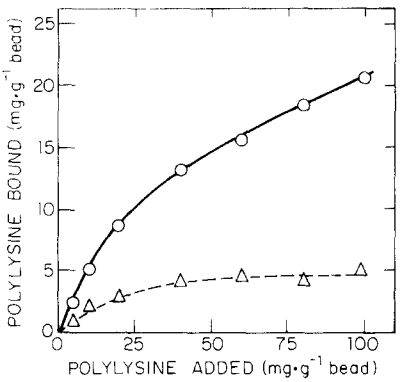

Fig 1. The amount of polylysine bound to polyacrylamide beads as a function of the amount initially present. The upper curve (○—○) is the amount covalently bound by carbodiimide activation and the lower curve (△— —△) is the amount adsorbed to beads in the absence of carbodiimide. Beads with adsorbed material were not useful for membrane isolation. The beads had a mol fraction of carboxyl groups of 0.05 and the polylysine was 68 000 daltons. The volume of the reaction medium was 2 ml/g bead. Similar results were found with polylysine 185 000 daltons. The polylysine was made radioactive by reductively formylating 1/2500 of the ϵ-amino groups (Jacobson, B.S., Cronin, J. and Branton, D., unpublished).

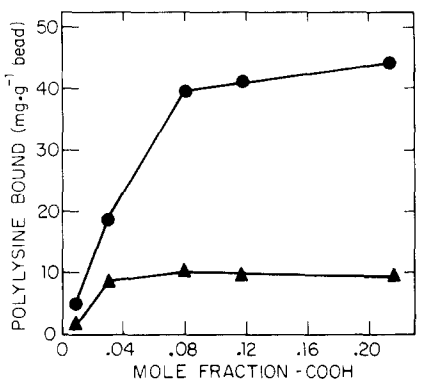

Fig 2. The amount of polylysine covalently bound to polyacrylamide beads with different mol fractions of carboxyl groups. The upper curve (●—●) had an initial amount of polylysine of 100mg/g bead and the lower curve (▲—▲) had 20 mg/g. Polylysine was 68 000 daltons and the volume of the reaction medium was 2 ml/g bead.

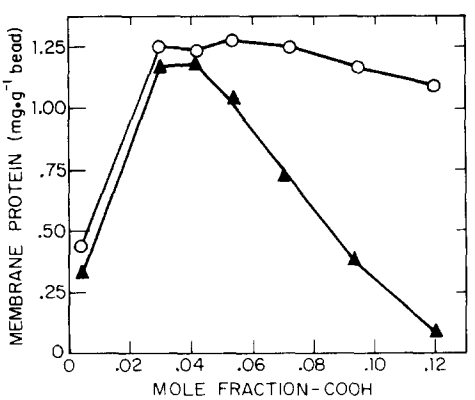

Fig 3. Plasma membrane isolated from erythrocytes on polylysine-coated beads with different mol fractions of carboxyl groups. Beads coated with polylysine (30 mg/g bead in 2 ml) either 185 000 daltons (○—○) or 68 000 daltons (▲—▲).

ed here the polylysine with the high molecular weight (186 000) was preferred over the low one (68 000) for isolation of erythrocyte membranes.

How much polylysine must be coupled to beads for maximum membrane purification depends upon the cell type. Plasma membranes from HeLa cells and *D. discoidium* were not purified as well by the beads with the low amounts of polylysine as they were by beads with the greater amounts (Table I). Erythrocyte plasma membranes could be purified by beads with 5.2 or 15 mg polylysine/g bead (Table I). Membrane purification by beads is no doubt related to an electrostatic interaction between the negatively-charged cell surface and the positively-charged beads. The greater this interaction the more firmly would the plasma membrane adhere to the bead. If membrane is not firmly held in place, it will be dislodged when attached cells are lysed thereby creating vacant sites on the bead which become contaminated by cytoplasmic components. In this connection, the HeLa cells which required beads with the greatest amount of polylysine for membrane purification (Table I) probably had the lowest negative surface charge as judged by their electrophoretic mobility [7] and *D. discoideum* which required less polylysine had a greater surface charge than the HeLa cells, but slightly less than that of the erythrocytes [8].

In addition to the intrinsic physical and chemical properties of the beads, other extrinsic factors may also affect membrane isolation. With HeLa cells, these include ionic strength of medium, pH, and temperature [5]. With *D. discoideum*, these factors are now being investigated with regard to plasma membrane isolation from cells at various stages of development. Thus, although preliminary experiments indicate that polylysine coated polyacrylamide beads can be used to isolate the plasma membrane from a variety of different cells, the exact conditions that optimize yield and purity should be determined for each type.

Acknowledgments

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