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COUPLING POLYLYSINE TO GLASS BEADS FOR PLASMA MEMBRANE ISOLATION

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Summary

Solid glass beads for use in isolating cell membranes were coated with a stable, covalently attached layer of polylysine. The optimal conditions for coating the bead surface were established and the beads were tested by measuring the attachment of human erythrocyte plasma membranes. When compared to other beads, such as those with absorbed polylysine or protamine, none retained red-cell membranes as well as glass beads with covalently linked polylysine.

Introduction

The membrane of any negatively-charged cell adheres tenaciously to an appropriately prepared, positively-charged surface [1—4]. Such a surface is an excellent substrate on which to isolate cell membranes because the cell and its contents can be sheared away, leaving behind, attached to the surface, a coherent piece of the plasma membrane [5,6]. Because spherical beads provide a large surface area which can be contained in a small convenient volume, work in this laboratory has focused on the development and use of charged beads to isolate the plasma membrane of eukaryotic cells [7,8]. This approach provides a rapid method of isolating plasma membranes and has the advantage that, while the extracellular membrane surface is apposed to the beads, the protoplasmic surface is fully exposed and accessible to biochemical probes. Thus, when on the beads, the asymmetric disposition of membrane components can be determined [8].

Solid and porous beads of various materials are available. Although each may

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have certain advantages, asymmetric labelling of attached membrane is favored by a solid support which is not permeable to the labelling reagent and which blocks access to the apposed extracellular membrane surface. We have found that solid glass beads are advantageous because they are dense, mechanically stable and biologically inert.

Procedures for modifying the surfaces of porous glass have been described [9—11], but the special problems encountered when preparing solid glass beads for membrane isolation have not been addressed. Because porous glass provides such a large internal surface area per unit of glass, complete and homogeneous alteration of the outer surface, which is necessary for good cell attachment to solid beads, has not been considered. Similarly, optimum conditions for bonding functional groups to the glass surface and the lifetime of these groups in aqueous media have received scant attention. In this paper we demonstrate how solid glass beads can be covered by an extensive, stably bonded meshwork of polylysine and show that such a meshwork provides a better surface for membrane isolation than any other we have tested.

Materials and Methods

Glass beads. Large stocks of glass beads, Class IVA, size no. 3200, were purchased from Cataphote corporation, Jackson, Miss. 39205, U.S.A. The manufacturer specified their diameter as 13—44 μm. They were composed of 60—70% SiO_2 and 14, 13 and 0.5% oxides of sodium, calcium and aluminum, respectively. The refractive index was 1.51 and the density reported by the manufacturer was 2.42 g/cm^3. The observed density calculated by water displacement was 2.36 g/cm^3. The wet packed volume was 0.64 ml/g with a void volume of 34%.

The beads were cleaned by incubating 600 g of beads in 600 ml of 10% nitric acid at 90°C for 1 to 2 h. The beads were stirred periodically with a glass rod, then washed 8 times with 800 ml of water and 3 times with 800 ml of acetone. They were then dried in air, first at room temperature to remove most of the acetone and its vapors, then in an oven at 110°C. During the cleaning procedures we used, most of the smaller beads were lost and mostly those ranging in size from 20 to 44 μm remained. After 12—24 h the beads were cooled and stored in sealed containers where they remained as a free-flowing powder when they were not hydrated. Before using the washed glass beads for chemical modification they were further treated as follows: 10 g of glass beads in 20 ml of water in a 150 × 25 mm culture tube were sonicated for 1 min with the semimicroprobe of a Heat Systems Sonifier model W185D set at 50 W. After the beads settled, the water was withdrawn with a water aspirator and the sonication procedure repeated once more. The beads were washed once again in 40 ml water and then 3 times in 40-ml portions of acetone. After air drying, they were stored for 16—20 h at 110°C before being used. This treatment removes excess water but does not affect the surface silanol groups which are dehydrated at approximately 400°C [12].

Silanization. The experiments detailed in Results indicate that the following procedures for silanization, succinylation and polylysination were optimal. To 10 g of prepared glass in a 150 × 25 mm culture tube, 40 ml 3.5% 3-aminopro-

pryltriethoxysilane in anhydrous toluene was added. The toluene was made anydrous by storage for at least 24 h over $CaSO_4$ granules and filtered before use. Culture tubes were sealed with teflon-lined, rubber-backed caps and then placed in a water bath at 85—90°C. After 15 min the tube was withdrawn and the cap retightened. Since the glass culture tubes sometimes broke while tightening the caps, gloves were worn to prevent injury. Silanization was continued for 6—7 days at 85—90°C. The tubes were shaken at least twice a day during the incubation period. After 6 or 7 days, the glass beads were washed 3 times with toluene and 3 times with acetone, dried at room temperature with a water aspirator, and stored in a lyophilizer for at least 24 h before use. Beads which have been subject to this silanization procedure will be referred to as aminopropyl glass beads.

Determination of amino groups attached to glass beads. 2,4,6-trinitrobenzene sulfonic acid ($N_3B_2SO_3^-$) was used as a qualitative assay for the presence of aminopropyl groups on the silanized glass. 0.5 g of amino glass was suspended in 1 ml of saturated sodium borate containing 20 g/l trinitrobenzene sulfonate. Beads turned yellow in color when aminopropyl groups were present. For quantitative determination, radioactive acetic anhydride was used. To 0.5 g of aminopropyl glass was added 1 ml anhydrous toluene, 0.05 ml anhydrous pyridine and 5 μl [1-^{14}C]acetic anhydride (0.02—0.03 μCi/mol) in a 4 ml flat bottom culture tube. A disc of teflon sheeting placed inside a plastic cap over the cardboard cap liner was used to seal the culture tubes. The tubes were placed in a 50°C water bath and shaken every 10—15 min for 1 h. The reaction was 85% complete in 5 min and complete in 30 to 60 min. The reaction was terminated by adding 3 ml of 95% ethanol. The beads were then washed twice in 10% acetic acid in 95% ethanol and 4 times in 95% ethanol. Following the last ethanol wash 2.8 ml of Aquasol scintillation fluid (New England Nuclear) was added and, after dispersing the beads, 1 ml of water was added and the tube quickly capped and rapidly shaken. In this way the beads were dispersed in a gel of scintillant. Counting efficiency was 90%. Background due to adsorption of [^{14}C]acetic anhydride was estimated by using beads prepared and treated in the same manner except that aminopropyltriethoxysilane was omitted.

Succinylation of aminopropyl glass beads. Aminopropyl glass beads, 10 g in a 150 × 5 mm culture tube, were washed once in 0.1 M sodium acetate buffer, pH 5.0, then once in 95% ethanol and finally twice in acetone and once in anhydrous acetone. The beads were mixed with 40 ml of anhydrous acetone containing 50 g/l succinic anhydride (w/v) and 5% pyridine (v/v). Acetone was made anhydrous by storage over $CaSO_4$ and passed through Whatman no. 1 filter paper just prior to use. Tubes containing the beads and the reaction mixture were kept at 50—55°C for 4 h. Succinylation was completed in 4 to 6 h. The beads were washed twice in 40 ml of 95% ethanol, twice in acetone. Six acetone washes were used when radioactive succinic anhydride was used. The beads were dried and stored in a lyophilizer at least overnight before further use.

Polylysination of succinylaminopropyl glass. 20 g of succinylaminopropyl glass beads were placed in a 150 ml beaker and washed twice with water. 6 ml of a solution containing 4 ml of H_2O, 2 ml of 4 M pyridine and 980 mg of

poly-L-lysine was then added. The polylysine was purchased from Sigma Chemical Co. and used without further purification. The stated average molecular weights given by Sigma varied from lot to lot, but were in all cases greater than 85000 and in no case greater than 250000. There was no consistent correlation between molecular weight and membrane retention although we did get the impression that the larger molecular weight polylysine may have been more effective for cell adherence. The beads were kept in suspension by agitating the beaker on a rotary shaker set at 175—225 rev./min. After 15 min, 4 ml of 5.2 M pyridine-HCl, pH 5.0 (determined at room temperature), was added followed by the slow (0.2 ml every 2 min) addition of 1 ml of freshly prepared 1.0 M 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide-HCl. The mixture was kept in suspension for 2 h on the rotary shaker. We have observed that the pyridine-HCl buffer enhanced the coupling reaction. Pyridine is also bactericidal and should prevent polylysine degradation. After 2 h of agitation the beads were allowed to settle and the supernatant was withdrawn and stored in the cold so that the polylysine could be reclaimed for future use. Beads were washed once with 20 ml water and the supernatant from this wash was combined with the first supernatant for reclamation. The beads were then washed once in 80 ml of 4 M NH_4Cl and then 5 times in water. They bore approximately 330 μg polylysine/g bead. The beads were lyophilized for storage.

The polylysine was reclaimed from 20—60 ml of used reaction mixture by exhaustive dialysis, first against 20 mM HCl, followed by a 2 h dialysis against water. The polylysine-HCl was then centrifuged at 30000 $\times g$ for 30 min. The supernatant was lyophilized and reused. We have reclaimed and reused polylysine methylated at its carboxyl terminus as often as 4 times. The polylysine is methylated if the polymerization reaction used by the manufacturer is initiated by sodium methoxide.

Synthesis of [^{14}C]N-ϵ-methyl-poly-L-lysine. 500 mg of poly-L-lysine-HBr or HCl were dissolved in 10 ml of prechilled saturated sodium borate. The temperature of the solution was brought to 50°C until the poly-L-lysine was completely dissolved and then lowered by placing the container in ice. Once the poly-L-lysine solution was chilled, 100 μl of water containing 100 μCi of [^{14}C]-formaldehyde (57 μCi/μmol) was added. The tube was kept on ice and shaken every 5—10 min for a total of 45 min, at which time 5 mg of sodium borohydride was added. After 1 h the solution was transferred to dialysis tubing and dialyzed against 3 changes of 6 l of 0.2 M NaCl for 24 h, and then against one change of 0.2 M NaCl in 0.1 N HCl for 24 h, and finally 16 h against water. The radioactive poly-L-lysine was removed from the dialysis tubing and centrifuged at 30000 $\times g$ for 1 h. The supernatant contained about 90—95% of the starting poly-L-lysine. One in 2500 ϵ-amino groups were labeled with [^{14}C]methyl groups. The procedure was 70% efficient with regard to [^{14}C]formaldehyde incorporation.

Dye adsorption to glass. Methyl red (*O*-(*p*-(dimethylaminophenyl azo)-benzoic acid) was mixed with anhydrous toluene (dried with $CaSO_4$) and stirred for 24 h at room temperature. Enough methyl red dissolved to make the solution 10^{-4} M, as determined spectrophotometrically using an observed molar extenction coefficient of $2.85 \cdot 10^4$ M^{-1} at 480 nm. In initial experiments, this extinction coefficient was determined by measuring the absorbance of a methyl

red toluene solution, then evaporating the toluene and determining the amount of methyl red colorimetrically after taking it up in 1 M HCl in 95% ethanol. Methyl red is freely soluble in acidic ethanol, and acidic ethanol solutions made up gravinometrically were used as standards.

The amount of dye adsorbed by glass was routinely determined as follows: 0.5 g of glass beads previously dried overnight at 110°C were added to 2 ml of the methyl red toluene solution in a 150 × 15 mm test tube. The tube was shaken on a rotary shaker for 1 h at room temperature before the absorbance of the supernatant was determined.

Attachment of red blood cell membranes to beads. Human erythrocytes from outdated bank blood were washed twice in 10 volumes of phosphate buffered saline, twice in 310 mosM phosphate buffer, pH 7.5, and twice in a solution containing 7 parts 310 mosM sucrose, and 3 parts 310 mosM sodium phosphate, pH 7.5. The cells were packed by centrifugation for 5 min at 3000 × *g* and 0.5 ml of the packed, washed red blood cells were added to 0.5 ml of buffer containing 0.5 g of beads which had also been washed twice in the sucrose/phosphate solution. For some experiments, packed erythrocyte ghosts prepared by standard methods [13] were used instead of intact erythrocytes. In this case, the packed ghosts, in 20 mosM phosphate buffer, pH 7.5, were added to the beads which had also been washed in the 20 mosM phosphate buffer. The suspension was very gently mixed by slowly rotating the containing tube. After 10 min, excess erythrocytes (or ghosts) were gently washed away from the beads using the sucrose/phosphate solution. After sufficient washing to free the solution above the beads of all unbound erythrocytes, 10 ml of 20 mosM phosphate buffer, pH 7.5, was added and the mixture sonicated 10 s with a Heat Systems sonicator set at 20 W. The material released from the beads was washed away in 2 14 ml washes with the 20 mosM phosphate buffer. The amount of membrane lipid that remained on the beads was determined after extraction first in 5 ml and then in 2 ml of chloroform/methanol/HCl (2 : 1 : 0.012, v/v). The combined extracts were mixed with 0.25 volumes of 9 g/l NaCl. After phase separation, the lower organic phase was mixed with 1/4 its volume of water and, again after phase separation, the lower phase containing the phospholipids was transferred to a new 13 × 100 ml test tube. After evaporating the solvent with a stream of dry N_2 while on a heating block at 60—80°C, the residue was analyzed for phosphate [14]. Protein on the beads was determined after it was extracted in 30 g/l sodium dodecyl sulfate. An aliquot of the extract was added to 1 ml of 20 g/l Na_2CO_3 in 0.1 M NaOH followed by the addition of 0.4 ml of 1 M Folin-phenol Reagent (Fisher Chemicals). After 30 min, the absorbance of the solution was determined at 750 nm and compared with a standard curve made from serum albumin. Polylysine does not produce a colored product in the presence of Folin-phenol.

Results

Bead surface area. To calculate whether or not the beads were completely covered by a monolayer of aminopropyl groups or by adherent plasma membrane, we had to determine the bead surface area. Two methods were used. In the first, surface area was calculated from bead diameters measured by light

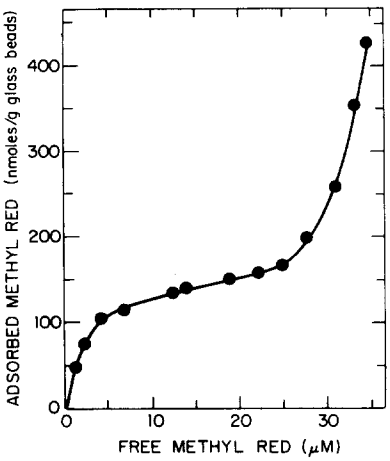


Fig. 1. Adsorption of methyl red by glass beads. Various quantities of beads were added to 2 ml of 10^{-4} M methyl red in a toluene solution. The amount of free methyl red remaining in the solution after incubation for 24 h at room temperature was determined spectrophotometrically. The amount adsorbed to the bead was calculated from the difference between the initial and post-incubation methyl red concentration. The equation for the curve best fits a function for a saturable adsorption isotherm plus an exponential component and is: $D_A = (D_M)\ (D)/2.5 + D + e^{0.15\ (D)}$; where D_A is the observed amount of adsorbed dye in nmol/g glass bead and D is the free dye concentration in μmol/l. The amount adsorbed as a monolayer is D_M in nmol/g. That D_M represents a continuous monolayer is based upon the rationale reviewed by Unger [15].

microscopy. Surface area averaged 840 $cm^2 \cdot g^{-1}$ depending upon how the glass was washed and from which manufacturer's lot number it originated. The second method used dye adsorption measurements. Dye adsorption is a more complex method for determining surface area but gives more information regarding submicroscopic surface topography [15]. The dye methyl red was chosen for our purposes.

Dye adsorption measurements from a toluene solution produced a sigmoidal isotherm (Fig. 1) characteristic of adsorption of water [16], ammonia [17] and N_2 [15]. It is generally agreed that the initial rectangular hyperbolic portion of the isotherm represents the formation of a monomolecular layer [15,17]. Rearranging the equation in the legend of Fig. 1 and incorporating the information that one molecule of methyl red occupies 116 $Å^2$ [16] gives the following formula for calculating the surface area for solid glass beads: $S = (D + 2.5)\ (D_A - e^{0.15\ D})\ (6.98/D)$; where S is the surface area in cm^2/g glass bead, D_A is the observed amount of adsorbed dye in nmol/g bead and D is the free dye concentration in μmol/l.

The surface area calculated from dye adsorption averaged 950 $cm^2 \cdot g^{-1}$ with the average ranging from 900 to 1200 $cm^2 \cdot g^{-1}$ depending on which lot number was used. The adsorption isotherm for methyl red (Fig. 1) was similar to that for N_2 adsorbed to a glass surface with minute pores [15] and was consistent with the notion that while the surface of our glass beads appears perfectly smooth in the light microscope, it must be interrupted by many pits of nanometer size. Hence, small molecules such as those used for chemical modification of the bead would see the surface area measured by dye adsorption

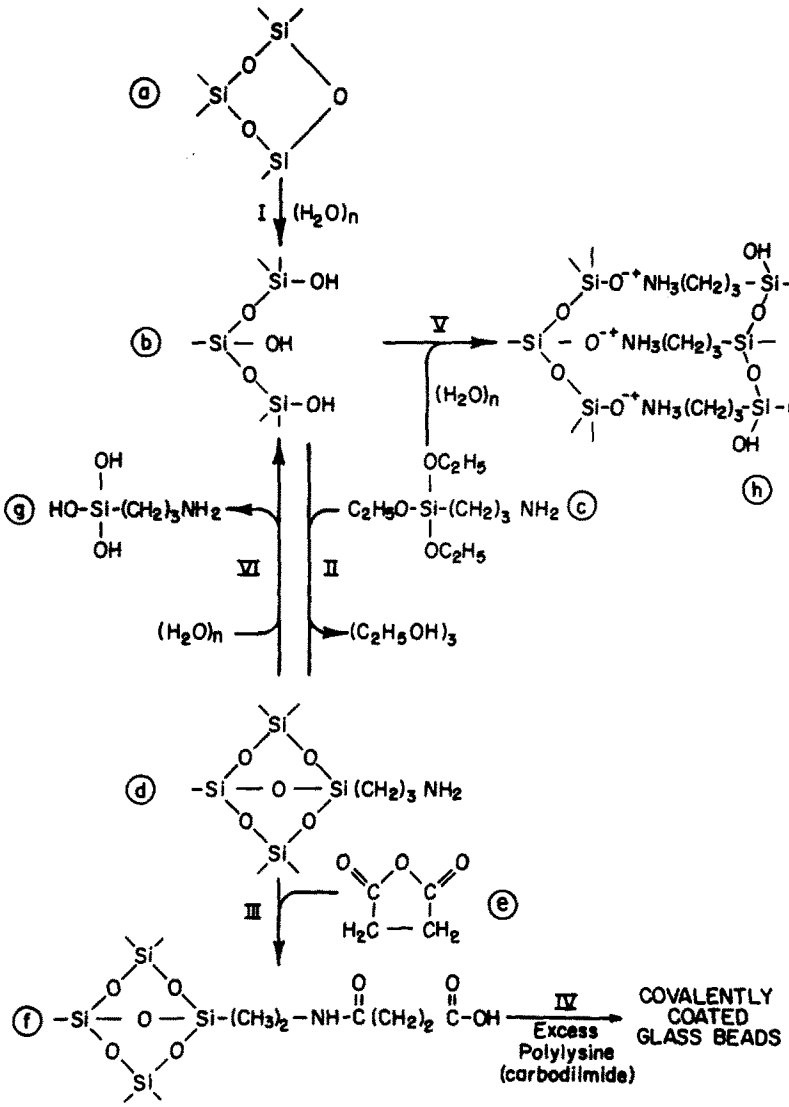


Fig. 2. Chemical reactions involved in the modification of glass beads. The compounds depicted are: (a), $[SiO_2]_n$, surface of native, anhydrous glass beads; (b), silanol groups of hydrated glass beads; (c), 3-aminopropyltriethoxysilane; (d), aminopropylsilane covalently bound to glass by three siloxane bonds; (e), succinic anhydride; (f), succinylaminopropyl-silane bound to glass; (g), aminopropylsilanol; (h), ionic interaction of polymerized aminopropylsilane with the glass surface silanol groups. The pK of the silanol is 6—8 and that for the amino group is 9—10.

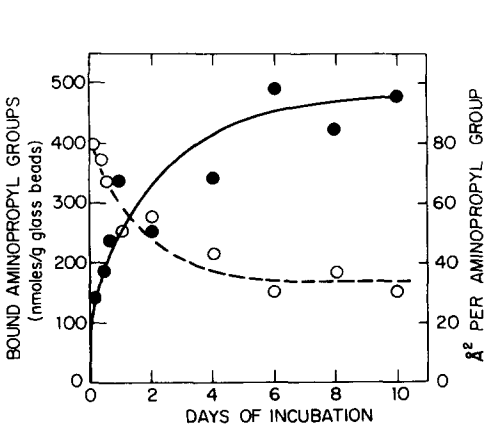
whereas large macromolecular domains of a membrane would be restricted to the surface seen with the light microscope.

Sialanization. We selected 3-aminopropyltriethoxysilane as an inexpensive reagent which could modify the bead to provide a cationic, primary amine group to which membranes would attach directly, or to which other polycationic groups could be covalently linked. The salient reactions (Fig. 2) have been studied with silica gels [18—20]; we have studied the reactions with solid

glass and ascertained which conditions assure optimal membrane adherence.

Beads were hydrated (Fig. 2, I) and surface silanols (Fig. 2, b) formed during the bead-cleaning procedure. However, the beads were treated with aminopropyltriethoxysilane (Fig. 2, II) in anhydrous toluene to minimize unwanted side reactions with water (Fig. 2, V and VI). The time required for completion of the reaction (Fig. 3) was greater than the reaction time used by other investigators [9,19,10,11] who either did not distinguish between loosely- and covalently-bound material or did not demonstrate saturation kinetics. When reacted to completion each aminopropyl group occupied about 32 $Å^2$ (Fig. 3), an area that is in good agreement with the area occupied by other silanizing reagents [15] and consistent with the formation of a monolayer of aminopropylsilane. Scanning electron microscopy confirmed that no aminopropylsilane aggregates were visible on the silanized bead surface and reaction with $N_3B_2SO_3^-$ followed by observation in the light microscope indicated that the beads were uniformly colored.

Two observations indicated that using aqueous solvents or wet organic solvents produced beads that were not covered by a uniform coating of aminopropyl groups. First, the polymerization of the silane reagent was proportional to the water content of the solvent (Fig. 4). Second, when glass beads were silanized with water in the solvent, clumps of material were found adhering to the surface (Fig. 5A). The clumps (and bead-bound amino groups measured by $N_3B_2SO_3^-$) were washed away by incubating the beads for 15—30 min in sodium acetate buffer (Fig. 5B). When excessive water was present (e.g., 0.5% in tolu-



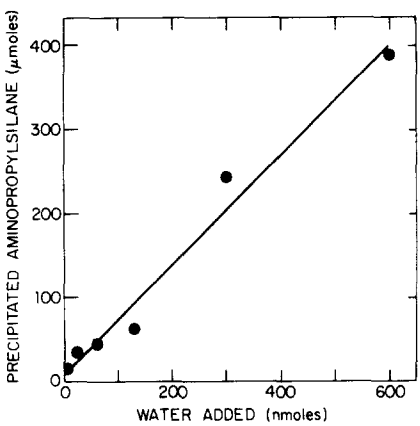


Fig. 3. Time course for the attachment of stably bonded 3-aminopropylsilane to glass beads (solid line). The beads were silanized and the amount of aminopropylsilane on the beads was measured (see Materials and Methods) at various times after the beads were washed once by incubating them for 20 min in 0.1 M sodium acetate buffer, pH 5.0, to remove loosely adsorbed aminopropylsilanol groups. The surface area occupied per aminopropyl group (dashed line) was calculated from the surface area of the beads determined from methyl red adsorption and the amount of amino groups determined as above.

Fig. 4. Effect of water on aminopropyltriethoxysilane. The reaction mixture consisted of 0.5 g aminopropyltriethoxysilane in 10 ml toluene with various amounts of water. The reaction was run in sealed tubes for 6 days at 85—90°C. After cooling, the tubes were centrifuged for 5 min at 3000 rev./min in a table-top clinical centrifuge. The supernatant was decanted and the precipitate washed once with anhydrous toluene. The precipated aminopropylsilane was dissolved by boiling in 0.2 M sodium borate for 10 min and the number of amino groups determined colorimetrically using $N_3B_2SO_3^-$.

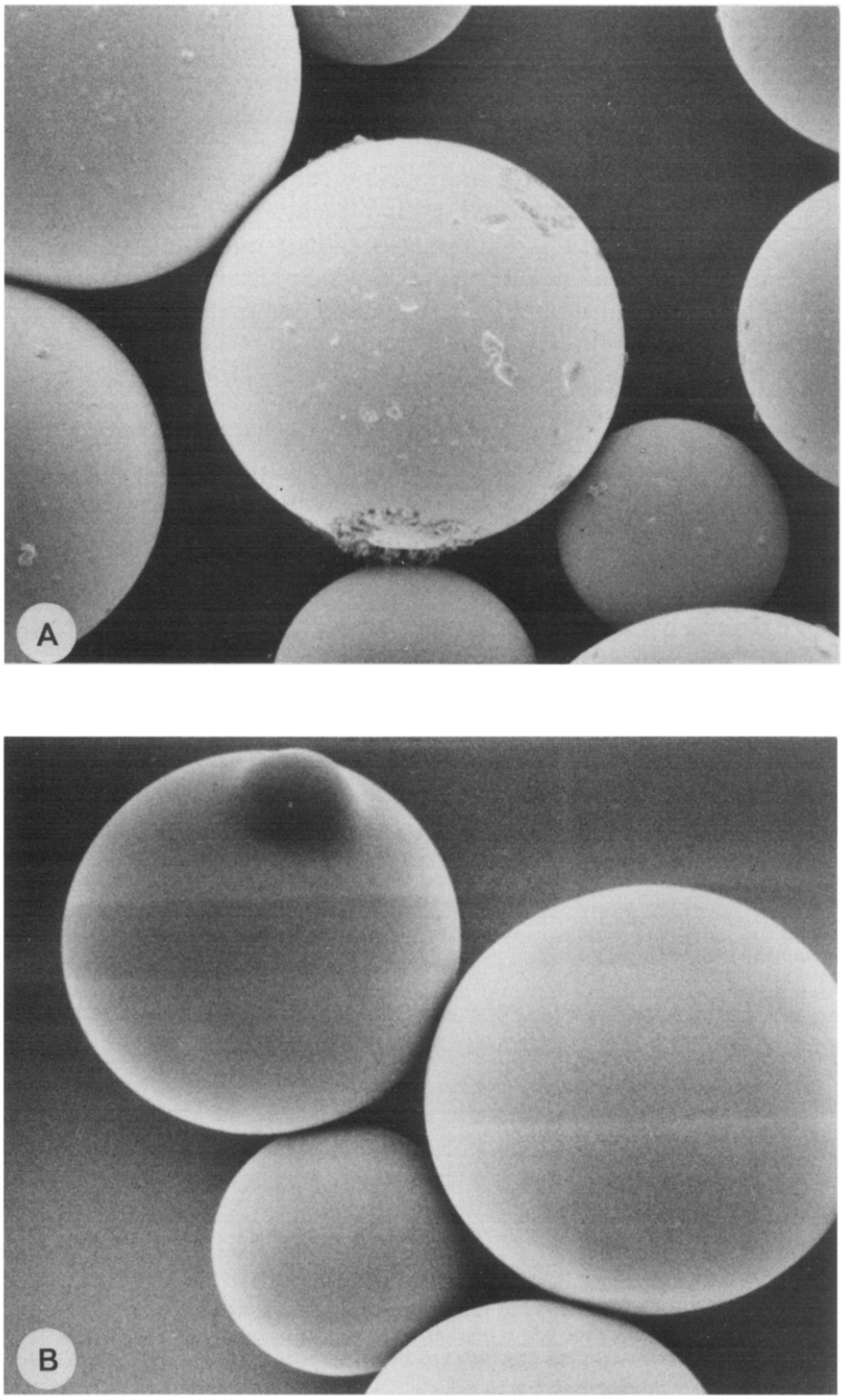


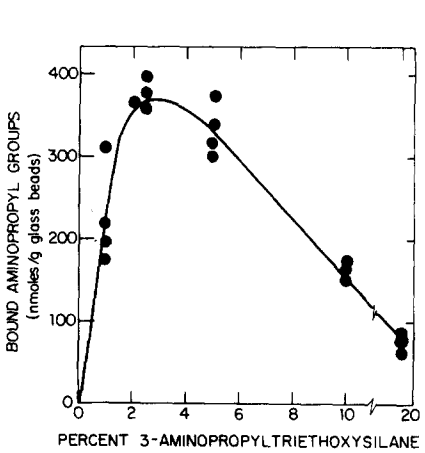
Fig. 5. Scanning electron micrographs of glass beads reacted for 6 days at 85—90°C with 3-aminopropyltriethoxysilane (50 g/l) in toluene with 0.1% water. The beads were coated with gold-paladium before examination. (A), Beads not washed after silanization; (B), washed by incubation for 20 min in ten times their weight of 0.1 M sodium acetate, pH 5.0. Both X 2000.

ene) some of the clumps of aminopropylsilanol could not be completely solubilized. Strictly anhydrous solvents and reagents were therefore always used for silanization. (Even unopened bottles of aminopropyltriethoxysilane sometimes produced clumps. Water may not have been rigorously excluded during their packaging.)

As anticipated, the concentration of aminopropyltriethoxysilane exhibited an optimum (Fig. 6) since one aminopropyl group attaches by 3 siloxane (Si-O-Si) bonds. Thus, high concentrations would favor either one and two point attachments that are easily broken during washing, or adsorption of amino groups to the ionizable silanol groups (Fig. 2, V) would compete with the more stable siloxane bond formation. The high concentrations (10%) of silane reagent previously used with porous glass [11] are clearly supra-optimal with solid beads.

Just as the anhydride siloxane bonds of the native glass bead can be hydrolyzed to the corresponding silanols (Fig. 2, I; ref. 17) so too can the siloxane bonds holding the aminopropyl groups be hydrolized with concomitant loss of positively charged aminopropyl groups (Fig. 2, VI). The measured half-time for loss of the presumed monolayer of animopropyl groups (Fig. 7) was approximately 12 h. Aminopropyl triethoxysilane attached at suboptimal concentrations (Fig. 6) were labile. The only way we have found to reduce the lability of the aminopropylsiloxane on the glass is by subsequent attachment of other groups such as succinate or polylysine.

Succinylation. Because the aminopropyl groups were unstable in water, the beads were succinylated (Fig. 2, III) while suspended in acetone (Fig. 8). At 40 g/l succinic anhydride the reaction was complete in 2 h and the number of



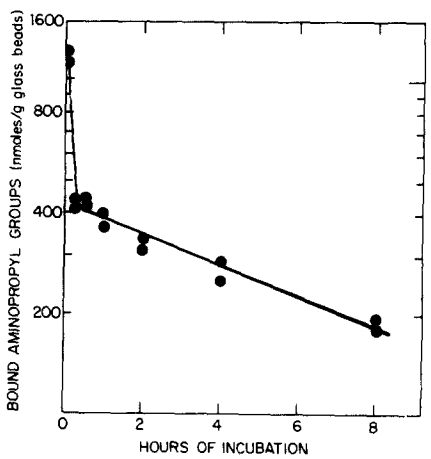


Fig. 6. Aminopropyl groups on glass beads silanized in various concentrations of 3-aminopropyltriethoxysilane in anhydrous toluene. The reaction time was 6 days at 85–90°C. The bound aminopropyl groups were measured (see Materials and Methods) after the beads had been incubated 20 min in ten times their weight of sodium acetate buffer, pH 5.0.

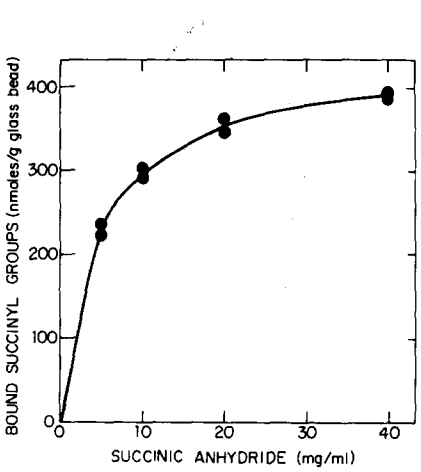
Fig. 7. Loss of aminopropyl groups from silanized glass beads during incubation in 0.1 M sodium acetate buffer, pH 5.0. Beads were incubated for various times in ten times their weight of buffer at room temperature. The aminopropyl groups remaining on the beads were determined (see Materials and Methods) after removing the buffer. The initial rapid loss is interpreted as desorption of noncovalently attached aminopropyltriethoxysilane.

succinate groups bound was approximately equal to the number of aminopropyl groups initially present.

The stability of the succinylated beads was tested in aqueous media. Loss of succinate groups was biphasic with a half time of 4.4 days for 25% of the succinate and 36 days for the remaining 75%. The important conclusion that emerged from these experiments is that the aminopropyl group was far more stable after succinylation than before succinylation.

Attachment of polylysine. Polylysine was covalently coupled to the succinylaminopropyl glass using 1-ethyl-3(3-dimethylaminopropyl)carbodiimide-HCl to activate the formation of an amide bond between the carboxyl groups on the glass beads and the amino groups of polylysine (Fig. 2, IV). The reaction was concentration dependent (Fig. 9, solid line) and critically dependent on the order of reagent addition. Because the activated *O*-acylisourea formed between succinate and a carbodiimide can rearrange to the stable *N*-acylisourea [21] and because the positively charged 1-ethyl-3(3-dimethylaminopropyl)carbodiimide-HCl can interfere with adsorption of polylysine, the succinylated beads were always preincubated with the polylysine before carbodiimide addition. Substantially less polylysine was bound when the carbodiimide was added first. The reaction was essentially complete in 1 h; only 3% more polylysine bound in the next 23 h.

The half-life of covalently-attached polylysine was indeterminately long; no polylysine loss was detected after incubating the beads for 60 days in 0.15 M phosphate buffer, pH 7.5, containing 0.02% NaN_3. The covalently bonded polylysine was also stable in 10% sodium dodecyl sulphate, 10% dodecyl tri-



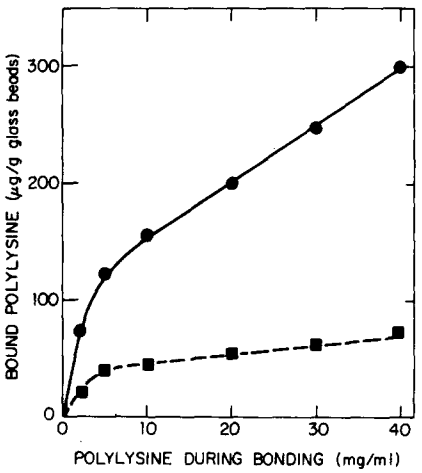


Fig. 8. Attachment of succinyl groups to aminopropyl glass beads as a function of succinic anhydride concentration. The beads were suspended in an actetone solution containing 5% pyridine with various concentrations of succinic anhydride labelled with 1,4[^{14}C]succinic anhydride and incubated for 4 h at 50—55°C.

Fig. 9. Attachment of polylysine to succinylaminopropyl glass beads. The reaction mixture consisted of 0.5 g of succinylaminopropyl glass beads in 0.4 ml buffer, with various concentrations of [^{14}C]polylysine, 70 000 daltons, either with (solid line) or without (dashed line) carbodiimide (see Materials and Methods). The reaction was terminated after 2 h by repeatedly washing the beads in 4 M NH_4Cl.

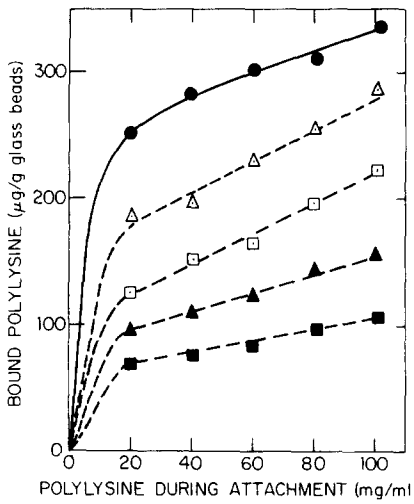
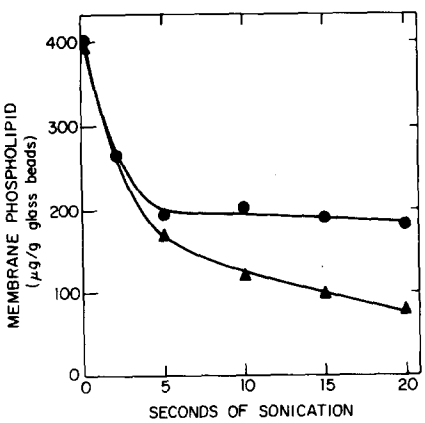


Fig. 10. The amount of polylysine on beads. Polylysine was either covalently bonded to succinylaminopropyl glass beads (solid circles and line) or adsorbed to washed beads that had not been silanized (dashed lines). For covalent bonding and washing conditions see Fig. 9 and Materials and Methods. Excess polylysine was removed from the beads with adsorbed polylysine by washing them once in 7 mM phosphate buffer followed by 5 H_2O rinses (open triangles and squares) or once in 4 M NH_4Cl followed by 5 H_2O rinses (solid triangles and squares) either after (triangles) or before (squares) drying down the beads by lyophilization. For adsorption, 0.5 g of washed beads were incubated in 0.4 ml of water containing the stated concentrations of 150000 dalton [^{14}C]polylysine. The amount of adsorbed polylysine remaining after buffer washing varied greatly from batch to batch; the amount remaining after NH_4Cl washing was more consistent.

methyl amonium bromide, 8 M urea, 6 M guanidine-HCl and acidic $CHCl_3$: CH_3OH (2 : 1).

Polylysine adsorption. Surfaces with adsorbed rather than covalently bonded polylysine have been used to retain cells [4,22]. Adsorbing polylysine to glass requires less work than covalently bonding the polylysine to glass, but, for any given concentration of polylysine used, the amount adsorbed was always less than the amount that could be covalently bonded and generally about 50% of that which was adsorbed was relesed by 4 M NH_4Cl treatment (Fig. 10).

Membrane attachment. To evaluate and compare plain beads with adsorbed polylysine and aminopropyl beads with and without covalently bound polylysine, adherence and retention of human erythrocyte membranes was measured by assaying phospholipid and protein on the beads. The best surface on which to harvest the erythrocyte membranes was provided by the aminopropyl beads with covalently bound polylysine. In comparison to others, these beads were more stable during sonication (Fig. 11) and retained more membrane phospholipid (Fig. 12). Furthermore, beads with covalently bound polylysine retained membranes whose phospholipid to protein ratio more closely resembled that in the native membrane than did beads with adsorbed polylysine (Table I). In fact, only beads with large amounts of covalently bound polylysine (i.e. beads prepared in the presence of 100 mg/ml polylysine) retained membranes whose phospholipid : protein ratios approached that in the native membrane (Table I), whereas on other beads substantial amounts of membrane protein are lose during the sonication and washing steps. Thus, retention of an



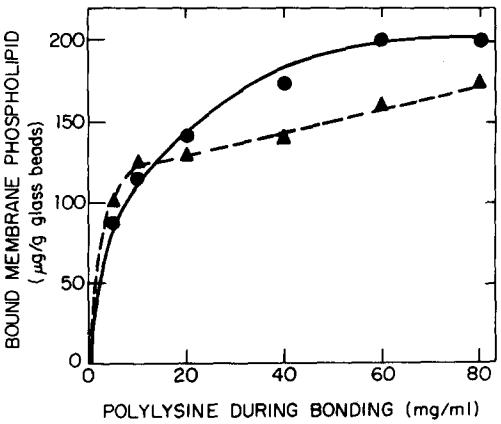


Fig. 11. Adherence of the erythrocyte plasma membrane to positively-charged glass beads. Erythrocyte ghosts were attached to either aminopropyl glass beads (triangles) or to beads with covalently bound polylysine (circles). After excess ghosts were removed, the beads with attached ghosts were sonicated for various times, rinsed twice in buffer, and the amount of phospholipid that remained on the beads was determined. Assuming complete coverage by membrane and knowing the lipid content of an erythrocyte [13], maximum lipid was calculated to be 220 μg for the particular batch of beads used. The initial rapid loss of phospholipid is interpreted as rupture and loss to the supernatant of regions of ghost membranes not in immediate contact with the bead surface.

Fig. 12. Attachment of erythrocyte membranes to glass beads. Intact erythrocytes were added to either beads coated with covalently-bonded polylysine (circles) or washed unsilanized beads coated with adsorbed polylysine (triangles). Both types of bead had been washed with 4 M NH_4Cl after the polylysine had been attached and the beads had been dried. Membrane attachment was monitored by measuring phospholipid after sonication and washing as described in Materials and Methods.

intact membrane with all of its components requires that careful attention be paid to the preparation of the bead surface. In general, for a variety of membranes, we have found that retention of membrane components is maximized by applying the polylysine using the more concentrated solutions. Even when

TABLE I

PHOSPHOLIPID : PROTEIN RATIO OF ERYTHROCYTE MEMBRANES ATTACHED TO BEADS COATED WITH EITHER ADSORBED OR COVALENTLY-BONDED POLYLYSINE

Human erythrocytes were added to the beads and the attachment monitored by measuring phospholipid and protein after sonication and washing as decribed in Materials and Methods. Polylysine was adsorbed to beads by incubating 20 g of washed beads in 10 ml of a polylysine solution at the concentrations stated. These beads were used after removing excess polylysine and after removing loosely-bound polylysine by washing once in 80 ml of 4 M NH_4Cl and 5 times in 80 ml H_2O. Polylysine was covalently bonded to the beads as described in Materials and Methods. The values shown are the average of 3 determinations with standard deviations no greater than 10%.

Polylysine concentration used during attachment (mg/ml)	Phospholipid: Protein on beads (g/g) *	
	Adsorbed polylysine	Covalently bound polylysine
25	1.70	1.40
50	1.60	0.82
100	1.06	0.68

* The phospholipid : protein ratio of control erythrocyte ghosts reduced by hemolysis [13] and not placed on beads ranged from 0.5 to 0.6.

TABLE II

MEMBRANE ATTACHMENT TO BEADS COATED WITH EITHER ABSORBED OR COVALENTLY-BONDED POLYLYSINE

Erythrocytes were added to the beads and lysed in 10 mM Tris · Cl. The beads with attached membranes were then either sonicated for 8 s with the sonicator set at 20 W or subject to vigorous swirling on a small table-top vortexer for 8 s. Attachment of membrane was monitored by measuring phopholipid. Phospholipid values shown are the average of 3 determinations + the standard deviation.

	Phospholipid (μg/g of beads)			
	Covalent polylysine *		Adsorbed polylysine **	
	Sonicated	Vortexed	Sonicated	Vortexed
Water-washed beads	105 ± 31	221 + 12	171 ± 35	210 ± 28
NH_4Cl-washed beads	202 ± 16	253 ± 10	122 ± 15	132 ± 20

* Polylysine was covalently bonded to these beads as described in Materials and Methods.

** Polylysine was adsorbed to these beads by incubating 20 g of washed beads in 10 ml of a 80 mg/ml solution of polylysine. These beads were used either after washing off excess polylysine in water or after washing off excess and loosely bound polylysine in 4 M NH_4Cl as described in Table I.

adherent cells were gently disrupted by vortexing instead of sonication, beads with covalently bound polylysine generally retained more membrane than those with adsorbed polylysine (Table II).

In addition, we tried a number of other bead coatings including adsorbed protamine sulfate, protamine chloride and aluminum clad beads (Table III). None retained red cell membranes as well as glass beads with covalently linked polylysine even when vortexing was used to disrupt the cells instead of sonication (Table II).

Discussion

Our results demonstrate that glass beads can be coated with a stable, covalently-bound layer of polylysine to which cells and their membranes stick tena-

TABLE III

MEMBRANE ISOLATION ON DIFFERENT TYPES OF POSITIVELY-CHARGED GLASS BEADS

Aminopropyl and polylysine glass beads were prepared by covalent bonding as described in Materials and Methods. Protamine was adsorbed to the glass by incubating 10 g of beads in 5 ml of solution containing 500 mg of either protamine/SO_4 or protamine/Cl. Excess solution was removed and the beads were lyophilized. Beads were clad with alumina by incubating 10 g of glass beads in a 50% solution of aluminum hydroxycholoride. All bead types were rinsed 2 times in the cell attachment buffer before (see Materials and Methods) being used. Cells were attached and vortexed as described in Table II.

Bead type	Phospholipid (μg/g bead)	
	pH 5.0	pH 7.5
Aminopropyl-	196 ± 14	187 ± 15
Poylysyl-	235 ± 10	242 ± 12
Protamine/Cl	176 ± 22	51 ± 26
Protamine/SO_4	183 ± 17	84 ± 20
Alumina	186 ± 18	—

ciously. Although the aminopropyl and succinylated aminopropyl glass were not fully stable in aqueous conditions, the polylysine bound to the succinyl-aminopropyl glass was stable both in water and in a variety of protein denaturing reagents. Because the carbodiimide reaction used can link one long polylysine molecule to several carboxyl groups, it is probable that multiply-linked polylysine molecules formed an interlocking meshwork. The existence of such a meshwork would stabilize the entire complex of bead surface components and readily accounts for the indeterminately long staying time of polylysine on the beads.

Although surfaces with adsorbed polylysine have been used for qualitative observations of immobilized cells and their components [1—4,22] maximal adherence and quantitative recovery of membrane components on beads requires a stable, covalently-bound layer of polylysine. Firm attachment will be particularly important for experiments such as those involving asymmetric labelling [8] where it is essential that all loose pieces and edges of membranes be removed by sonication.

Polylysine-coated glass beads are somewhat harder to prepare than similarly coated polyacrylamide beads [6] but they do have advantages which may be crucial for certain kinds of experiments. Because they are solid, they will not include small molecules in a porous space such as would sepharose or polyacrylamide beads. Furthermore, freeze-fracturing of lipid bilayers and membranes on glass slides has been demonstrated [23—25] and preliminary experiments suggest that fracturing can also be accomplished on glass beads. We are exploring the possibility of using beads to collect and purify large amounts of half-membrane.

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