Simple Centrifugation Method for Efficient Pelleting of Both Small and Large Unilamellar Vesicles That Allows Convenient Measurement of Protein Binding†

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ABSTRACT: Separation of unilamellar model membrane vesicles from external solution is often an important step in quantitation of vesicle bound or entrapped materials. An efficient method that allows pelleting of both small and large model membrane vesicles by centrifugation is described in this report. In this method streptavidin is added to vesicles containing a trace amount of biotinylated lipid. The resulting aggregation allows pelleting of the vesicles using an ordinary high-speed centrifuge. Control experiments show that the addition of streptavidin does not induce substantial vesicle fusion or leakage of substances trapped in the internal aqueous compartment of the vesicles. The method can accommodate different phospholipid compositions and lipid concentrations. Experiments with proteins that switch between hydrophilic and hydrophobic states show that the method can readily be used to monitor protein binding to vesicles.

Unilamellar model membrane vesicles are an important tool in the study of membrane proteins. Often it is desirable to assess the amount of a protein that is vesicle bound or the amount of a substance that has been transported into or out of the vesicle. Such experiments require separation of vesicles and the molecules associated with them from the surrounding solution. Centrifugation can be a powerful technique for achieving this separation. For example, the ability to make use of centrifugation and pelleting of Triton X-114 has proven to be a major advance in the study of protein–detergent interactions (Bordier, 1981).

Use of centrifugation and pelleting in connection with model membrane vesicles has proven to be more problematical than with Triton X-114. Multilamellar vesicles can be readily pelleted but make poor model membranes for the vast majority of applications. Pelleting unilamellar vesicles is difficult because of their size and low density. A density marker can be introduced into *large* unilamellar vesicles (LUV)[1] to increase the ease of pelleting (Dawidowitz & Rothman, 1976), but this involves a major change either in the composition of the bilayer or the internal content of the vesicles and requires ultracentrifugation. In the case of small unilamellar vesicles (SUV), even ultracentrifugation has been insufficient to achieve pelleting (Barenholz et al., 1977).

In this paper, we describe a convenient and efficient procedure that allows pelleting of *both* small and large unilamellar vesicles by ordinary high speed centrifugation at 10000*g*. The method takes advantage of the tenacity and tolerance of biotin–streptavidin interactions. This interaction is both temperature stable and pH independent over a broad range (Green, 1990). It can be used as a means to cross-link vesicles into large lipid/streptavidin conglomerates which can be readily removed by centrifugation. This approach can be used in a wide variety of situations and could prove valuable in many procedures calling for isolation of model membrane vesicles.

EXPERIMENTAL PROCEDURES

Materials. DOPC, DOPG, BrPC, and NBD-PE were obtained from Avanti Polar Lipids (Pelham, AL). Rhodamine-DHPE, pyrene-DHPE, pyrene-PC, biotin-X-DHPE, biotin-DHPE, and streptavidin were obtained from Molecular Probes (Eugene, OR). The streptavidin was rehydrated to give (based on manufacturers data) a 5 mg/mL solution in 10 mM phosphate and 150 mM NaCl, pH 7.2–7.4. SDS–PAGE gels, PhastSystem supplies, Q-Sepharose Fast Flow, Sephacryl S-200 and Sepharose CL-4B, and phenyl-Sepharose were obtained from Pharmacia/LKB. FITC-dextran (average molecular mass, 4.4 kDa) and octyl glucoside were obtained from Sigma Chemical. Matrex Gel Green A and PM10 ultrafiltration membranes were obtained from Amicon. [^{3}H]-Lysine was purchased from Amersham.

Lipid purity was confirmed by TLC on Adsorbosil H silica gel plates (Alltech Associates, Deerfield, IL) using a solvent system of 65:25:4 (v/v) $CHCl_3$/methanol/water. Upon detection by charring with H_2SO_4 the DOPC, DOPG, NBD-PE, and rhodamine-DHPE were found to be virtually pure. Charring revealed small amounts of impurities in the biotinylated lipids, probably under 10%. The exact concentrations of the phospholipids were measured by phosphate analysis (Bartlett, 1959; Morrison, 1964). In some cases significant differences were found between the concentrations reported by the manufacturer and those determined by phosphate analysis. The phosphate corrected values were used.

Cytochrome b_5 (218 μM in 10 mM Tris-acetate, 1 mM EDTA, pH 8.1) was a gift of Dr. Peter Holloway (University of Virginia). It showed only one band on a 10–15% SDS–PAGE after Coomassie blue staining.

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[1] Abbreviations: A_pU_p, adenylyl(3′–5′)uridine 3′-phosphate; biotin-DHPE, *N*-(biotinoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; biotin-X-DHPE, *N*-(6-(biotinoyl)aminohexanoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; BrPC, 1,2-di(9,10-dibromostearoyl)-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; FITC-dextran, fluorescein isothiocyanate dextran; pyrene-DHPE, *N*-(1-pyrenesulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; LUV, large unilamellar vesicle; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; PAGE, polyacrylamide gel electrophoresis; pyrene-PC, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine; rhodamine-DHPE, *N*-(lissamine Rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; SUV, small unilamellar vesicle.

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Purification of Diphtheria Toxin. Partially purified diphtheria toxin was obtained from Connaught Laboratories (Ontario, Canada). The toxin (100 000 Lf) was thawed in a refrigerator and phenylmethanesulfonyl fluoride (Sigma) was added to a concentration of 0.7 mM. The sample was dialyzed overnight against 10 mM potassium phosphate, 1 mM EDTA, and 0.02% NaN_3 (w/v), pH 7.0, at 4 °C using a 6–8-kDa molecular mass cutoff dialysis membrane (Spectrapor). The sample was then applied to a Q-Sepharose Fast Flow column (2.5 × 25 cm) equilibrated in 10 mM potassium phosphate, 1 mM EDTA, and 0.02% NaN_3, pH 7.0. The protein was eluted using a linear gradient from 10 mM potassium phosphate, 1 mM EDTA, and 0.02% NaN_3, pH 7, to 200 mM potassium phosphate, 1 mM EDTA, and 0.02% NaN_3, pH 7 using a total volume of 1.5 L of buffer. Two major peaks were eluted from the column. The first peak corresponded to monomeric diphtheria toxin as judged by native gel electrophoresis on a 8–25% gradient gel. The pooled fractions from this peak were concentrated by ultrafiltration using a PM10 membrane in an Amicon stirred cell concentrator and dialyzed against 10 mM Tris, 1 mM EDTA, and 0.02% NaN_3, pH 7.0. The samples were applied to a Matrex Green A column (1.5 × 35 cm) equilibrated in 10 mM Tris, 1 mM EDTA, and 0.02% NaN_3, pH 7.0. The column was then washed until the absorbance was at the baseline (approximately 1.5 column volumes). The free (i.e., without bound A_pU_p) toxin bound to the column was eluted by a single salt step of 10 mM Tris, 750 mM NaCl, and 0.02% NaN_3, pH 7.0, and then pooled and concentrated by ultrafiltration as described above.

Additional purification was performed by using a phenyl-Sepharose column. The protein was applied to the phenyl-Sepharose column (1 × 20 cm) in 50 mM Tris, 1 mM EDTA, and 0.5 M $(NH_4)_2SO_4$, pH 7.9. The protein was eluted using a descending salt gradient from 50 mM Tris, 1 mM EDTA, and 0.5 M $(NH_4)_2SO_4$, pH 7.9, to 50 mM Tris, 1 mM EDTA, and 0.25 M $(NH_4)_2SO_4$, pH 7.9. All purified samples were dialyzed against 5 mM Tris, 1 mM EDTA, and 0.02% NaN_3, pH 7.0, and stored at 4 °C.

Purification of SecA. SecA was purified from a whole cell lysate of *Escherichia coli* strain BL21(DE3)/pT7-SecA as previously described (Ulbrandt et al., 1992).

Preparation of LUV. LUV were prepared by detergent dialysis (Mimms et al., 1981; Jiang et al., 1991). A 7.75 mg mixture was prepared consisting of (w/w) 68% DOPC, 31% DOPG, 0.06% pyrene-DHPE, 0.48% biotin-DHPE, and 0.22% biotin-X-DHPE (0.7% total biotinylated PE) dissolved in $CHCl_3$. The mixture was dried under a stream of N_2 and then further dried under high vacuum for 1 h. Next, it was suspended in 0.32 mL of a buffer containing 15 mM Tris-HCl, and 150 mM NaCl pH 7.1 (buffer A). To the lipid mixture 100 μL of 200 mg of octyl glucoside dissolved in 1 mL of buffer A and 20 μL of 5 mg/mL FITC-dextran dissolved in buffer A were added. Once this mixture clarified, the solution was placed in Spectrapor 12–14-kDa cutoff dialysis tubing and dialyzed at 4 °C against 1 L of buffer A for 16 h. (There were two buffer changes, with the first after 4 h.) After dialysis, the LUV were separated from the untrapped FITC-dextran using a Sepharose 4B-CL column (1 cm × 26.5 cm) eluted with buffer A. Fractions of 0.54 mL were collected, with dextran (via fluorescein) and lipid (via pyrene-DHPE) elution monitored by fluorescence. Two peaks were seen. The first contained LUV with entrapped FITC-dextran, and the second contained unentrapped dextran. The lipid concentration in peak fractions was determined from comparing fluorescence intensity to that of the stock sample.

LUVs with different lipid compositions were prepared by octyl glucoside dialysis in a similar fashion except the Sepharose 4B-CL chromatography was omitted for samples lacking FITC-dextran.

Pelleting of LUV. LUV containing entrapped FITC-dextran were used to determine pelleting efficiency. The lipid compositions examined were (1) 3:7 DOPG/DOPC (w/w): 68% DOPC, 31% DOPG, 0.06% pyrene-DHPE, and 0.7% total biotinylated PE (with 0.48% biotin-DHPE); (2) DOPC: 99% DOPC, 0.06% pyrene-DHPE, and 0.7% total biotinylated PE (with 0.48% biotin-DHPE); (3) 3:7 DOPG/DOPC without biotin: 69% DOPC, 31% DOPG, and 0.06% pyrene-DHPE. To vesicles that had been diluted with buffer A to the desired concentration, a small aliquot of 5 mg/mL streptavidin was added to give a streptavidin to biotin mole ratio of 1:4.3 and a total volume of 300 μL. (In a control experiment streptavidin was omitted, and an equivalent volume of buffer A was added instead.) The final concentrations of 3:7 DOPG/DOPC LUV were 0.15, 0.76, and 3.0 mg/mL. The final concentrations of the DOPC vesicles were 0.13, 0.67, and 2.7 mg/mL. The samples were allowed to incubate for the desired period of time, and 150-μL aliquots were placed in 500-μL microcentrifuge tubes and centrifuged in a Hermle Z230M table-top microcentrifuge at the high-speed setting (10000*g*) for the desired time using a 220.59 V01 rotor. After centrifugation, the top third of the supernate was diluted 20-fold with buffer A and analyzed by fluorescence for the presence of lipid via pyrene-DHPE and FITC-dextran. In experiments in which the lower part of the supernate was examined we generally found only a minute increase in vesicle concentration.

Coprecipitation of LUV with and without Biotinylated Lipid. To determine whether vesicles without biotinylated lipids would coprecipitate with biotin-labeled vesicles, 15 μL of 12.8 mg/mL 3:7 DOPG/DOPC LUV containing 0.07% pyrene-DHPE and 0.7% total biotinylated PE was mixed with 15 μL of 12.8 mg/mL 3:7 DOPG/DOPC vesicles with 0.53% (w/v) rhodamine-DHPE and diluted to 0.295 mL with buffer A. Then this vesicle mixture was incubated with 4.5 μL of 5 mg/mL streptavidin (giving a 1:3.6 mole ratio of streptavidin to biotin) for 30 min at room temperature and centrifuged for 10 min. An aliquot of the supernatant was removed, diluted 20-fold with buffer A, and analyzed for pyrene-DHPE and rhodamine-DHPE fluorescence.

Preparation of SUV. For pelleting experiments a 6.52 mg 2:8 DOPG/DOPC mixture containing (w/w) 80% DOPC, 19% DOPG, 0.57% biotin-DHPE, 0.26% biotin-X-DHPE, and 0.19% rhodamine-DHPE was prepared. Another 6.57 mg 2:8 DOPG/DOPC mixture containig (w/w) 79% DOPC, 19% DOPG, 1.1% biotin-DHPE, 0.5% biotin-X-DHPE, and 0.19% rhodamine-DHPE was also prepared. After being dried under nitrogen and high vacuum, the lipid mixtures were suspended in 15 mM Tris and 150 mM NaCl, pH 7.0, to a concentration of 13 mg/mL lipid and flushed with argon. SUV were prepared in a bath sonicator (Laboratories Supply Co., Hicksville, NY) until near optical clarity.

For entrapment of [^{3}H]lysine the dried mixture containing 1.6% total biotinylated lipid was suspended in 15 mM Tris-HCl and 150 mM NaCl, pH 7.0, in the presence of 0.1 mCi of [^{3}H]lysine prior to sonication. Removal of untrapped lysine was performed by passing the solution over a Sephacryl S-200 (1 × 18 cm) column equilibrated in 15 mM Tris and 150 mM NaCl, pH 7.0. Radioactivity was quantified by scintillation counting. Lipid elution position was determined by visual inspection of rhodamine fluorescence. The efficiency of

entrapment of [^{3}H]lysine into the SUV was found to be approximately 1% of the total added lysine.

For protein pelleting experiments, a 5 mg 2:8 DOPG/DOPC mixture containing (w/w) 78% DOPC, 20% DOPG, 1% biotin-DHPE, 1% biotin-X-DHPE, and 0.1% pyrene-PC or containing (w/w) 98% DOPC, 1% biotin-DHPE, 1% biotin-X-DHPE, and 0.1% pyrene-PC was prepared. These samples were then dried under nitrogen and high vacuum, suspended to a concentration of 10 mg/mL in water, flushed with argon, and sonicated to near optical clarity.

Pelleting of SUV. Ten microliters of the 2:8 DOPG/DOPC SUV containing either 0.8 or 1.6% biotinylated lipid was added to 90 μL of 15 mM Tris-HCl and 150 mM NaCl, pH 7.0, to yield a solution containing 1.3 mg/mL lipid. To this, varying amounts of 5 mg/mL streptavidin were added. Samples were incubated at room temperature for 30 min prior to centrifugation. Then they were subjected to centrifugation in a Beckman airfuge at 24 psi (110000*g*) for 1 h in an A-100 rotor. Samples containing 1.6% (w/w) biotin lipid were also tested for the ability to pellet at lower centrifuged force using the Hermle microcentrifuge on the high-speed setting (10000*g*) for 20 min. Pelleting efficiency was determined by measuring the rhodamine fluorescence of 25 μL of the top part of the supernate diluted into 1 mL of 15 mM Tris-HCl and 150 mM NaCl, pH 7.0. Percent of vesicles unpelleted was quantitated relative to an uncentrifuged sample which had not been exposed to streptavidin.

Pelleting of SUV with entrapped [^{3}H]lysine was performed to determine the percentage of leakage of SUV upon streptavidin treatment. Pelleting of a solution containing approximately 1.66 mg/mL SUV at a streptavidin to biotin ratio of 1:8 was performed as described above. Chromatography of a vesicle sample neither treated with streptavidin nor subjected to centrifugation was performed in parallel to the pelleting experiment to determine the percent of [^{3}H]lysine that was not initially entrapped. The lipid sample was applied to a Sepharose S-200 (0.7 × 13 cm) equilibrated in 15 mM Tris and 150 mM NaCl, pH 7.0.

Preparation of Vesicles for Fusion Assay. To measure fusion, populations of vesicles were prepared containing both NBD-PE and rhodamine-DHPE in addition to DOPG, DOPC, and biotinylated lipid. For the standard curve of NBD/rhodamine fluorescence vs percent NBD/rhodamine, 3:7 (w/w) DOPG/DOPC LUV were prepared in buffer A as described above containing (assuming no significant concentration changes upon dialysis) 10.5 mg/mL DOPC, 4.7 mg/mL DOPG, 0.074 mg/mL biotin-DHPE, 0.034 mg/mL biotin-X-DHPE, and one of the following combinations of NBD-PE and rhodamine-DHPE: (1) no NBD-PE and no rhodamine-DHPE, (2) 0.025 mg/mL [0.16% (w/w)] NBD-PE and 0.040 mg/mL [0.26% (w/w)] rhodamine-DHPE, (3) 0.052 mg/mL (0.32%) NBD-PE and 0.08 mg/mL (0.51%) rhodamine-DHPE, (4) 0.104 mg/mL (0.64%) NBD-PE and 0.16 (1.0%) rhodamine-DHPE, or (5) 0.21 mg/mL (1.25%) NBD-PE and 0.32 mg/mL (2.0%) rhodamine-PE.

For the standard curve for SUV, 2:8 (w/w) DOPG/DOPC SUV were prepared in buffer A as described above and contained 10.4 mg/mL DOPC, 2.5 mg/mL DOPG, 0.148 mg/mL biotin-DHPE, 0.068 mg/mL biotin-X-DHPE, and one of the following combinations of NBD-PE and rhodamine-DHPE: (1) no NBD-PE and no rhodamine-DHPE, (2) 0.042 mg/mL (0.32%) NBD-PE and 0.066 mg/mL (0.5%) rhodamine-PE and (3) 0.085 mg/mL (0.64%) NBD-PE and 0.13 mg/mL (1.0%) rhodamine-DHPE, or (4) 0.169 mg/mL (1.25%) NBD-PE and 0.27 mg/mL (2.0%) rhodamine-PE.

In the first step for assaying for LUV fusion, initial fluorescence was measured in a 1-mL sample of 2% rhodamine-DHPE LUV diluted with buffer A to a concentration of 0.048 mg/mL total lipid. Energy transfer was evaluated by the enhancement of rhodamine fluorescence relative to NBD fluorescence as given by the ratio of emission at 590 relative to 535 nm, with the excitation set at 460 nm. Then a 19.3-fold excess of 0% rhodamine-DHPE vesicles was added (60 μL of 15.4 mg/mL). Fluorescence was measured every 10 min for 30 min. Next, 16 μL of 5 mg/mL streptavidin was added to give a mole ratio to biotin of 1:5.3. The fluorescence of the samples was measured every 5 min for 40 min after streptavidin addition. A control sample containing only rhodamine-labeled vesicles was prepared similarly except 60 μL of 16 mg/mL 2% rhodamine-DHPE was added instead of the 0% rhodamine-DHPE.

SUV fusion was measured similarly. Initial fluorescence was measured in a 1-mL sample of 2% rhodamine-DHPE SUV diluted with buffer A to a concentration of 0.048 mg/mL total lipid. Then a 19.3-fold excess of 0% rhodamine-DHPE vesicles was added (70.4 μL of 13.1 mg/mL). Fluorescence was then measured every 5 min for 35 min. Next, 23.3 μL of 5 mg/mL streptavidin was added to give a streptavidin to biotin ratio of 1:8. The fluorescence of the samples was measured every 5 min for 50 min following streptavidin addition. A control sample containing only rhodamine-labeled vesicles was prepared similarly except 68.1 μL of 13.6 mg/mL 2% rhodamine-DHPE was added instead of the 0% rhodamine-DHPE.

Fluorescence Measurements. Fluorescence was measured with a Spex 212 Fluorolog spectrofluorometer. Unless otherwise noted, the intensity of 1-mL samples was measured in quartz cuvettes having 1-cm excitation and 0.4-cm emission path lengths. All measurements were made in the ratio mode against the internal standard of rhodamine B to minimize intensity fluctuations. Unless otherwise noted the excitation and emission wavelengths used were 330 nm excitation and 380 nm emission for pyrene, 495 nm excitation and 515 nm emission for fluorescein, and 535 nm (LUV experiments) or 570 nm (SUV experiments) excitation and 590 nm emission for rhodamine.

Diphtheria Toxin Model Membrane Insertion. Five microliters of diphtheria toxin (0.295 mg/mL) was added to a solution containing 10 μL of sonicated SUV [10 mg/mL containing (w/w) 78% DOPC, 20% DOPG, 1% biotin-DHPE, 1% Biotin-X-DHPE, 0.1% pyrene-PC] and 90 μL of each of the following buffers: 10 mM sodium phosphate and 150 mM NaCl, pH 7.0, 10 mM sodium phosphate and 150 mM NaCl, pH 6.0, 10 mM sodium acetate 150 mM NaCl, pH 5.5, 10 mM sodium acetate and 150 mM NaCl, pH 5.0, 10 mM sodium acetate and 150 mM NaCl, pH 4.5, and 10 mM sodium acetate and 150 mM NaCl, pH 4.0. The samples were incubated for 30 min prior to the addition of 2.8 μL of 5 mg/mL streptavidin. Following streptavidin addition, the samples were incubated for a further 30 min. The samples were pelleted at 10000*g* in the microcentrifuge for 20 min. The supernate was withdrawn and assayed for percent pelleting as measured by pyrene fluorescence. The pellets were suspended in 20 μL of nonreducing SDS sample buffer, boiled for 5 min, and separated on a 10–15% SDS–PAGE gel. Control samples containing no toxin showed that the pelleting was not affected by the differences in pH. The samples pelleted to an average of 98% efficiency over the pH range tested. In addition, control samples lacking added streptavidin were tested for pelleting efficiency since low pH induced incor-

poration of diphtheria toxin into the membrane results in some fusion and pelleting without the addition of streptavidin. The pelleting in the absence of streptavidin was dependent on low pH and maximal at pH 4.0, resulting in 58% of the SUV pelleting.

SecA Model Membrane Insertion. Ten microliters of LUV, made by freeze thawing of 10 mg/mL SUV (Kasahara & Hinkle, 1977) and containing either (w/w) 78% DOPC, 20% DOPG, 1% biotin-DHPE, 1% biotin-X-DHPE, 0.1% pyrene-PC or (w/w) 98% DOPC, 1% biotin-DHPE, 1% biotin-X-DHPE, 0.1% pyrene-PC, was mixed with 77 μL of 15 mM Tris-HCl, 150 mM NaCl, and 2 mM DTT, pH 7.2 and then 13 μL of SecA protein (25 μg) dissolved in 15 mM Tris-HCl and 150 mM NaCl, pH 7.2, was added. The SecA was allowed to interact with the vesicles for 30 min at 22 °C prior to the addition of 2.8 μL of 5 mg/mL streptavidin and subsequent incubation at 22 °C for 30 min. The vesicles were then pelleted by centrifugation at 10000*g* at 4 °C. The pellets were suspended in 120 μL of SDS running buffer, and 1 volume of the supernates was added to 0.2 volumes of SDS running buffer for analysis on 8–25% SDS–PAGE. Turbidity increases showed that incorporation of SecA into the lipid membranes resulted in some fusion and/or aggregation of vesicles. Pelleting of 15% of the lipid sample was found under these conditions without the addition of streptavidin.

Cytochrome b_5 Membrane Association. Seven microliters of 218 μM cytochrome b_5 (or in control samples 7 μL of buffer A) and a small aliquot of 13.1 mg/mL SUV composed of 79% BrPC, 19% DOPG, 1.1% biotin-PE, 0.5% biotin-X-PE, and 0.11% rhodamine-DHPE (w/w) in buffer A were diluted with buffer A to 0.6 mL. Final lipid concentrations were 0, 0.1, 0.3, or 0.5 mg/mL. Samples and controls were incubated for 30 min at room temperature, and then protein fluorescence was measured at 280 nm excitation and 330 nm emission, subtracting values for the controls. To a 0.5-mL aliquot of each sample, streptavidin was added to a streptavidin to biotin ratio of 1:8. After a further 30-min incubation, the samples were centrifuged at 10000*g* for 30 min. A 450-μL aliquot of the supernate was diluted to 600 μL with buffer A, and both rhodamine-DHPE and protein fluorescence were measured. Background fluorescence values due to streptavidin and lipid were obtained from the samples lacking cytochrome b_5 and were subtracted. A 400-μL aliquot of each diluted sample was then further diluted to 1 mL and the percent of cytochrome remaining in the supernate was additionally assayed by heme absorbance at 412 nm minus absorbance at 480 nm. In all cases, corrections were made for incomplete pelleting (as judged from rhodamine-DHPE fluorescence).

RESULTS

Effect of Incubation and Centrifugation Time upon LUV Pelleting. In order to define conditions suitable for efficient pelleting, preliminary studies were performed to determine an adequate time of preincubation of LUV with streptavidin. The LUV were composed of 3:7 DOPG/DOPC (w/w) with 0.7% (w/w) biotinylated lipids. Figure 1A shows a plot of percent lipid in the supernate versus the time of vesicle preincubation with streptavidin at a streptavidin to biotin mole ratio of 1:4.3.[2] The amount of lipid in the supernate was measured by analyzing the fluorescence of the trace pyrene-DHPE incorporated into the vesicles. The results show that

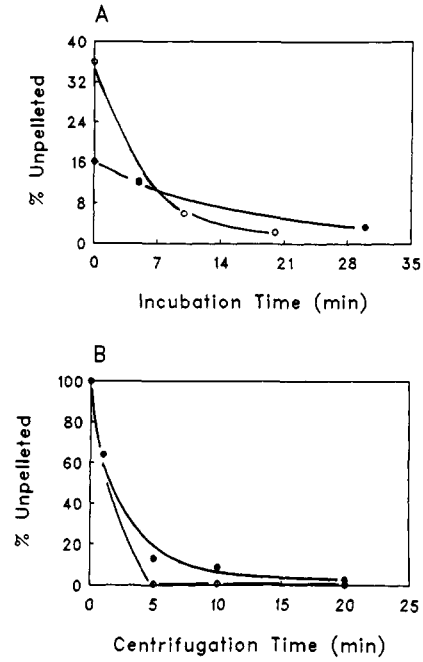

FIGURE 1: Effect of varying incubation and centrifugation times upon vesicle pelleting. (A) Effect of varying time of preincubation with streptavidin on pelleting of 3:7 DOPG/DOPC LUV containing 0.7% biotinylated lipid at total lipid concentrations of 0.15 mg/mL (●) and 0.77 mg/mL (○). The 0.15 mg/mL lipid sample was centrifuged for 25 min. The 0.77 mg/mL was centrifuged for 10 min. Note that even at zero time of preincubation samples aggregated sufficiently to start to pellet. (B) Effect of varying centrifugation time on pelleting for LUV of the same composition as in panel A for total lipid concentrations of 0.15 mg/mL (●) and 0.77 mg/mL (○). Samples in panel B were preincubated with streptavidin for 30 min.

by 10 min there is a sharp decrease in the percent lipid in the supernate for lipid concentrations of both 0.15 and 0.77 mg/mL. However, a 20-min preincubation was required to obtain 97–98% pelleting of lipid. On this basis, an incubation time of 30 min was chosen for subsequent experiments.

The effect of centrifugation time upon pelleting was also examined. Figure 1B shows a plot of percent lipid in the supernatant versus centrifugation time. The streptavidin to biotin ratio was again fixed at 1:4.3. At a lipid concentration of 0.15 mg/mL, a sharp decrease in percent lipid in the supernate was seen by 5 min, but a 20-min centrifugation was necessary to obtian 97% pelleting. At 0.77 mg/mL, pelleting was 99% complete after a 5-min centrifugation. On the basis of these data, subsequent 0.15 mg/mL samples were centrifuged for 30 min, and samples having a lipid concentration of 0.77 mg/mL or higher were centrifuged for 10 min. It is interesting that the rate of pelleting increases at higher lipid concentrations. Presumably, this results from greater vesicle aggregation as lipid concentration increases.

We also examined whether streptavidin-dependent pelleting via high-speed centrifugation at 10000*g* was superior to LUV pelleting using ultracentrifugation at 120000*g*. Ultracentrifugation of 0.77 mg/mL 3:7 DOPG/DOPC LUV was significantly slower than the streptavidin-dependent pelleting, with pelleting being 80% complete after 6 min and 94% complete after 30 min. We estimate that it would take 1 h to obtain complete pelleting, where 5 min is more than sufficient at high speed using streptavidin. At 0.15 mg/mL ordinary ultracentrifugation was even slower, with there being only 66% pelleting at 5 min and 88% pelleting after 30 min.

Efficiency of Pelleting of LUV under Various Conditions. Using the incubation and centrifugation times determined above and a streptavidin to biotin ratio of 1:4.3, the efficiency

[2] This is the total streptavidin to biotin ratio. About half the biotins are likely to be in the inner leaflet of the vesicles and thus be inaccessible to streptavidin.

Table I: Pelleting of LUV by High Speed Centrifugation[a]

	percent in pellet[b]			
	3:7 DOPG/DOPC		DOPC	
[lipid] (mg/mL)	lipid[c] (%)	dextran[d] (%)	lipid[c] (%)	dextran[d] (%)
+SA +biotin				
0.15/0.13[e]	99	94[f]	81	75
0.77/0.67	99	98[f]	99	98
3.10/2.7	99	98[f]	99	97
–SA +biotin				
0.77	6.5	8	ND	ND
+SA –biotin				
0.77	2	3	ND	ND

[a] ND, not determined; SA, streptavidin; biotin, biotinylated lipids. [b] Experimental conditions are described under Experimental Procedures. [c] As judged by pyrene fluorescence. [d] As judged by fluorescein fluorescence. These pyrene and fluorescein values are for the top two-thirds of the supernate. [e] The first concentration is for the DOPG/DOPC vesicles, and the second is for the DOPC vesicles. [f] About 8% of the dextran was not entrapped at the outset of these experiments as judged by rechromatography of vesicles upon Sepharose 4B-CL, and this was subtracted to derive the value of pelleted dextran.

of pelleting was examined in more detail. In order to examine whether vesicle integrity was compromised by streptavidin binding (and/or centrifugation), the release of vesicle-entrapped FITC-dextran (4.4 kDa) was monitored. Table I shows that vesicles comprised of 3:7 DOPG/DOPC and 0.7% biotinylated lipid display about 99% pelleting efficiency at the concentrations studied. For the entrapped FITC-dextran, 94% of the dextran pelleted with 0.15 mg/mL lipid, and 98% dextran pelleted with both 0.77 and 3.1 mg/mL lipid. These results show that it is possible to obtain almost complete vesicle pelleting with very little leakage of entrapped contents.

Controls were performed to determine if both streptavidin and biotin are required for pelleting. In one experiment streptavidin was added to 0.77 mg/mL 3:7 DOPG/DOPC LUV lacking biotinylated lipid. Only 2% of the vesicles pelleted (Table I). Another control involved trying to pellet 0.77 mg/mL 3:7 DOPG/DOPC LUV containing 0.7% biotinylated lipid without adding streptavidin. This resulted in only 6.5% of the vesicles pelleting (Table I). These results show that both biotin and streptavidin are necessary for efficient pelleting.

To see if lipid composition affected pelleting, the behavior of DOPC LUV containing 0.7% (w/w) biotinylated lipid was examined. Table I shows that pelleting of vesicles and entrapped FITC-dextran was efficient at lipid concentrations of 0.67 and 2.7 mg/mL. At a lipid concentration of 0.13 mg/mL about 19% of the vesicles were present in the supernatant. This indicates that DOPC vesicles do not pellet quite as well as the 3:7 DOPG/DOPC LUV at very low concentrations. On the other hand, the dextran results indicate DOPC LUV maintain vesicle integrity as well as the 3:7 DOPG/DOPC LUV.

To determine whether the pelleting of biotinylated vesicles would affect the pelleting of unbiotinylated vesicles, the pelleting of mixed vesicle populations was examined. The pelleting of a 1:1 mixture of 3:7 DOPG/DOPC LUV containing 0.7% biotinylated lipid and a trace amount of pyrene-DHPE mixed with 3:7 DOPG/DOPC LUV containing a trace amount of rhodamine-DHPE but lacking biotinylated lipids was measured. As judged by pyrene fluorescence, 98% of the biotinylated LUVs pelleted, consistent with previous experiments. However, 52% of the LUVs lacking biotinylated lipids also pelleted, as judged by rhodamine fluorescence. It is possible that nonspecific association of vesicles to streptavidin weakly attaches vesicles lacking biotin to the biotinylated

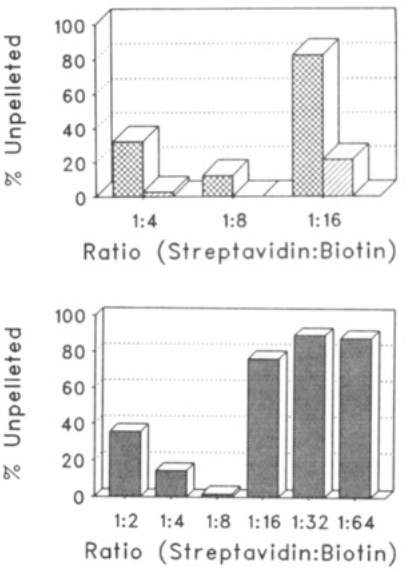

FIGURE 2: Pelleting efficiency of SUV at varying streptavidin and biotin concentrations. (Top) 2:8 DOPG/DOPC SUV (1.3 mg/mL) containing either 0.8% (cross-hatched) or 1.6% (hatched) biotinylated lipids were incubated with varying amounts of streptavidin for 30 min. The samples were pelleted at 110000*g* for 1 h. (Bottom) Pelleting efficiency of SUV at varying streptavidin concentrations using low-speed centrifugation. 2:8 DOPG/DOPC SUV (1.3 mg/mL) containing 1.6% biotinylated lipid were incubated with varying amounts of streptavidin for 30 min. The samples were pelleted at 10000*g* for 20 min.

vesicles. Alternately, the large aggregates formed by the biotinylated vesicles may somehow tend to trap many of the unbiotinylated vesicles.

Efficiency of SUV Pelleting under Various Conditions. Next, the method was extended to small unilamellar vesicles. Because SUV do not ordinarily pellet (Barenholz et al., 1997), ultracentrifugation was used in the first experiments, and both the streptavidin/biotin ratio and biotinylated lipid content were varied to obtain optimal pelleting conditions. Figure 2 (top) illustrates the effect of varying streptavidin concentration on pelleting for 1.3 mg/mL 2:8 DOPG/DOPC (w/w) SUV containing either 0.8% or 1.6% (w/w) biotinylated lipid and a trace amount of rhodamine-DHPE. SUV pelleting shows a sharp concentration dependence with an optimum streptavidin to biotin mole ratio near 1:8.

Visual inspection of the SUV solution following streptavidin addition showed that the SUV containing the greater percentage of biotinylated lipid (1.6%) became more opaque. This suggested that at this higher content of biotinylated lipids the SUV formed larger aggregates and could possibly be removed from the solution by lower centrifugal force than used during ultracentrifugation. Therefore, the SUV containing 1.6% biotinylated lipid were studied for their ability to pellet using ordinary high-speed centrifugation in the presence of varying concentrations of streptavidin. The results are shown in Figure 2 (bottom). Pelleting occurred, although generally slightly less efficiently than pelleting by the longer ultracentrifugation (compare Figure 2 top and bottom). Pelleting was again dependent upon the streptavidin concentration with the greatest pelleting observed at a ratio of 1:8. At this ratio virtually all of the SUV could be pelleted. These results indicate that with a modestly higher concentration of biotinylated lipids SUV can be removed from solution in a short time and at a reasonably low centrifugal force. They also indicate that the optimal ratio of streptavidin to biotin is not strongly dependent on the concentration of biotinylated lipid in the membrane. However, it should be noted that

there was a less pronounced dependence of pelleting upon the streptavidin to biotin ratio and a shift in the optimal ratio at higher lipid concentrations (data not shown).

To determine if the SUV lost their integrity upon pelleting with the streptavidin, the release of SUV-entrapped [^{3}H]lysine was examined using the SUV sample containing 1.6% biotinylated lipid and centrifugation at 10000*g* at a 1:8 streptavidin/biotin ratio. Rhodamine-DHPE fluorescence indicated that the efficiency of vesicle pelleting was greater than 98%. In the same sample about 86% of the [^{3}H]lysine pelleted. However, chromatography of an aliquot of the SUV preparation used in this experiment on a Sephacryl S-200 column in the absence of streptavidin showed that 14% of the lysine present was not SUV-entrapped even prior to streptavidin addition, indicating that almost none of the SUV-entrapped lysine leaked out due to streptavidin binding or centrifugation.

Assay for Vesicle Fusion upon Streptavidin Binding. Another area which was addressed was whether biotinylated vesicles fuse with each other after binding streptavidin. Energy transfer was chosen to monitor bilayer fusion via lipid mixing (Struck et al., 1981). In this experiment, vesicles with a small amount of both NBD and rhodamine-labeled lipids are mixed with unlabeled vesicles. Fusion of the labeled and unlabeled vesicles induces a dilution of labeled lipids within the membrane such that the average NBD to rhodamine distance within a vesicle increases. This increased distance results in decreased NBD to rhodamine energy transfer, and thus an increase in NBD fluorescence at 535 nm and a decrease in rhodamine fluorescence at 590 nm (Struck et al., 1981).[3]

Figure 3 panels A and B show the dependence of the ratio of fluorescence at 590 nm to that at 535 nm as a function of NBD and rhodamine density within the membrane for vesicle composed of 3:7 DOPG/DOPC (w/w) LUV with 0.7% biotinylated lipid, or 2:8 DOPG/DOPC SUV with 1.6% biotinylated lipid, and various amounts of a 1:1.6 (w/w) mixture of NBD-PE/rhodamine-DHPE. As expected, the 590/535 ratio decreases as the content of labeled lipid within the vesicle decreases. The dependence of this ratio upon concentration acts as a standard curve for assessing fusion. One round of fusion between labeled vesicles and excess unlabeled vesicles will dilute the rhodamine and NBD concentration in half, and Figure 3 shows this would result in an easily detected decrease in the 590/535 ratio by about half.

When 3:7 DOPG/DOPC LUV containing 1.25% NBD-PE, 2% rhodamine-DHPE, and 0.7% biotinylated lipid (w/w) were mixed with a 19.3-fold excess of 3:7 DOPG/DOPC vesicles containing 0.7% biotinylated lipid but no NBD or rhodamine lipids, there was no significant effect on the 590/535 ratio over a 30-min period. More importantly, incubation of this mixture with streptavidin at a ratio to biotin of 1:5.3 for 40 min induced no change in the 590/535 ratio (Figure 3A). Therefore, there is no evidence of significant streptavidin-induced fusion on the time scale used in the centrifugation experiments. There was also no change in 590/535 ratio in a control containing only labeled 3:7 DOPG/DOPC LUV with 0.7% biotinylated lipids, 1.25% NBD-PE, and 2% rhodamine-DHPE (w/w) after streptavidin was added to a ratio to biotin of 1:5.3.[4] This shows that streptavidin binding itself has no effect on energy transfer.

[3] NBD and/or rhodamine self-quenching could also affect this ratio at high concentrations.

[4] At this ratio complete pelleting could be obtained (data not shown).

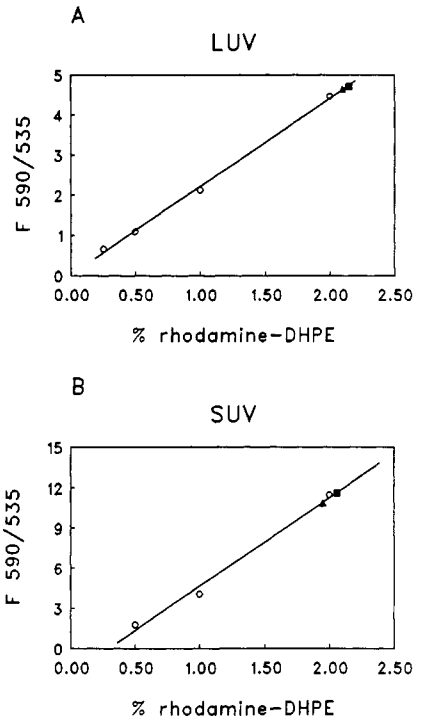

FIGURE 3: Assay for streptavidin-induced vesicle fusion using NBD to rhodamine energy transfer. A standard curve (○) of the ratio of emission intensity at 590 nm relative to 535 nm (F_{590}/F_{535}) vs the percent of NBD and rhodamine lipid content in the vesicles was composed for (A) LUV and (B) SUV. The ratio of NBD-PE to rhodamine-DHPE was 1:1.6 (w/w) in each case. (■) Fluorescence of mixtures of 0% NBD-PE/rhodamine-DHPE samples and 1.25% NBD-PE/2% rhodamine-DHPE samples before addition of streptavidin. (▲) Fluorescence 40–50 min after the addition of streptavidin as described in the text.

When 2:8 DOPG/DOPC SUV containing 1.25% NBD-PE, 2% rhodamine-DHPE, and 1.6% biotinylated lipid (w/w) were mixed with a 19.4-fold excess of 3:7 DOPG/DOPC vesicles containing 1.3% biotinylated lipid but no NBD or rhodamine lipid, there was again no significant effect on the 590/535 ratio over 40 min.[5] Furthermore, incubation of this mixture with streptavidin at a ratio to biotin of 1:8 for 50 min induced only a small decrease in the 590/535 ratio, indicative of at most only a few percent fusion, if significant at all (Figure 3B). Upon addition of streptavidin to a ratio to biotin of 1:8, very little change in 590/535 ratio was found in a control having only 2:8 DOPG/DOPC LUV containing 1.6% biotinylated lipids, 1.25% NBD-PE, and 2% rhodamine-DHPE (w/w), again showing that streptavidin binding itself had no strong effect on energy transfer.

Assessment of Protein-Vesicle Association by the Centrifugation Method. The degree of vesicle association with proteins having well-characterized interactions with membrane bilayers was used to demonstrate that the streptavidin–biotin method could be used to measure protein–vesicle interaction. One protein examined was diphtheria toxin. This protein binds and inserts into lipid bilayers in a low pH dependent manner (Kagen et al., 1981; Donovan et al., 1981; Hu & Holmes, 1984; Zalman & Wisnieski, 1984). To examine vesicle binding, the toxin was incubated at varying pH with 2:8 DOPG/DOPC SUV. Streptavidin was then added, and the

[5] We are not certain why the energy transfer was larger in the case of SUV. It is possible that the distribution of NBD-PE and rhodamine-DHPE between inner and outer leaflet is SUV is different from that in LUV. If they were crowded into the inner or outer leaflets instead of being evenly distributed, it should cause increased energy transfer.

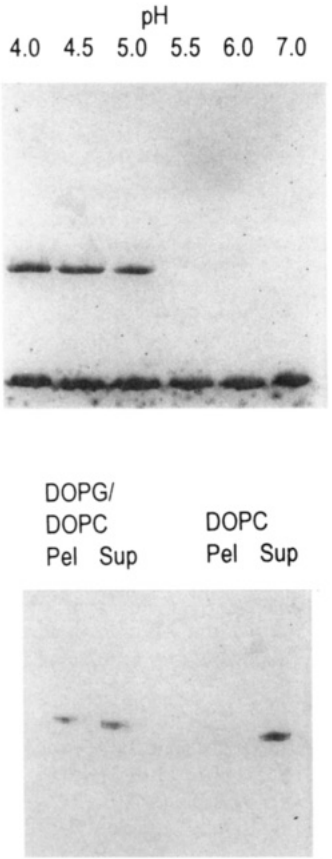

FIGURE 4: Assay of protein binding to vesicles by centrifugation. (Top) pH dependence of diphtheria toxin insertion into SUV. PAGE of proteins pelleting with vesicles is shown after Coomassie staining. The pH of buffer in which toxin and vesicles were incubated is indicated above each lane. The upper band is diphtheria toxin; the lower band is streptavidin. (Bottom) Interaction of SecA with 2:8 DOPG/DOPC LUV or DOPC LUV at 22 °C. A PAGE analysis of SecA in pellet and supernate is shown. Pel, pellet; Sup, supernate.

vesicles were pelleted. Lipid pelleting was found to be at least 98% complete as judged by the residual amount of pyrene-PC in the supernatant. As can be seen in Figure 4 (top), the diphtheria toxin associated with the vesicles only at pH 5 and below. This is agreement with previous studies of diphtheria toxin–lipid interaction (Hu & Holmes, 1984; Chung & London, 1988).

The lipid dependence of SecA interaction with vesicles was also studied using the streptavidin–biotin method. Interaction of SecA with model and real membranes has previously been shown to be dependent upon lipid composition (Cabelli et al., 1991; Breukink et al., 1992; Ulbrandt et al., 1992). SecA inserts into anionic vesicles containing DOPG at room temperature and above but will only insert into zwitterionic DOPC vesicles when the temperature exceeds 35 °C (Ulbrandt et al., 1992). Consistent with these observations, the biotin streptavidin method showed significant binding of SecA to the 2:8 DOPG/DOPC LUV but little interaction with DOPC LUV (Figure 4, bottom). Therefore, results obtained by the centrifugation method agree with literature results for SecA as well as diphtheria toxin.

In order to see whether the amount of protein binding is perturbed by the binding of streptavidin and centrifugation, the binding of cytochrome b_5 to vesicles was examined. Previous studies have shown that cytochrome b_5 binding to vesicles can be followed spectroscopically by the degree of quenching of its Trp residues by brominated lipids incorporated into SUV (Markello et al., 1985). Therefore, binding measured by quenching before addition of streptavidin was compared to binding determined from the percent of protein that pelleted after streptavidin addition. The results are shown in Figure 5. Binding is half-maximal at roughly 0.1 mg/mL both before and after streptavidin addition and pelleting. This shows that the binding of streptavidin and pelleting of the vesicles has relatively little effect upon cytochrome b_5 binding.

DISCUSSION

It had previously been noted that biotinylated lipids tend to aggregate when streptavidin is added (Plant, 1989). This report demonstrates it is possible to make use of streptavidin-induced aggregation to obtain efficient pelleting of small and large unilamellar vesicles under nondestructive conditions. One advantage of this system over pelleting of MLVs is that the leakage of vesicle contents can be correlated with binding. This cannot be done in MLV due to their multiple internal compartments. Furthermore, the amount of lipid available for binding in MLV is difficult to determine, because it will depend on how many layers are present and MLV size, and may vary with lipid composition. None of these problems exist in unilamellar vesicles. The amount of lipid available for binding will be the amount in the outer leaflet, 50% of the total in LUV and about 66% of the total in SUV. Finally, SUV are of particular use because they often bind proteins more strongly than large vesicles (Greenhut et al., 1986; Chung & London, 1988), which facilitates the detection of weak protein binding.

As an assay for protein hydrophobicity measuring protein binding to vesicles by streptavidin-dependent vesicle pelleting has certain advantages to the Triton X-114 procedure. The Triton X-114 method does not allow one to look at interactions which also involve contributions from interactions with the lipid polar headgroups. In addition, the Triton X-114 procedure cannot be used to look at temperature-dependent interactions since it requires fixed exposures to both 4 °C and a temperature that must be above 30 °C (Bordier, 1981).

It should be noted that we have not explored the performance of the centrifugation procedure under all possible conditions. For example, although preliminary results show vesicles containing either types of biotinylated lipid used in this study can be effectively pelleted (D. Cullinan and E. London, unpublished observations), the best combination of biotinylated lipids has not been explored. It is conceivable that the assay could be extended to shorter times and/or lower lipid concentrations by further adjusting the biotin content of the vesicles. It is also possible that there will be a problem with vesicle fusion or leakiness under some conditions we have not examined. This should be kept in mind when applying the method to a new system for the first time. However, it is already clear that the method will be useful under a wide variety of conditions.

One question that arises from experiments involving varying streptavidin concentration is why pelleting is so sensitive to the streptavidin to biotin ratio. Our rough interpretation of what is happening is summarized in Figure 6. At low streptavidin to biotin ratios it is likely that all four biotin binding sites on a streptavidin molecule are linked to the biotins of a single vesicle. This might arise if the lateral diffusion of the biotin lipids within a single vesicle allows them to bind all four biotin binding sites on a streptavidin before vesicle–vesicle collisions allow the streptavidin to come into contact with biotin groups on other vesicles. At the other extreme of high streptavidin to biotin ratios, the excess of streptavidin may result in each streptavidin binding only a single biotin,

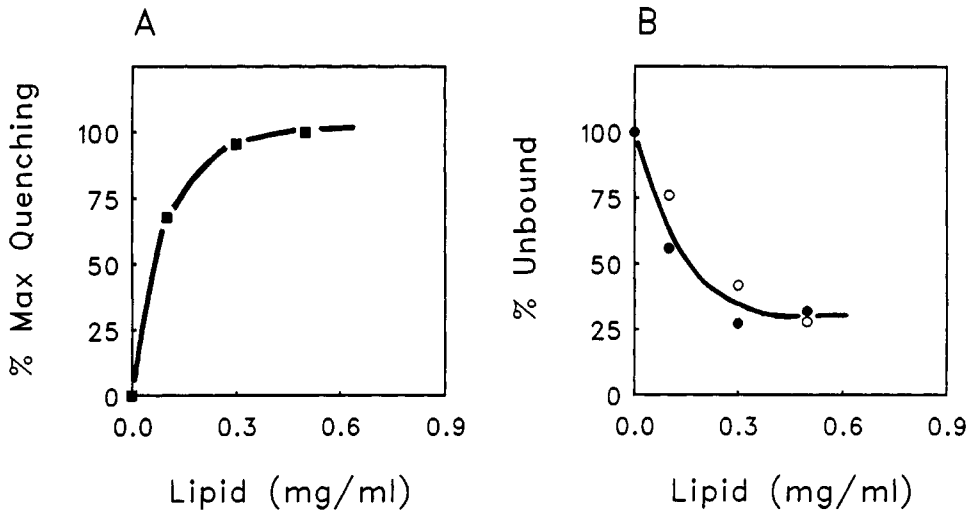

FIGURE 5: Cytochrome b_5 binding to SUV composed of 2:8 DOPG/BrPC and 1.6% biotinylated lipid. (A) Binding determined by the degree of quenching of cytochrome b_5 Trp fluorescence upon binding to brominated lipids *before* addition of streptavidin and centrifugation. Since there is only partial quenching of bound cytochrome b_5 fluorescence by brominated lipids (Markello et al., 1985), the 27% quenching found at 0.5 mg/mL lipid was defined as representing 100% of maximal binding. (B) Binding determined *after* streptavidin addition and centrifugation. Binding was assessed by the amount of cytochrome heme absorbance (○) and by the amount of cytochrome Trp fluorescence in the supernate (●) after pelleting at 10000*g* for 30 min.

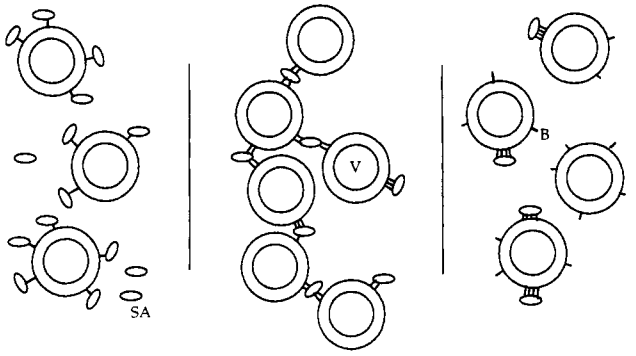

FIGURE 6: Schematic: illustration of the effect of streptavidin to biotin ratio on vesicle aggregation. (Left) Situation at a high streptavidin to biotin ratio. (Middle) Intermediate streptavidin to biotin ratio. (Right) Low streptavidin to biotin ratio. B, biotinylated lipid; SA, streptavidin; V, vesicle.

preventing cross-linking. Therefore, the best pelleting would occur at intermediate streptavidin to biotin ratios, as is observed. Due to the sensitivity of pelleting to vesicle concentration and the streptavidin to biotin ratio (which was only approximately known in our study), we recommend that empirical determination of the optimal ratio should be performed before using the method.

One potential problem with using this method to detect protein binding is interference from the vesicle-bound streptavidin when analyzing binding from gel electrophoresis. We found that under most conditions this was not a problem with analysis except where high streptavidin concentrations are present and when the mobility of the protein being examined was close to that of streptavidin. Even in these cases, streptavidin interference can be eliminated by shifting the mobility of the streptavidin band through omission of the boiling of samples in loading buffer (Jiang et al., 1991). (This allows streptavidin to retain its oligomeric structure.) Of course, use of detection by Western blotting or some other species-specific staining also totally avoids this problem.

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