

FREE ENERGY POTENTIAL FOR AGGREGATION OF MIXED PHOSPHATIDYLCHOLINE/PHOSPHATIDYLSERINE LIPID VESICLES IN GLUCOSE POLYMER (DEXTRAN) SOLUTIONS

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ABSTRACT The energetics of lipid vesicle-vesicle aggregation in dextran (36,000 mol wt) solutions have been studied with the use of micromechanical experiments. The affinities (free energy reduction per unit area of contact) for vesicle-vesicle aggregation were determined from measurements of the tension induced in an initially flaccid vesicle membrane as it adhered to another vesicle. The experiments involved controlled aggregation of single vesicles by the following procedure: two giant ($\sim 20\ \mu\text{m}$ diam) vesicles were selected from a chamber on the microscope stage that contained the vesicle suspension and transferred to a second chamber that contained a dextran (36,000 mol wt) salt solution (120 mM); the vesicles were then maneuvered into position for contact. One vesicle was aspirated with sufficient suction pressure to create a rigid sphere outside the pipette; the other vesicle was allowed to spread over the rigid vesicle surface. The aggregation potential (affinity) was derived from the membrane tension vs. contact area. Vesicles were formed from mixture of egg lecithin (PC) and phosphatidylserine (PS). For vesicles with a PC/PS ratio of 10:1, the affinity showed a linear increase with concentration of dextran; the values were on the order of 10^{-1} ergs/cm² at 10% by weight in grams. Similarly, pure PC vesicle aggregation was characterized by an affinity value of 1.5×10^{-1} ergs/cm² in 10% dextran by weight in grams. In 10% by weight in grams solutions of dextran, the free energy potential for vesicle aggregation decreased as the surface charge (PS) was increased; the affinity extrapolated to zero at a PC/PS ratio of 2:1. When adherent vesicle pairs were transferred into a dextran-free buffer, the vesicles did not spontaneously separate. They maintained adhesive contact until forcibly separated, after which they would not readhere. Thus, it appears that dextran forms a "cross-bridge" between the vesicle surfaces.

INTRODUCTION

Interactions between phospholipid vesicles continue to be studied as models for cellular and subcellular adhesion, aggregation, and fusion processes (Wu et al., 1981; Wong and Thompson, 1982; Cohen et al., 1982; Duzgunes et al., 1981; Wilschut et al., 1981). Additional motivation for such studies is to evaluate lipid vesicles as potential encapsulation systems for drug delivery and release in humans. All previous studies have involved optically invisible preparations of small (200–500 Å diam) and not so small (1,000–2,000 Å diam) vesicles; however, no direct observation or quantitation of the energetics of vesicle-vesicle aggregation (much less fusion) has been accomplished. The studies referred to here have relied on measurements of changes in population properties (e.g., light scattering, fluorescence quenching by chemical mixing, etc.). Likewise, the physical state of vesicles in such suspensions is difficult if not impossible to regulate, i.e., the level of stress in the vesicle membranes is not uniform. Indeed, there is evidence that demonstrates that membrane stress is signifi-

cant in modulating vesicle aggregation and fusion (Wong and Thompson, 1982; Cohen et al., 1982). Also, theoretical analysis of the mechanics of vesicle-vesicle aggregation clearly exposes the opposition of vesicle membrane stress to the forces of chemical attraction between vesicle surfaces (Evans and Parsegian, 1983). In this study, we have set out to demonstrate the mechanics of vesicle-vesicle adhesion and to evaluate the free energy reduction (surface affinity) associated with formation of adhesive contact in a controlled aggregation experiment. Specifically, we have measured the free energy potential (surface affinity) for aggregation of two "giant" (20×10^{-4} cm diam) lecithin vesicles in solutions of a glucose polymer (dextran) of 36,500 mol wt. In addition, we have used mixtures of egg phosphatidylcholine (PC) and bovine phosphatidylserine (PS) to quantitate the effect of electrostatic repulsion between vesicle surfaces.

The approach we took was to select two giant vesicles from a suspension, to transfer the chosen vesicles to a separate chamber that contained the appropriate solution, and finally to maneuver them into position for contact by

micromanipulation. The vesicles were allowed to adhere in a stepwise process controlled by the suction pressure in a micropipette. The vesicle membrane tension was measured as a function of contact area, and the free energy potential per unit area (surface affinity) of contact formation was derived with the use of simple mechanical principles.

Extensive studies of the aggregation of human red blood cells in dextran solutions have been carried out for broad range of molecular weights. Red cells form aggregates in dextran solutions that exhibit close, uniformly spread cell-cell contact over large surface areas. The important features of the aggregation have been well described by Brooks (1973 *b*), and Chien and associates (Jan and Chien, 1973), i.e., (*a*) normal red cells are not aggregated by dextrans with molecular weights less than $\sim 50,000$; (*b*) aggregation commences at low concentrations of $<1\%$ by weight in grams; (*c*) aggregation of normal red cells does not occur for dextran concentrations above 5–10% by weight in grams; and (*d*) aggregation depends on cell surface charge. Thus, there is a window of dextran concentrations within which red cells are aggregated. Outside this window, aggregation does not occur even though the concentration of dextran has not saturated the membrane surface (Brooks, 1973 *a*; and Chien, 1980). These features have been verified in controlled aggregation tests of single red blood cells; also, the level of chemical affinity between red cell membranes has been determined in such tests as a function of dextran concentration and molecular weight (Buxbaum et al., 1982). Values of the surface affinity for normal red cells range from low values characteristic of aggregation in blood plasma (10^{-3} ergs/cm²) to values above 10^{-2} ergs/cm² in solutions of 150,000 mol wt dextran (Buxbaum et al., 1982). Recently, we measured the surface affinity (free energy potential for aggregation) of a single red blood cell and a giant lipid vesicle ($2-3 \times 10^{-4}$ cm diam) of mixed lipid (PC/PS) composition (Evans and Kukan, 1983). The results of these tests showed that (*a*) for PC/PS ratios $<2.6:1$, no aggregation of vesicles with red cells was detected; (*b*) in the range of PC/PS ratios from 4:1 to 2.6:1, vesicle aggregation with red cells only occurred within a window of concentrations from 1–8% by weight in grams; (*c*) the peak affinity for aggregation increased with PC content; and (*d*) for pure PC vesicles, the affinity for vesicle aggregation with red cells appeared to increase without limit at higher dextran concentrations. Because the levels of affinity for vesicle–red cell aggregation were comparable to those of red cell–red cell aggregation but for much lower molecular weight dextran, the results indicated that dextran penetrates between the carbohydrate groups on the red cell surface. The observation that the aggregation process was quenched at higher dextran concentrations only for charged vesicle surfaces was also consistent with observations of red cell–red cell aggregation in dextran solutions. The quenching of the aggregation process at high dextran concentrations is not understood, although it has been

proposed (Brooks, 1973 *b*) that adsorption of dextran may act to expand the electric double layer. Thus, one of our aims has been to evaluate the potential for aggregation of lipid vesicles with charge densities comparable with those of normal red cells in solutions of dextran at high concentrations (5–10% by weight in grams).

EXPERIMENTAL METHODS

Lipid vesicles were made from mixtures of egg yolk lecithin (PC) and bovine phosphatidylserine (PS) dissolved in 10:1 chloroform methanol (PC and PS from Avanti Biochemical, Inc., Lakewood, NJ). The solvent was evaporated and the lipid mixture was rehydrated in sucrose solutions (200 mM). The resulting suspension was dialyzed against an osmotically equivalent sodium chloride solution to replace the external sugar with salt. Then a dilute suspension of these vesicles was injected into one side of a double microchamber on the microscope stage. Two vesicles were selected with a small suction micropipette and placed into a larger transfer pipette that spanned the air gap between the two chambers on the microscope stage. The microscope stage was then translated to leave both pipettes and the vesicles in the other chamber. The second chamber contained a more concentrated sodium chloride buffer (120 mM) to slightly dehydrate the vesicles. This solution also contained the dextran 36,500 mol wt polymer (kindly supplied by Dr. Kirsti Granath, Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ) in the concentration range of 2–10% by weight in grams. (Vesicles have to be slightly dehydrated to produce a geometric state where the membrane surface area is in excess of that of a sphere of equivalent volume. In this state, the vesicle is flaccid, essentially tension free [Kwok and Evans, 1981] and can be easily deformed into any shape within the constraints of the membrane area and internal volume. If the vesicles are not dehydrated, they are nearly spherical and essentially undeformable because of great resistance to membrane area expansion and volume reduction.) After the transfer, the lead vesicle in the transfer pipette was withdrawn by the small pipette and handed to another small pipette, which aspirated the vesicle with sufficient suction pressure to form a rigid spherical portion exterior to the pipette. Next, the second vesicle was withdrawn by the small micropipette and maneuvered into close proximity of the rigid vesicle surface (as shown in Fig. 1 *a*). Finally, the second vesicle was allowed to adhere to the rigid vesicle surface in steps controlled by the aspiration pressure in the micropipette. Examples of equilibrium configurations are shown in Fig. 1 *b* and *c*. Similarly, the vesicles were disassociated by stepwise increases in suction pressure so the reversibility of the adhesion process could be evaluated.

The multi-micromanipulator, microscope system is centered around a Leitz inverted microscope (E. Leitz, Inc., Rockleigh, NJ) with up to four small micromanipulators mounted directly on the microscope stage. The dimensions of each microchamber were typically $1 \text{ cm} \times 0.5 \text{ cm} \times 0.1 \text{ cm}$. The vesicles were aspirated and maneuvered by small glass suction pipettes attached to the micromanipulators. The pipettes were produced from 1 mm glass tubes pulled to a needle point and then broken by quick fracture to obtain flat tips in the range of $1-10 \times 10^{-4} \text{ cm}$. The pipette inner diameter was measured from the insertion depth of a tapered micro needle that was calibrated by scanning electron microscopy. The pipettes were coupled by a continuous water system to micrometer-positioned water manometers for zero pressure adjustment. The negative pressures were measured through the continuous water system connected to digital pressure transducers with a resolution of micro atmospheres. Pressures were simultaneously recorded on videotape with video multiplexing. A Hoffman (Ithaca, NY) phase optical system was used to enhance the vesicle image. All experiments were carried out at room temperature.

Method of Analysis

Adhesive contacts between membranes are mediated by a wide variety of agents, e.g., multi-valent ligands, plasma proteins and expanders, etc.

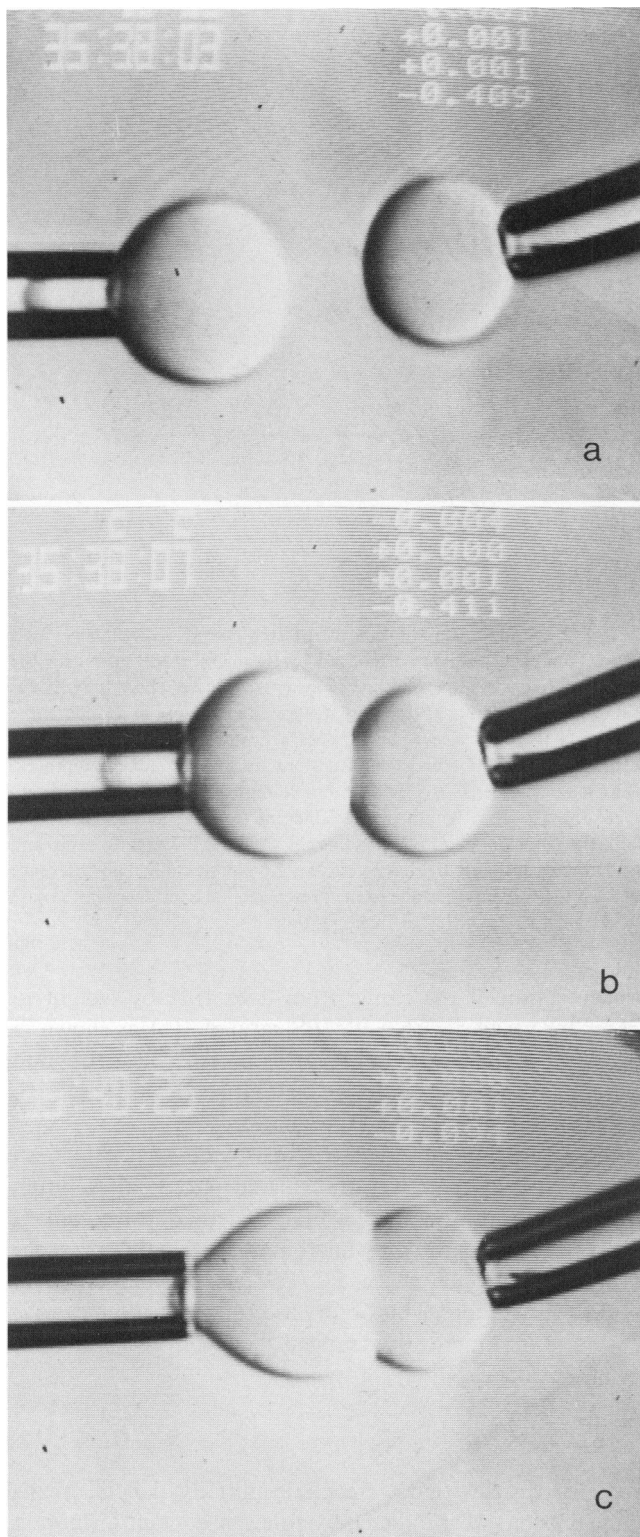


FIGURE 1 Video micrograph of controlled aggregation of two giant PC/PS vesicles in a 10% by weight in grams dextran (36,500 mol wt) and 120 mM salt solution. (a) The vesicles with diameters of 25 and 20×10^{-4} cm are first maneuvered into proximity for adhesion. The vesicle on the right is aspirated with sufficient suction pressure to form a rigid spherical test surface; the vesicle on the left, the adherent vesicle, is held with a low suction pressure that will permit formation of adhesive contact. (b) The

Such surface reactions, as well as the electrostatic and electrodynamic interactions of colloidal systems (Israelachvili, 1974; Parsegian, 1975), are prominent only over short range ($<100 \text{ \AA}$) in comparison with the scale of cellular dimensions. Hence, the spatial variations of adhesion forces between surfaces are essentially inaccessible and are best cumulated into an integral of force times displacement. This integral is the work involved in formation of an adhesive contact between locally flat surfaces and is represented thermodynamically as a free energy reduction per unit area. This intensive free energy potential defines the surface affinity, γ . Stable aggregation of cells or vesicles is promoted by the chemical affinity between outer membrane surfaces but is opposed by the work required to deform the cell or vesicle as contact progresses. Mechanical equilibrium is established when small (virtual) decreases in free energy due to contact formation plus small (virtual) increases in work of cellular deformation just balance the work of displacement of external forces at the boundaries, i.e.,

$$-\gamma \cdot \delta A_c + \delta W_d \approx \delta W_s \quad (1)$$

where W_d is the work of deformation, W_s is the work of displacement of external forces at the boundaries (e.g., the displacement of the suction force in the micropipette), and A_c is the area of contact. Hence, the variational statement of equilibrium yields the relationship that the surface affinity equals the derivative of the work of deformation with respect to contact area minus the derivative of the work of external forces with respect to contact area formation:

$$\gamma = \frac{\partial W_d}{\partial A_c} - \frac{\partial W_s}{\partial A_c} \quad (2)$$

As noted previously, vesicle lipid bilayers greatly resist surface area expansion. Likewise, when the osmotic strength of the vesicle contents is in excess of 10 mosM, giant vesicles ($>10^{-3}$ cm diam) greatly resist displacement of water across the membrane (i.e., volume changes). Consequently, for membrane tensions <0.5 dyn/cm, the surface area and volume remain constant to better than 0.5%. Furthermore, within the restrictions of constant surface area and volume, vesicles easily deform to any shape with negligible amounts of work required to deform the vesicle (Kwok and Evans, 1981). As the vesicle shape becomes spherical (or approaches a state of uniform total curvature), the internal pressure and membrane tension built up in opposition to further deformation. Thus, the limit of encapsulation in an adhesion process is set by the surface area and volume of the vesicle in relation to the geometry of the substrate to which it adheres. One useful feature of these geometric restrictions is that it is possible to force one of the vesicles to form an essentially rigid spherical surface (i.e., the "test" surface); hence, only the mechanics of the deformable, adherent vesicle need be considered. This situation has been treated theoretically by Evans (1980) with application of the variational principle given in Eqs. 1 and 2. The simple result is that the surface affinity is directly related to the displacement of the pipette suction force:

$$\gamma = -\left(\pi R_p^2\right) \cdot P \cdot \left(\frac{\partial L}{\partial A_c}\right) \quad (3)$$

where P is the pipette suction pressure; L is the aspirated length of the vesicle inside the pipette; and R_p is the pipette radius. If we were able to move the adherent vesicle such that the shape of the outer body always

left vesicle adheres spontaneously to the rigid vesicle surface and forms a stable equilibrium geometry as shown here. (c) When the pressure is lowered, the left vesicle spreads to a new equilibrium configuration with greater contact area. The magnitude of the suction pressure is indicated by the lower, negative digital volt meter reading recorded in the video image.

remained an exact sphere, then there would be no net axial force between micropipettes. In this conceptual case, the surface affinity is simply related to the membrane tension, \bar{T} , through an equation analogous to Young's equation for liquid droplet adhesion (Evans and Parsegian, 1983), i.e.,

$$\gamma = \bar{T} \left[1 - \sqrt{1 - \left(\frac{r}{R_s}\right)^2} \cdot \sqrt{1 - \left(\frac{r}{R_c}\right)^2} + \frac{r^2}{R_c \cdot R_s} \right] \quad (4)$$

where r is the circular radius of the contact zone; R_c and R_s are the radii of the spherical segments of the adherent vesicles. Experimentally, however, it is very difficult to move the pipettes such that the adherent vesicle surface is an exact sphere. It is preferable to leave both pipettes in fixed positions and change the suction pressure to alter the extent of encapsulation, then no work is done by displacement of axial forces. In this situation, the shape of the adherent vesicle is not an exact sphere but is given by a surface of uniform total curvature. As such, the geometry is too complicated to permit a closed form analytical solution; but it is a simple matter to solve the problem numerically subject to the constraints of constant area and volume. Eq. 3 can be rearranged to give a dimensionless form:

$$\left(\frac{2\gamma}{R_p \cdot P} \right) = - (2\pi R_p) \left(\frac{\partial L}{\partial A_c} \right) \quad (5)$$

where the surface affinity is normalized by the suction pressure times the pipette radius, which is related to a function only of the geometry. Similarly, Eq. 4 can be arranged to give,

$$\left(\frac{\gamma}{\bar{T}} \right) = \left[1 - \sqrt{1 - \left(\frac{r}{R_s}\right)^2} \cdot \sqrt{1 - \left(\frac{r}{R_c}\right)^2} + \frac{r^2}{R_c \cdot R_s} \right] \quad (6)$$

Fig. 2 presents a sample comparison of these two normalized affinity relations as functions of the fractional extent of encapsulation of the rigid vesicle surface. These curves are for a specific geometric situation and cannot be generalized. The fractional extent of encapsulation, x_c , is the contact area divided by the area of a sphere with a diameter equivalent to that of the rigid vesicle. Note the difference between the two curves is due to the presence of an axial force in the case where the pipettes were left at fixed positions; it is apparent that a greater level of surface affinity was required to produce an equivalent extent of encapsulation in the case where the axial force is present.

RESULTS

Fig. 3 presents examples of the fractional extent of encapsulation of the rigid vesicle surface vs. the reciprocal of the tension in the adherent vesicle membrane for 2 and 10% dextran (36,500 mol wt) solutions; observations of both the formation and separation of adhesive contact are presented to indicate the level of hysteresis in the experiment. Because there was no perceptible level of hysteresis associated with aspiration of a single vesicle into the micropipette, any hysteresis in the adhesion test was totally attributable to the process of separation of the adhesive contact. We emphasize that the calculation of surface affinity must be derived from the phase of the experiment where the adhesive contact is being formed; the correlation of this phase with the theoretical model is shown in Fig. 3 *a* and *b*. The free energy potential for aggregation of vesicles in dextran was found to a linearly increasing function of

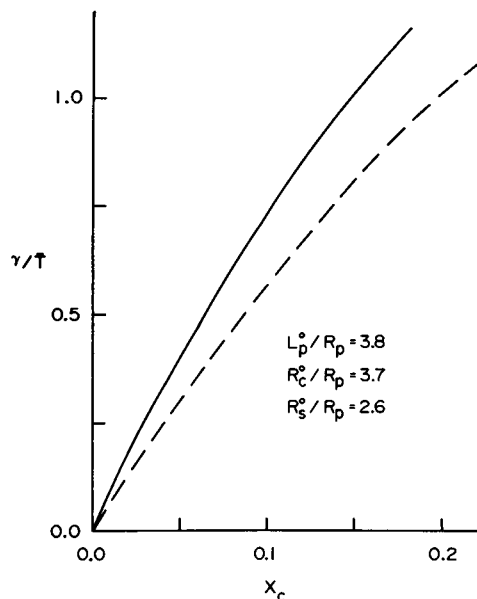


FIGURE 2 Sample computation of the free energy reduction per unit area of contact (affinity) normalized by the membrane tension for the following situations: the solid line represents the case where the pipettes maintain fixed positions which creates an axial force; the dashed curve represents the case where the pipettes are moved so that the vesicles maintain perfect spherical conformations during the adhesion process with no axial force. This specific example represents an initial geometry where the aspirated length of the adherent (*left*) vesicle is 3.8 pipette radii, the outer diameter of the adherent vesicle is 3.7 pipette radii, and the diameter of the rigid (*right*) vesicle is 2.6 pipette radii. The difference between the two curves is due to the presence of an axial force in the case where the pipettes are left at fixed positions.

concentration in the range from 2 to 10% by weight in grams for vesicles with a PC/PS ratio of 10:1 as shown in Fig. 4. Also, the vesicle aggregation potential was found to decrease as the surface charge content (PS) was increased, as shown in Fig. 5 for the 10% solution data.

A significant observation was made that when an adherent vesicle pair was transferred into a salt solution without dextran, the vesicles maintained adhesive contact until forcibly separated by micromanipulation. After separation, the vesicles (with PC/PS composition of 10:1) would not readhere. This observation shows that dextran is present and kinetically trapped between the adherent vesicle surfaces.

CONCLUSIONS

Even for vesicles that were made initially flaccid, membrane tensions were induced by adhesive contact formation that represented the opposition to further enlargement of the contact zone. The induced tensions ranged from the order of 10^{-2} to 10^{-1} dyn/cm and characterized the scale of the chemical affinity between membrane surfaces (i.e., the free energy potential for aggregation). The affinities for aggregation of PC/PS vesicles, derived from the mem-

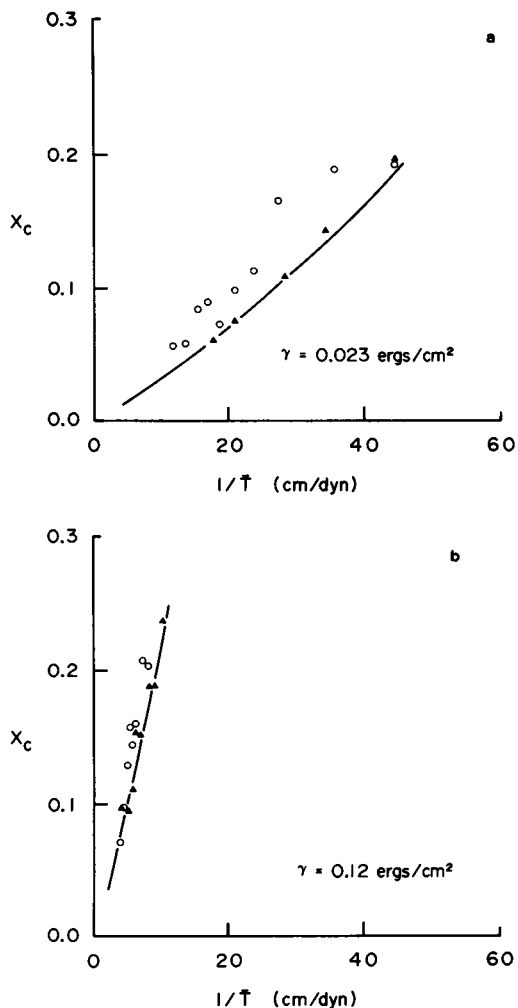


FIGURE 3 Examples of data for single vesicle-vesicle aggregation experiments. The fractional extent of encapsulation of the rigid vesicle is plotted vs. the reciprocal of the tension in the adherent vesicle membrane. (a) A vesicle-vesicle aggregation experiment in 2% by weight in grams dextran (36,500 mol wt and 120 mM salt solution); (b) similar experiment in 10% by weight in grams dextran, salt solution. The closed triangles (\blacktriangle) represent the formation of adhesive contact whereas the open circles (\circ) represent separation of contact.

brane tension vs. contact area, showed a linear increase with concentration of dextran in the range of 2 to 10% by weight in grams. The values of 10% by weight in grams were $\sim 1.5 \times 10^{-1}$ ergs/cm² for pure PC and decreased, as the surface charge (PS) was increased, to zero when the vesicle PS concentration reached $\sim 33\%$. It should be noted that pure PC vesicles in salt solutions exhibited very weak aggregation potential ($\sim 2-3 \times 10^{-2}$ ergs/cm²) which was promoted by the long-range attractive forces (van der Waal's) between the membrane surfaces. The low levels of affinity in the absence of the aggregating polymer were more difficult to measure and are the subject of a separate paper. Also, studies of the equilibrium spacing of lipid multibilayers have yielded free energy reductions per unit

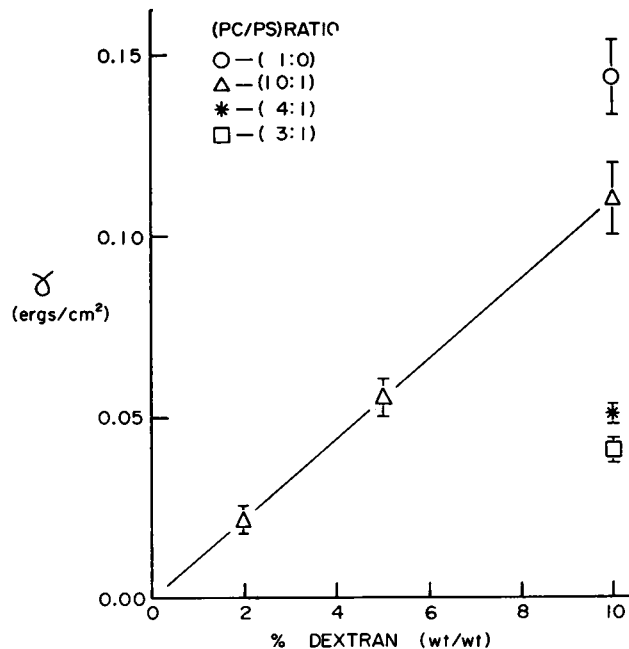


FIGURE 4 The free energy potential for aggregation of mixed PC/PS vesicles in dextran (36,500 mol wt), 120 mM salt as a function of dextran concentration. The PC/PS ratios are shown by the respective data points. The points are the averages of 5-10 vesicle-vesicle aggregation experiments and the brackets represent the total range of affinities derived from the data.

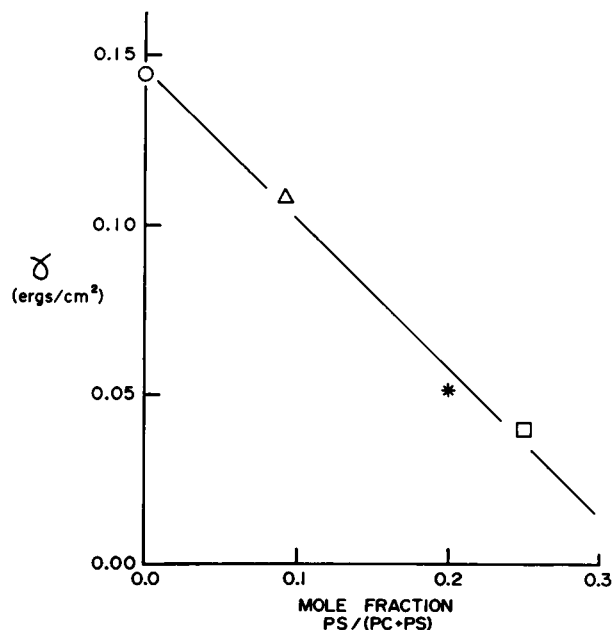


FIGURE 5 The free energy potential for aggregation of vesicles in 10% by weight in grams dextran (36,500 mol wt), 120 mM salt, as a function of mole fraction of PS contained in the surface.

area due to long range van der Waal's attraction on the order of 10^{-2} ergs/cm² or less (Loosley-Millman et al., 1982). There was no perceptible level of affinity between vesicles with PC/PS mixtures of 10:1 in the 120 mM salt solution. Based on the observed levels of affinity between neutral PC surfaces and between vesicles with 10:1 (PC/PS) in 10% by weight in grams dextran, the surface charge density for 10:1 vesicles, and the theory for electrostatic potential energy between charged planes in salt solution (Verwey and Overbeek, 1948), we have estimated that a separation distance between the membrane surfaces of ~ 30 Å would account for the difference in affinity levels. This calculation indicates that, unless the polymer unfolded, there would only be a single molecular layer between the surfaces. With the observation that the vesicle remains adherent even after transfer to a dextran-free medium, these results strongly support the hypothesis that dextran forms molecular "cross-bridges" between the membrane surfaces.

An interesting feature of the concentration dependence of the surface affinity between vesicles was that there appeared to be no quenching effect at high concentrations, characteristic of red cell-red cell or red cell-vesicle aggregation, even for charge compositions comparable to that of the human red cell (i.e., PC/PS ratios of 3:1 to 4:1). This data indicated that, at least for lipid vesicle surfaces, the proposed model of expansion of the electric double layer repulsion by adsorption of high concentrations of dextran is inappropriate. It is not clear as to the origin of the quenching effect of dextran on red cell aggregation. One obvious difference between the red cell surface and a vesicle surface is that the red cell surface is covered with a superficial layer of carbohydrates that both "roughen" the membrane surface and distribute the charge normal to the surface. Because it appears that dextran penetrates between the surface sugars (Evans and Kukan, 1983) to form adhesive contact, one speculation is that perhaps the interstitial regions between the sugars saturate with dextran and further dextran adsorption interferes with the adhesion process.

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