Avidin-Biotin Interactions at Vesicle Surfaces: Adsorption and Binding, Cross-Bridge Formation, and Lateral Interactions

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ABSTRACT Densely packed domains of membrane proteins are important structures in cellular processes that involve ligand-receptor binding, receptor-mediated adhesion, and macromolecule aggregation. We have used the biotin-avidin interaction at lipid vesicle surfaces to mimic these processes, including the influence of a surface grafted polymer, polyethyleneglycol (PEG). Single vesicles were manipulated by micropipette in solutions of fluorescently labeled avidin to measure the rate and give an estimate of the amount of avidin binding to a biotinylated vesicle as a function of surface biotin concentration and surface-grafted PEG as PEG-lipid. The rate of avidin adsorption was found to be four times less with 2 mol% PEG^{750} than for the unmodified surface, and 10 mol% PEG completely inhibited binding of avidin to biotin for a 2-min incubation. Using two micropipettes, an avidin-coated vesicle was presented to a biotinylated vesicle. In this vesicle-vesicle adhesion test, the accumulation of avidin in the contact zone was observed, again by using fluorescent avidin. More importantly, by controlling the vesicle membrane tension, this adhesion test provided a direct measure of the spreading pressure of the biotin-avidin-biotin cross-bridges confined in the contact zone. Assuming ideality, this spreading pressure gives the concentration of avidin cross-bridges in the contact zone. The rate of cross-bridge accumulation was consistent with the diffusion of the lipid-linked "receptors" into the contact zone. Once adherent, the membranes failed in tension before they could be peeled apart. PEG^{750} did not influence the mechanical equilibrium because it was not compressed in the contact zone, but it did perform an important function by eliminating all nonspecific adhesion. This vesicle-vesicle adhesion experiment, with a lower tension limit of 0.01 dyn/cm, now provides a new and useful method with which to measure the spreading pressures and therefore colligative properties of a range of membrane-bound macromolecules.

INTRODUCTION

Densely packed domains of membrane proteins are important in a variety of intra- and intercellular processes (Alberts et al., 1994). For example, i) as a prelude to endocytosis, the binding of extracellular ligands to certain membrane receptors leads to the accumulation of ligand-receptor complexes in clathrin-coated pits; ii) multivalent antibody binding to proteins on cell surfaces produces capping on leukocytes; iii) in phagocytosis and cell attachment to the extracellular matrix, the formation of specific receptor-counter structure bonds leads to the accumulation of receptors in the contact zone and the formation of focal contacts; iv) gap junctions, which are specialized protein-rich domains of connexons, provide direct electrical and chemical communication between many different cell assemblies. Another important feature of cell surface interactions involves the influence of a polysaccharide-rich layer, the glycocalyx, which is attached to the core lipid bilayer membrane (Leblond and Bennett, 1974). The molecular architecture of this layer expresses both repulsive and attractive features, i.e., a ubiquitous property of steric stabilization is modulated by an ability to form specific adhesive molecular bonds. Thus, questions central to our understanding of receptor-ligand interactions, receptor-mediated cell adhesion, and membrane protein segregation include i) To what extent do polymeric steric repellers oppose both the process of ligand-receptor bond formation at a crowded membrane interface and the formation of adhesive contact zones? ii) What are the consequences of receptor-mediated bond formation, including equilibrium and dynamic aspects of receptor accumulation in the contact zone? and iii) What are the colligative characteristics of lateral interactions between packed macromolecules, i.e., do the confined molecules interact with each other and possibly form multimers?

To begin answering these questions by experiment, certain "ideal" conditions are required. For example, the membrane should be smooth; cross-bridges should be strong; the range and influence of steric repellers should be readily changed; receptors should be mobile; the total number of receptors should be constant (no up-regulation of receptors); and it is absolutely essential to be able to measure the tension in the membranes to quantitate mechanical equilibrium. Although many features of cell-cell and cell-substrate adhesion have been and continue to be studied using cells (Bongrand, 1988; Evans, 1994; Sung et al., 1986; Tozeren et al., 1992), the above idealized conditions are not easily controlled in cellular systems. Thus, to mimic ligand-receptor binding, receptor-mediated adhesion, and macromolecule aggregation in model experiments, we have begun the process of developing a synthetic lipid vesicle system incorporating the well-characterized avidin-biotin interaction as the "ligand-receptor" pair (Loughrey et al., 1987;

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Wilchek and Bayer, 1990). In the experiments to be described, micropipette manipulation is used to study the binding of avidin to a single biotinylated vesicle, and the adhesion between two biotinylated lipid vesicle membranes that are bridged by avidin. The biotin is linked to the headgroup of DPPE lipid and so can easily be incorporated into a lipid bilayer at defined concentrations. The biotin group is attached to the lipid via an extension arm that makes it more accessible to binding up and into the relatively deep binding β-barrel pocket of the avidin molecule. Detection of avidin on the biotinylated vesicle surface and in adherent contact zones is made possible by using fluorescently labeled avidin. To provide the repulsive steric barrier, poly(ethyleneglycol) (PEG) linked to the headgroup of distearoylphosphatidylethanolamine (DSPE) lipid is co-incorporated into the lipid vesicle membranes.

In this paper, then, we present data that i) show how surface-grafted PEG retards the diffusion-limited binding of avidin from aqueous solution to biotinylated lipids present in the lipid vesicle membrane; ii) demonstrate a direct measurement of the spreading pressure that results from the accumulation of biotin-avidin-biotin cross-bridges in an adherent contact zone between two vesicles; and iii) extend a simple model that describes the confinement of macromolecules in an adherent zone for the general case where chemical equilibrium can be attained (Bell, 1978; Bell et al., 1984; Evans, 1985). In this model, mechanical equilibrium is established at each point in the adhesion process when the surface pressure of confined cross-bridges in the contact zone is balanced by the tension in the spreading vesicle membrane, i.e., the pressure due to confined macromolecules can be sensed as a membrane tension (Evans, 1985, 1993, 1994). For suitable concentrations and binding energies of cross-bridge formation, this method then provides a surprisingly versatile and sensitive technique with which to study the lateral interactions between a variety of membrane-bound macromolecules.

MATERIALS AND METHODS

Materials

Vesicles were prepared from egg phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL). Various amounts of biotin-X-dihexadecylphosphatidylethanolamine (biotin-X-DHPE) (Molecular Probes, Eugene, OR), a lipid with a biotin linked to the headgroup via a 10–15-Å hydrocarbon spacer, were included. To ensure that nonspecific adhesion due to van der Waals attraction between the lipid surfaces and self-adhesion for a single vesicle did not occur, PEG^{750}-DSPE (MW.750) (Liposome Technology, Menlo Park, CA) and negatively charged dioleoylphosphatidylglycerol (Avanti Polar Lipids) were incorporated into the lipid vesicles. The amount of charge present in the membrane was always kept at 7 mol%, because this amount is in excess of that shown previously to overcome van der Waals attraction between lipid vesicles (Evans and Needham, 1987). Furthermore, because the biotin-X-DHPE and PEG^{750}-DSPE are both negatively charged, dioleoylphosphatidylglycerol was used to either make up the 7 mol% surface charge in their absence or to top up the charge to 7 mol% in their presence. Avidin Neutralite was chosen as the form of avidin to use because it lacks any glycosylated groups and therefore has an isoelectric point near neutral pH (Molecular Probes). Therefore, it is not expected to show any long-range stabilization at the interface. This is important because the conventional avidin molecule is positively charged at neutral pH and the biotinylated lipid is negatively charged and so electrostatic interactions would compromise the binding (Leckband et al., 1992, 1994). Furthermore, avidin Neutralite exhibits significantly less nonspecific binding than pure avidin. Unlabeled and fluorescein labeled avidin Neutralite (Molecular Probes) was used in a 0.1 mg/ml PBS solution (1.7×10^{-6} molar) to coat the surface of the biotinylated vesicles with avidin. According to Molecular Probes, the fluoroscein-labeled avidin has, on average, 6.3 fluorophores per molecule.

Methods

Vesicle preparation

The preparation of giant vesicles (20–40 μm) was performed as described elsewhere (Needham, 1993, 1995; Needham and Evans, 1988). Briefly, lipid mixtures were made up in chloroform. Vesicles were formed by gentle rehydration of dried lipid lamellae in a glass tube using sucrose solution (160 mOsm) and were resuspended in glucose solution at the same or slightly higher (170 mOsm) osmolarity. The vesicle suspension was then added to a manipulation microchamber on the interference contrast videomicroscope.

Avidin adsorption and binding to biotinylated vesicles

Characterizing the adsorption and binding of avidin to a biotinylated vesicle surface is of direct relevance to molecular recognition at surfaces. Here we are interested in the rate at which avidin binds under diffusion controlled conditions to the biotinylated surface in the absence and presence of a grafted PEG layer. (The PEG layer will later be used in vesicle-vesicle adhesion experiments to provide a sufficient barrier to nonspecific adhesion between the vesicle membranes. This is an important feature of these experiments because, in mimicking receptor-ligand interactions, we must make sure that adhesion is only due to biotin-avidin-biotin cross-bridges.) The relative dimensions of avidin, biotin-X-DHPE, PEG^{750}-DSPE, and the lipid bilayer are shown in Fig. 1.

Avidin binding as a function of time for fixed biotin concentration, with and without PEG-lipid

For diffusion controlled transport, relative measures of the diffusion constant were determined for the approach of avidin to a bare surface and to one that was decorated with PEG. To allow adsorption of avidin to the vesicle surface for a given period of time, a vesicle with a given amount of incorporated biotin was aspirated into a micropipette and transferred into an adjacent microchamber containing 0.1 mg/ml avidin. After the desired time, the vesicle was then transferred back to the avidin-free chamber, and the image of the fluorescent vesicle was observed by switching to a 40× oil immersion objective, viewed under epifluorescence illumination, and recorded on videotape. The images were then analyzed on a Macintosh II supplemented with a frame grabber card (Neotech, Easleigh, Hampshire, England). Image-processing software Image 1.43 (Wayne Rasband, National Institutes of Health) was used to determine the relative fluorescence intensity by evaluating the gray values (0 for black, 255 for white) along a line drawn through the video image of the vesicle as shown in Fig. 2. For a given camera setting and Image gray scale, the maximum value at the circumference of the vesicle was taken to be directly proportional to the amount of bound avidin. Each data point was the mean value of three to eight experiments. The avidin binding experiment was extended to 9 min (measurements made at 2, 4, 5, and 9 min) for a fixed biotin concentration of 5 mol%, both with (2 mol%) and without PEG-lipid.

Avidin binding as a function of PEG-lipid

To determine how the presence of surface grafted PEG (incorporated as PEG-lipid, DSPE-PEG^{750}) might affect the binding of avidin to a biotiny-

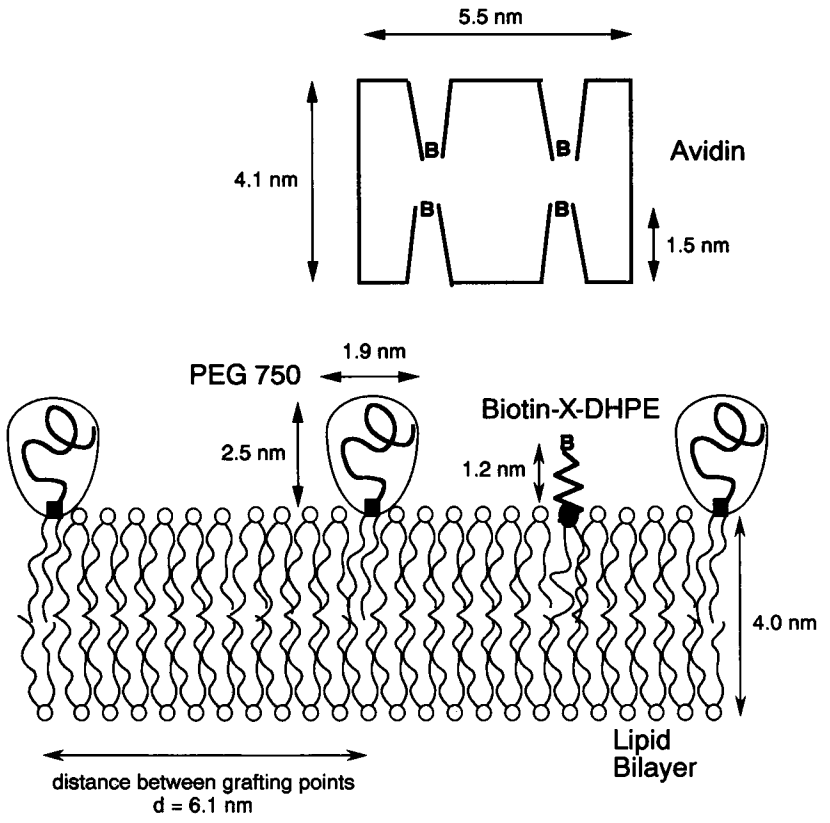

FIGURE 1 Sketch showing the dimensions of the vesicle lipid membrane with incorporated Biotin-X-DHPE, PEG^{750} lipid, and the avidin molecule with its four biotin-binding sites.

lated vesicle, the avidin binding experiment was carried out as a function of the amount of PEG-lipid incorporated into the vesicle membrane. Thus, for a 2-min incubation time, the relative fluorescence intensity was measured for vesicles containing 0, 0.5, 1, 2, 3, 5, and 10 mol% PEG-lipid.

Micromechanical test of vesicle-vesicle adhesion

An important aspect of the present work was to be able to measure the tension in the avidinated vesicle membrane as it spread on the biotinylated vesicle surface. This spreading tension along with the contact angle between vesicles was used to determine the spreading pressure of accumulated receptor cross-bridges. As described later, from this we have estimated the concentration of the cross-bridges in the region where adhesion between two vesicle membranes occurs.

Briefly, three microchambers on the microscope stage were filled with sodium chloride solution (170 mOsm), glucose solution (160 mOsm), and avidin in phosphate-buffered saline solution (160 mOsm), respectively. All solutions contained 0.2 g% albumin to prevent vesicles from sticking to the glass micropipette and chamber surfaces. Vesicles expressing PEG and biotin at their surfaces were added to the glucose and sodium chloride solutions. Three micropipettes were used to transfer the lipid vesicles between chambers and to manipulate them in the adhesion experiment: one 10-μm-diameter measuring pipette, one 10-μm-diameter holding pipette, and one 100-μm-diameter transfer pipette. To preadsorb avidin to one of the vesicles, a large vesicle from the glucose solution was first incubated in the avidin solution for 2 min to allow for avidin adsorption and was then transferred into the sodium chloride solution. Here, a second vesicle was aspirated in the holding pipette at a high suction pressure to form a rigid biotinylated test surface. Both vesicles were then brought into close contact for the adhesion test. The tension in the adhering vesicle membrane was set to a constant value and the adherent geometry was analyzed to give the change in contact angle with time at several points during the process of attachment (Evans, 1980; Needham, 1993). Adhesion of the avidin-coated vesicle to the biotinylated test surface (the second vesicle) was observed and recorded on video, as shown in Fig. 3. Note that, because the vesicle membranes are not expanded by the small tensions used in this experiment, as the left-hand vesicle spreads on the right-hand vesicle, the vesicle membrane projection length in the left-hand pipette reduces in proportion to the area of contact between the vesicles.

The geometry of the adherent vesicle throughout the adhesion process was calculated numerically from the initial diameters of the two vesicles and the initial projection length of the adherent vesicle in the pipette, assuming a constant vesicle area and volume. The membrane tension was obtained from the adherent vesicle and pipette geometry and the pipette suction pressure according to micropipette methods used previously to determine van der Waals, polymer-induced, and receptor-mediated adhesion as described elsewhere (Berk and Evans, 1991; Evans, 1980; Evans and Metcalfe, 1984; Evans and Needham, 1987; Needham, 1993). To directly observe the accumulation of avidin in the contact zone, fluorescein-labeled avidin was used in the adhesion experiment and viewed under epifluorescence. Unfortunately, progress to equilibrium adhesion could not be followed with fluorescence because, when the vesicles were illuminated by epifluorescence, the avidin was destroyed by photolytic degradation and the adhesion did not proceed any further.

RESULTS AND DISCUSSION

Avidin adsorption and binding to biotinylated vesicles

The influence of surface-grafted PEG on the adsorption and binding of avidin to biotinylated vesicles was examined under both diffusion-limited and convective conditions. The comparatively short chain-length PEG^{750} was chosen as the steric barrier because it was found not to completely inhibit

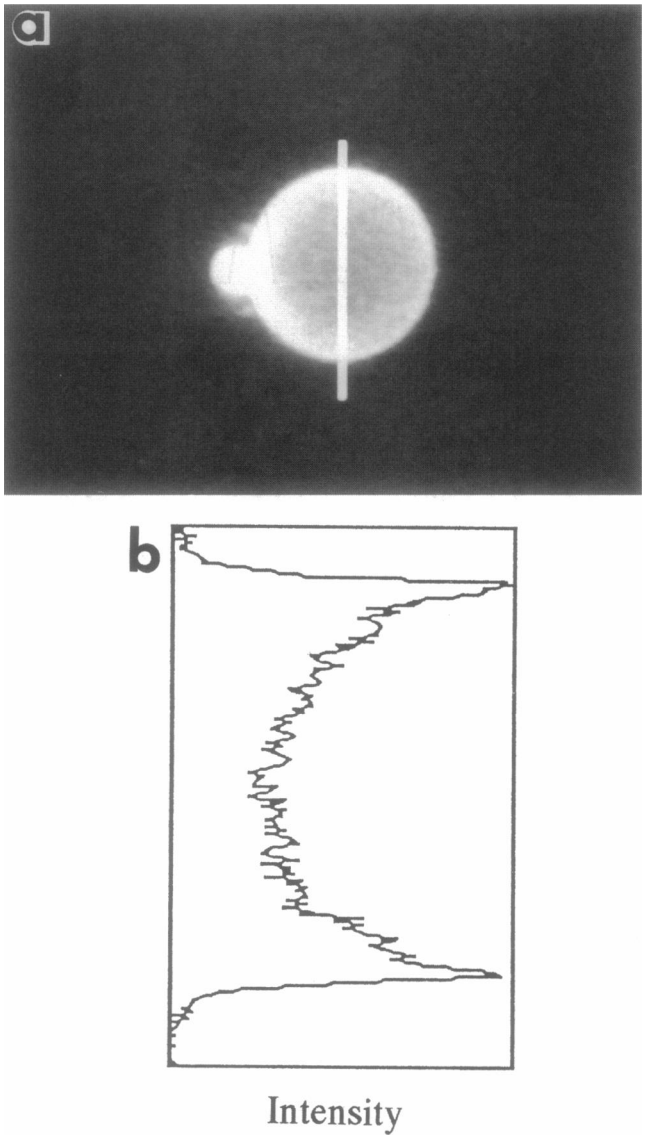

FIGURE 2 (*a*) Fluorescence video micrograph of vesicle held in a suction micropipette with bound fluorescent avidin. The gray values along the line are evaluated by image analysis. (*b*) The obtained intensity profile.

adsorption of avidin at low biotin concentrations. Also, of importance to our adhesion studies, it was found to prevent nonspecific van der Waals adhesion between the two vesicles in the absence of biotin-avidin-biotin cross-bridges.

Avidin binding as a function of time for fixed biotin concentration, with and without 2 mol% PEG-lipid

The time dependence for binding of fluorescent avidin to a biotinylated (5 mol% biotin) vesicle without PEG^{750} and with 2 mol% PEG^{750} is shown in Fig. 4, *a* and *b*. As we (Needham and Zhelev, 1995) and others (Evans et al., 1994) have observed previously for other adsorbing molecules, a layer of solution around the vesicle becomes depleted of avidin during the incubation, and the rate of adsorption depends on diffusion across this depletion layer. The number of avidin molecules adsorbed per unit area $n(t)$ for initial times is given by (Andrade, 1985)

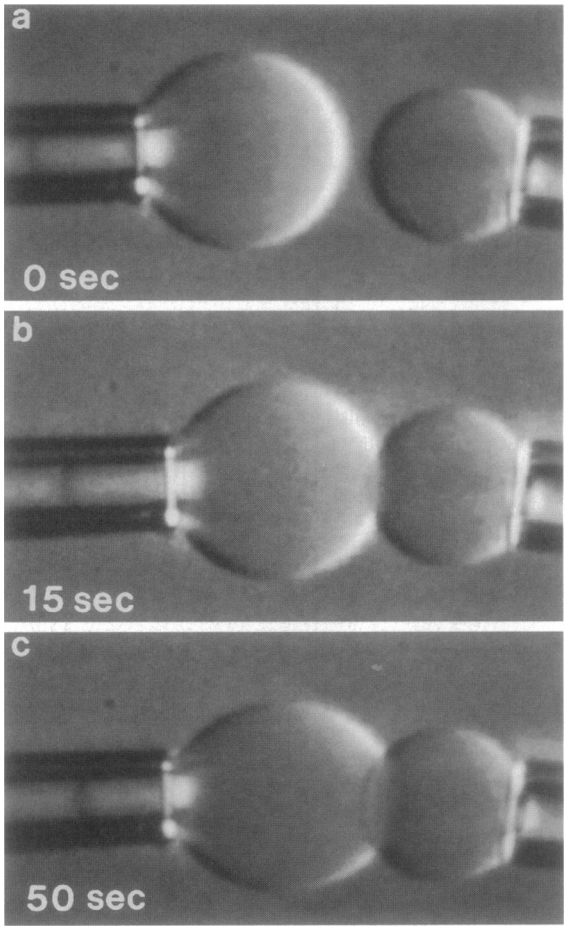

FIGURE 3 Vesicle-vesicle adhesion experiment. (*a*) Presentation (t = 0 s); (*b*) contact and spreading (t = 15 s); (*c*) further spreading (t = 50 s). The biotin concentration was 5 mol%.

$$n(t) = 2C_o(Dt/\pi)^{1/2}, \quad (1)$$

where C_o is the initial bulk concentration, D is the diffusion coefficient, and t is time. In Fig. 4 *b* the relative intensity was plotted against the square root of time. As the relative intensity is assumed to be directly proportional to the density of bound fluorescent avidin molecules per unit area, the slope of a linear fit to the data is given by the initial bulk concentration $\times D^{1/2}$ (Eq. 1). As shown in Fig. 4 *b*, the good agreement between the fit of a square root to the experimental data suggests that diffusion of avidin to the vesicle surface is the main influence on the time dependence for the binding of avidin from aqueous solution. The incorporation of 2 mol% PEG^{750} significantly reduced the amount of bound avidin and the rate of avidin binding. The constant is the same for both situations with and without 2% PEG^{750}, and so the ratio of the two different slopes yields the ratio of the two "effective" diffusion constants, i.e., $D_{0\%PEG}/D_{2\%PEG}$ = 4.8. Therefore, the presence of a thin (25 Å) PEG layer at the surface of the vesicle was sufficient to

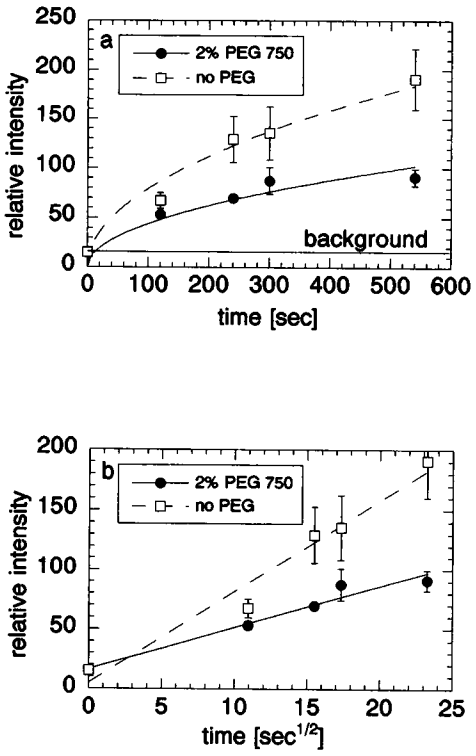

FIGURE 4 Avidin binding to biotinylated vesicle. (*a*) Relative fluorescent intensity plotted against incubation time in avidin solution. Results for vesicles with (●) and without (□) 2 mol% PEG^{750} are shown. The biotin content was 5 mol%. A square root was fitted to the data according to Eq. 1. (*b*) Relative fluorescent intensity plotted against the square root of incubation time. The slopes of the linear fit according to Eq. 1 are then a measure of the diffusion coefficients.

slow down the diffusion of avidin to the vesicle surface by a factor of about 5.

Under convective transport conditions, the amount of binding with and without 2 mol% PEG^{750} was the same. This would appear to indicate that the polymer layer plays a lesser role when the stagnant layer is minimized, so that the avidins are delivered directly to the surface. It is clear from these results that 2 mol% PEG^{750} is not sufficient to block binding under convective flow conditions but does slow down the adsorption when dominated by diffusion.

Avidin binding as a function of PEG-lipid

By adding increasing amounts of surface-grafted PEG^{750}-DSPE, the binding of avidin to a surface containing 5 mol% biotin decreased significantly, as shown in Fig. 5. For a 2-min incubation (i.e., under diffusion conditions), it was possible to block binding almost completely by incorporating up to 10 mol% PEG^{750} in the membrane. At this concentration, the polymer layer is close to the "brush regime" (Kenworthy et al., 1995), where the surface is completely covered with PEG. For a larger 2000 molecular weight polymer (PEG^{2000}-DSPE), the addition of only 4 mol%, which is also close to the brush regime, was also

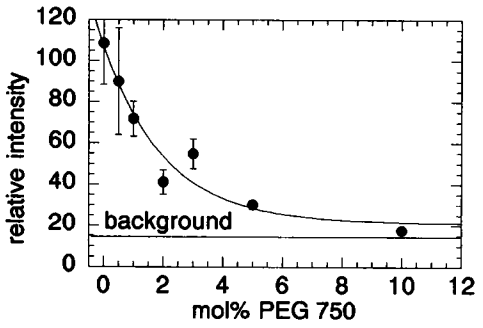

FIGURE 5 Dependence of avidin binding on molar PEG^{750} concentration. Biotin (5 mol%) was incorporated into the vesicle membranes, and the incubation time in 0.1 mg/ml avidin was 2 min. An exponential decay according to Eq. 2 was fitted to the data.

found to block binding completely for the same incubation time (results not shown).

This retardation behavior can be explained in terms of two parameters: an additional energy barrier that the surface pressure Π_p of PEG mushrooms provides against diffusion to the surface, and the cross-sectional area A_{Av} of the avidin molecule. Conceptually, the avidin has to "push" the polymer aside to create free area at the interface, and so the rate of adsorption and, therefore, the absolute number of adsorbed molecules in a given time, is decreased by a factor $\exp(-A_{Av}\Pi_p/kT)$ (Andrade, 1985). If it is assumed that the surface pressure of PEG is given by the ideal relationship, $\Pi_p = N/A \cdot kT$ and the surface density by $N/A = 0.01 \times$ mol% PEG/(area of lipid molecule A_L), then the following equation can be fitted to the data in Fig. 5:

$$\text{Relative intensity} = (\text{Factor}) \times \exp(-A_{Av}/A_L \times 0.01 \times \text{mol\% PEG}). \quad (2)$$

The obtained value for the area ratio A_{Av}/A_L of 48.9 is very close to the expected ratio of 46.5 for an area per avidin of 3025 Å^2 (Green, 1975) and a lipid area of 65 Å^2 (Lecuyer and Dervichian, 1969). The excellent agreement between the fit and the data, and the fact that the obtained area ratio matches the expected value, strongly indicate that the additional surface pressure that PEG provides is the reason for a reduced binding when a low concentration of PEG^{750} with its short chain length is present.

So far, models for adsorption of proteins to polymer covered surfaces have only been proposed for polymers in the brush regime (Jeon et al., 1991) that have fixed grafting points. The mechanism whereby a low density of highly mobile polymers grafted to a lipid membrane affects protein adsorption has not been investigated. The present results suggest that, at the low density of 2 mol% PEG^{750}, the binding of avidin to a biotinylated vesicle is hindered but not prevented by the surface pressure of the lipid-linked PEG^{750}.

Vesicle-vesicle adhesion via biotin-avidin-biotin cross-bridges

Fluorescence studies

We first used fluorescent avidin to demonstrate qualitatively that avidin accumulates in the contact zone between vesicles containing 1 mol% biotin-X-DHPE. After allowing several minutes for the biotin-avidin-biotin cross-bridges to accumulate, the vesicles were observed under epifluorescence. (Note that after switching to fluorescence no further accumulation occurs because the biotin-binding sites are destroyed by the intensive exciting light. Therefore, we could not observe the whole process under epifluorescence illumination, just the end point.) As shown in Fig. 6, a strong fluorescent adhesion zone, compared to the rest of the vesicle, indicated significant accumulation in this zone.

Chemical and mechanical equilibrium

As treated previously by others (Bell, 1978; Bell et al., 1984; Evans, 1985), ideal mixing relations establish equilibrium concentrations of cross-bridged avidin in the contact zone (c_c) compared to that for freely diffusing, surface-bound avidin (c_b) given by

$$kT \ln(c_c/c_b) = \Delta\mu_{B-Av}, \tag{3}$$

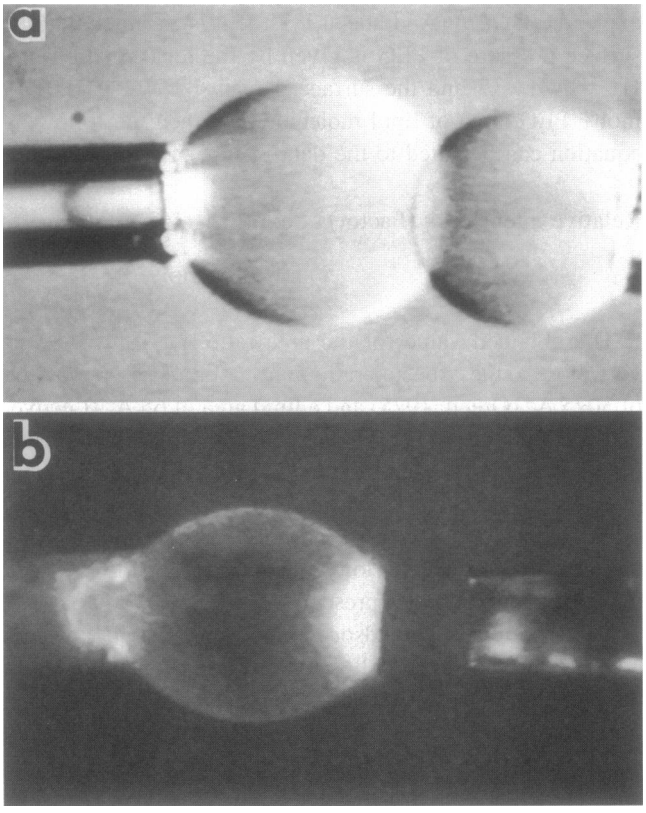

FIGURE 6 Video micrographs of adherent vesicles. (*a*) Vesicle pair in transmitted light and (*b*) different vesicle pair in epifluorescence. The accumulated fluorescently labeled avidin receptors form a very bright contact zone. The biotin concentration was 1 mol%.

where c_c and c_b are given by the following mass conservation relations:

$$c_c = n_c/A_c \tag{4a}$$

$$c_b = (n_t - n_c)/(A_t - A_c), \tag{4b}$$

in which n_t is the total number of avidin molecules per vesicle, n_c is the number of cross-bridged avidin molecules in the contact zone, and A_t and A_c are the areas of the whole vesicle and the contact zone, respectively.

This difference in chemical potential for cross-bridge formation $\Delta\mu_{B\text{-}Av}$ is equivalent to the binding energy of the biotin-avidin bond, which is on the order of $30kT$. Thus, $\ln(c_c/c_b) \cong 30$, and so there is an overwhelming tendency for all the surface-bound avidin to accumulate as cross-bridges in the contact zone. This is what we see in the fluorescent videomicrograph of Fig. 6 *b*, at least for 1 mol% biotin. Cross-bridged avidin is essentially irreversibly confined in the zone and so is not in equilibrium with surface-bound avidin in the rest of the vesicle. Any chemical equilibrium between surface-bound and cross-bridged avidin can therefore be ignored.

Mechanical equilibrium of the contact zone involves the spreading pressures of cross-bridged avidin in the contact zone, surface-bound avidin that is free to diffuse both outside and inside the zone, and the tension in the vesicle membrane (that is set by the micropipette suction pressure) (Evans, 1985, 1993, 1994). As depicted in Fig. 7, surface-bound avidin diffuses into the contact zone, becomes cross-bridged to a biotin on the opposite membrane, and is confined to the zone. Mechanical equilibrium throughout the adhesion process is therefore established when the membrane tension τ_m just opposes the excess spreading pressure of the confined, cross-bridged avidin $\Delta\Pi_{Av}$, according to the relation

$$\tau_m(1 - \cos\theta_c) = \Delta\Pi_{Av}. \tag{5}$$

During spreading, the avidin spreading pressure is the difference between the surface pressures exerted by avidin cross-bridges in the zone Π_{Av} (c_c) and that of surface bound (non-cross-bridged) avidin both inside Π_{Av} (c_{in}) and outside Π_{Av} (c_{out}) the zone:

$$\Delta\Pi_{Av} = \Pi_{Av}(c_c) - \Pi_{Av}(c_{out}) + \Pi_{Av}(c_{in}). \tag{6}$$

To obtain this pressure for different initial surface concentrations of avidin, micropipette adhesion experiments were carried out with vesicles containing 1, 2, and 5 mol% biotin incubated in avidin as described in Methods. As shown in the videomicrograph of the experiment (Fig. 3), for a biotin concentration of 5 mol%, no initial rapid spreading could be detected, but spreading did occur on the order of minutes. For a constant micropipette suction pressure, vesicle and pipette geometry during spreading gave the change in the contact angle between the vesicles and therefore the spreading pressure $\Delta\Pi_{Av}$ from Eq. 6. This spreading pressure is plotted as a function of time after vesicle-

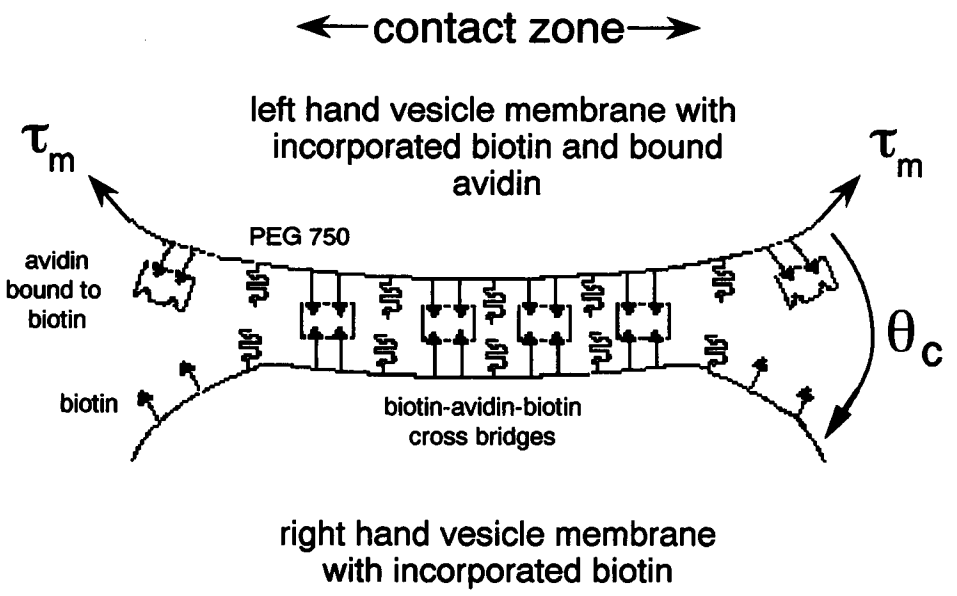

FIGURE 7 Schematic of the contact zone between two vesicles, showing the arrangement of avidin, biotin, and PEG^{750} on each vesicle, membrane tension, and contact angle.

vesicle contact has been made in Fig. 8 for the three initial concentrations of biotin. Several experiments at each concentration showed that the surface pressure approached or even attained a plateau; the average values for these maxima are given for each plot, arbitrarily shown at $t = 900$ s. These saturation results indicate that avidin accumulates and the spreading pressure increases until either i) all the avidin in the vesicle surface is in the contact zone, or ii) avidin reaches a maximum surface density that is equivalent to its minimum area per molecule. Because of the overwhelming tendency for all the avidin to be bound in the contact zone, which of these situations actually occurs will depend on the initial concentration of avidin bound to the surface before contact between the vesicles is made. For a 2-min incubation in avidin solution, fluorescence intensity measurements showed that the initial amount of avidin bound increased with increasing biotin concentration (results not shown). However, in the absence of a reliable and standardized fluorescence calibration for the amount of avidin on the surface and in the contact zone, we cannot make any stronger statement about the amount of accumulation except to say that the increasing surface pressure indicates an accumulation over and above the initial amount bound.

Under conditions of low initial avidin concentration (such that the surface bound avidin is free to come to equilibrium), all of the bound avidin would be expected to accumulate in the contact zone, and an estimate of the concentration of cross-bridges in the contact zone can be made from the ideal mixing relation (Bell et al., 1984; Evans, 1985):

$$\Delta\Pi_{Av} = \Pi_{Av} = c_c\, kT. \tag{7}$$

Here, the excess surface pressure is simply the spreading pressure due to the confined cross-bridges. Estimates of the density of avidin cross-bridges in the zone using this equation are given in Table 1. This ideal treatment of the data gives an estimate of how the concentration of cross-bridged avidin is increased in the contact zone. For nonideal behavior though, an independent measure of the cross-bridge density is required, and this can be obtained from mass conservation and areas of contact as presented below (under Confined Macromolecules).

Time dependence for contact formation

The data in Fig. 8 can now be used to obtain an indication as to the rate-determining step in the adhesion process. As discussed previously by Evans (1985), three time increments are important: t_b, the reaction time for binding and cross-bridge formation; t_D, the diffusion time for concentration equilibration; and t_m, the mechanical response time for contact area changes. The chemical reaction time is expected to be negligible compared to t_D and t_m, and for lipid vesicles, where surface viscosities are very small, the mechanical response time is expected to be less than a second. A first approximation for the diffusion coefficient $D_{B\text{-}Av}$ that determines the accumulation of biotin-avidin-biotin bridges is given by

$$D_{B-Av}{}^2 \sim r^2/t, \tag{8}$$

where r is the radius of the vesicle and t is the time needed to reach a significant accumulation. Thus, using typical data from our experiment ($r = 15$ μm and $t \sim 200$s), $D_{B\text{-}Av} \sim 10^{-8}$ cm^2/s, which is in close agreement with the diffusion coefficient for egg phosphatidylcholine lipid (Edidin, 1974). Because the biotin-avidin "receptors" are attached to freely diffusing lipid molecules in the lipid bilayer membrane, the approach to equilibrium is therefore simply determined by the rate at which the lipid-linked macromolecules can diffuse into the contact zone from the surrounding vesicle membranes and is not hampered by mechanical responses of the lipid membrane.

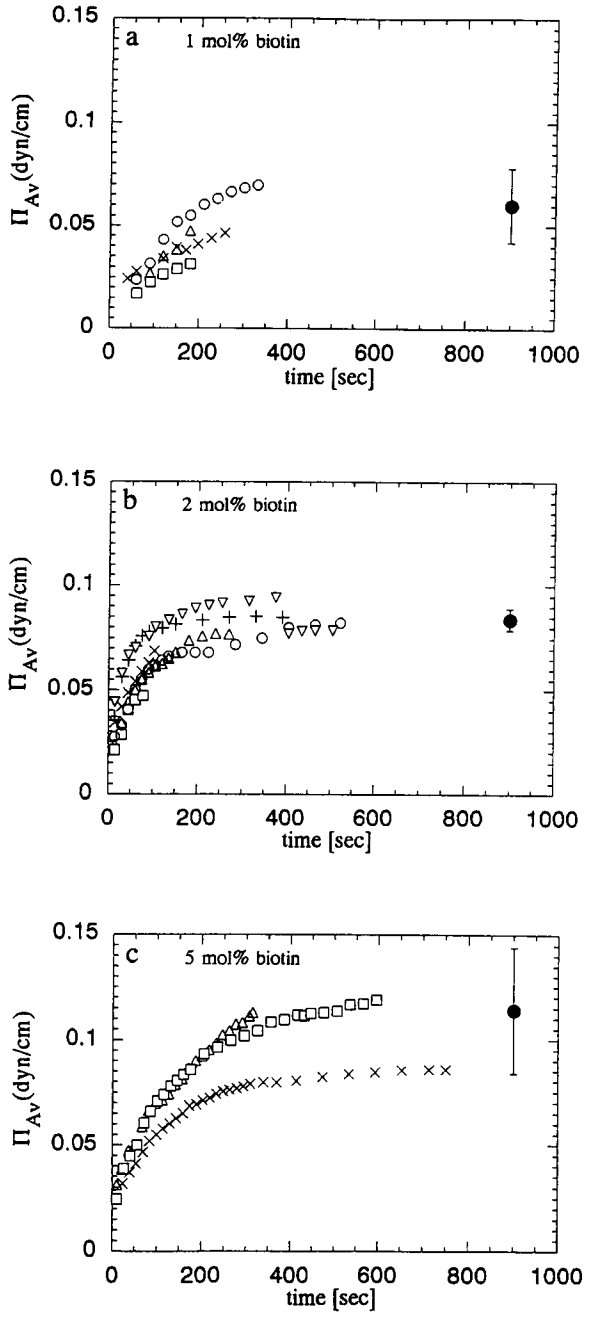

FIGURE 8 Increase of spreading pressure of bound receptors ($\Delta\Pi_{Av}$) in the contact zone as a function of time after adherent contact is made. The behavior for three different initial biotin concentrations is shown. (*a*) 1 mol% biotin-lipid; (*b*) 2 mol% biotin lipid; (*c*) 5 mol% biotin-lipid. Average maximum values of the spreading pressures are arbitrarily represented on the plots at t = 900 s.

Peeling of contact

Attempts to peel the membranes apart, by increasing the suction pressure on the pipette holding the left-hand vesicle, resulted in only a slight decrease of the contact zone, indicating very limited compression of bound receptors in the contact zone, which, as expected, appear to be packed fairly densely. With continued attempts at peeling, the vesicle membrane itself expanded and eventually failed, i.e., peeling tensions exceeded the tensile strength of the lipid membrane, even with equimolar amounts of cholesterol in the vesicle membranes. This result is in accordance with those of Leckband et al., in which supported bilayers bound together by biotin-avidin-biotin cross-bridges were also found to fail upon attempts at separation (Leckband et al., 1992, 1994). Thus, the biotin-avidin-biotin bonds were very strong and essentially irreversible under the tensions (~10 dyn/cm) that can be imposed on lipid vesicles (Needham and Nunn, 1990). With regard to theoretical predictions concerning peeling kinetics (Dembo, 1988; Evans, 1985, 1993; Kuo and Lauffenburger, 1993), it would be interesting to relate the critical tension that is necessary to start the peeling of an adhesive contact to the individual bond strength. Furthermore, the peeling velocity at a given tension relates to the rates of bond formation and dissociation and to the strain placed on individual bonds. These kinds of predictions are, unfortunately, not testable in the strongly bonded avidin-biotin model vesicle system. However, it should be possible to examine these aspects of peeling by using modified biotins that have a significantly lower binding affinity to avidin or various avidin mutants (Chilkoti et al., 1995).

TABLE 1 Surface concentrations of avidin cross bridges (c_c) for the initial concentrations of biotin of 1, 2, and 5 mol%

Biotin concentration in vesicle (mol%)	Cross-bridge density c_c from $\Pi_{Av} = c_c\, kT$ (Av/cm^2)
1	1.5×10^{12}
2	2.1×10^{12}
5	2.8×10^{12}

Cross-bridge density is calculated from the surface pressure Π_{Av} assuming ideality of mixing in the contact zone.

Confined macromolecules: colligative properties from true equilibrium

Up to now we have used the spreading pressure data and Eq. 8 to predict amounts of avidin in the contact zone from simple assumptions about ideal behavior. The power of this new technique, however, lies in an ability to detect small membrane tensions that oppose the spreading pressure of confined cross-bridges and to use this information to measure the true colligative behavior of the cross-bridged macromolecules, i.e., to determine whether avidin (or other macromolecules) behaves ideally or shows some lateral interactions and possibly mutual aggregation. Thus, to evaluate the colligative behavior of avidin cross-bridges an independent estimate is needed of the concentration of cross-bridges in the contact zone. Such an independent measure could come from a calibration of the fluorescence, although contact zone geometry, bleaching of fluorophores, self-quenching at high surface densities, and free radical formation that cross-links the proteins become major obsta-

cles to using fluorescence analysis. Instead, estimates of the amount of avidin in the contact zone are most easily achieved from a simple mass conservation of receptors. By using relatively low initial concentrations of avidin, it can be ensured that all the avidin in the vesicle will accumulate in the contact region. For this case, $n_c \approx n_t$, $c_c \gg c_b$.

This is an interesting and potentially very useful result, because it shows that we can measure the spreading pressure of avidin in the contact zone simply by measuring the tension in the vesicle membrane that just prevents spreading when all the avidin in the vesicle surface accumulates in the contact zone in the form of cross-bridges. Under these conditions, the concentration of avidin in the zone (c_c) is given independently by the initial avidin concentration in the vesicle c_i multiplied by the area ratio of contact formation,

$$c_c \approx c_i(A_c/A_t), \tag{9}$$

where A_c and A_t are the area of contact and the total area of the vesicle, respectively.

These estimates for the spreading pressure and concentration of avidin in the contact zone then provide a unique way to determine the colligative properties of the accumulated avidin by plotting Π_{Av}/kTc_c versus c_c. For ideal behavior, this plot should have zero slope and should intercept at $\Pi_{Av}/kTc_c = 1$. For the results shown in Fig. 8, estimates for c_c (obtained by assuming that all the avidins accumulated in the contact zone and that the area of contact A_c was typically 20% of the total adherent vesicle area) are plotted against Π_{Av}/kTc_c in Fig. 9. The negative slope of this plot appears to indicate that some aggregation of the avidin cross-bridges occurred and, furthermore, an intercept close to 0.5 suggests that avidins in the contact zone may have formed dimers. Such interpretations of these results, however, must be viewed with some caution because, in the present experiments, the initial concentrations of avidin were already relatively high. Except for the lowest concentration (for the 1 mol% biotin vesicles) the assumption that all of the avidins accumulated in the contact zone may not be correct, especially for the 5 mol% biotin vesicles. For these high initial concentrations, the limiting factor for final degree of spreading could be due to a saturating compaction of avidin in the zone.

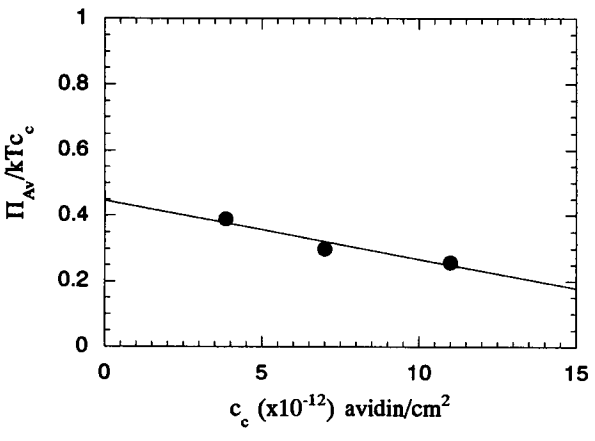

FIGURE 9 Π_{Av}/kTc_c versus c_c plot. Negative slope indicates aggregation of avidin, and an intercept of 0.5 indicates dimerization of avidin in the contact zone.

Influence of surface-grafted PEG

The influence of surface-grafted PEG on the above mechanical equilibrium can be simply represented by a negative surface pressure that the polymer layer exerts when the membranes are adherent. In the presence of such polymer, the mechanical equilibrium is now a balance between the membrane tension and the combined surface pressure differences for avidin ($\Delta\Pi_{Av}$) and PEG ($\Delta\Pi_p$),

$$\tau_m(1 - \cos\theta_c) = \Delta\Pi_{Av} + \Delta\Pi_p, \tag{10}$$

where, $\Delta\Pi_p$ is given by the chemical equilibrium,

$$\Delta\Pi_p = \Pi_p(c_o') - \Pi_p(c_c'), \tag{11}$$

where c_o' and c_c' are the surface concentrations of polymer outside and inside the contact zone, respectively. For two vesicles with the same total areas (A_t) and total number of polymer lipid molecules per vesicle (n_t'), these concentrations are

$$c_c' = n_c'/A_c \tag{12}$$

$$c_o' = (n_t' - n_c')/(A_t - A_c), \tag{13}$$

where n_c' is the number of polymer molecules in the contact zone, and A_c is the area of contact between the vesicles.

Because the polymer-lipids are free to move, ideal mixing of polymer molecules produces a new equilibrium according to

$$\ln(c_o'/c_c') = \Delta\mu_{steric}/kT, \tag{14}$$

in which $\Delta\mu_{steric}$ is the steric energy, i.e., work to compress a random coil polymer chain.

A significant redistribution of the PEG repellers will only occur if they are significantly compressed. The value for $\Delta\mu_{steric}$ is only on the order of kT, and so only when the repulsion energy per repeller reaches a value of $\sim kT$ will any significant redistribution occur. Because the extended length of the PEG^{750} is ~2.5 nm (Kenworthy et al., 1995), which is approximately half the avidin molecule height (as depicted in Fig. 1), such redistribution of repellers is not expected for this particular PEG^{750}-lipid, a conclusion supported by the observation that the fluorescence intensity of the zone (measured for vesicles in which 2 mol% PEG^{750} was incorporated in the membrane) was similar to that without the PEG^{750}. As mentioned earlier, in the absence of biotin-avidin-biotin cross-bridges, however, the presence of the lipid-linked PEG^{750} did prevent binding of vesicles due to nonspecific van der Waals attraction, and in this way it performed an important function. This analysis suggests that even if the steric repulsion from repellers is of longer

range than the size of the avidin molecule (for example, with 2000 or 5000 MW PEGs), the cross-bridges will still form (at equilibrium) because $\Delta\mu_{Av} \gg \Delta\mu_{steric}$, but the dynamics of contact formation may well be affected, as suggested by Fig. 4.

CONCLUDING REMARKS

These results and analyses therefore set guiding experimental boundaries for examining cross-bridge-dependent adhesion with the biotin-avidin system. Clearly, lower membrane concentrations of biotin than used in the present experiments are required to ensure total accumulation of avidin cross-bridges in maximally spread contacts. Only then can the surface pressure be used to determine the colligative properties of the confied avidin. In addition to establishing an experimental adhesion system that can represent features of diffusion-limited, receptor-mediated adhesion, we have discovered a uniquely sensitive method for measuring the spreading pressure of confined macromolecules. Thus, the surface pressure, and therefore colligative characteristics, for a range of macromolecules could now be assessed by attaching the molecule to a lipid anchor and then providing a second vesicle with lipid-anchored molecules that bind to the test molecule. For macromolecules that are larger than avidin, one might simply sandwich a biotinylated macromolecule between biotin-lipid/avidin complexes expressed on both vesicle surfaces. Alternatively, a biotinylated macromolecule (such as an antibody) can be bridged directly to a lipid-linked antigenic moiety (such as DNP). This method should complement more traditional studies in which the surface pressure of a monolayer of a given macromolecule, spread on a Langmuir trough, is determined by measuring the surface tension of the monolayer (Roberts, 1990). Our vesicle-vesicle adhesion experiment has a lower limit of ~0.01 dyn/cm and should therefore allow more sensitive measurements to be made. Thus, the presentation of two vesicles to each other with the ability to control and measure membrane tension has produced an unexpected method in which lateral interactions between a variety of macromolecules can be studied.

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