Electrostatic Nanotitration of Weak Biochemical Bonds

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We introduce a new method to measure the energetics and range of weak biochemical bonds using functionalized vesicles. Large bilayer regions are held in molecular proximity by osmotic depletion forces to enable rapid specific bonding. By fixing an electrical charge to the tethering site of the functional group on one surface, persistent adhesion of the vesicles after removal of the depletion stress is titrated against the clamped electrostatic potential of the opposite surface. We demonstrate the method with DNA bases and obtain new information on the range of their specific interactions. [S0031-9007(97)04018-0]

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Nature has chosen weak biochemical bonds to play important roles in biology—e.g., the interaction energy between a complementary pair of nucleic bases of DNA is perhaps only $\approx 5\ k_BT$. Yet, bonds of a few k_BT in energy are extremely difficult to probe by any of the existing force techniques (atomic force microscopy [1], surface forces apparatus [2], optical tweezers [3], bead force probe [4], etc.) because these bonds have very short lifetimes with little strength on the time scale of experiments. Thus, we have developed a new method to titrate the specific chemical interaction of a weak bond against a controlled electrostatic bias. The concept is based on forced confinement of the reactant groups to a molecularly thin layer. When the surfaces are sufficiently close, the specific bonds equilibrate rapidly; but if separated beyond the range of the specific interaction, no bonding occurs [5]. Simultaneously, specific bonds that form are armed with a controlled repulsive potential which is triggered simply by release of the confining stress. If this potential exceeds the binding energy, the surfaces unbind; if not, they remain adherent. The experimental approach is to chemically graft a functional group [6] to the headgroup of a membrane lipid bearing a single electrical charge, which is then mixed with neutral lipids into the surface of a lipid bilayer vesicle #1. The counter structure (receptor group) is grafted to a neutral lipid, which is mixed with neutral and electrically charged bare lipids in the surface of a second bilayer vesicle #2. The charged lipids clamp the surface potential of vesicle #2. Then, using a novel preassembly technique, vesicle #1 is brought into molecular proximity with vesicle #2 and released to test for adhesive bonding. By titrating the electrolyte concentration in the aqueous environment and the surface charge density in vesicle #2 against adhesion, both the range and magnitude of the specific chemical interaction can be established. Here, we demonstrate the nanotitration method for weak hydrogen bonding between *A* and *T* nucleosides.

Three new diacyl lipids were synthesized [6] by grafting nucleosides to lipid chains, a negatively charged adenosine lipid (DOSPA), a neutral adenosine lipid (DOSA), and a neutral thymidine lipid (DOST) (see Fig. 1). Negatively charged bare lipids stearoyl-oleoyl-phosphatidylserine (SOPS) and neutral stearoyl-oleoyl-phospatidylcholine lipids (SOPC), were also used. Large vesicles were formed by hydration of mixtures (the compositions are listed in Table I) of these nucleoside lipids with the bare lipids (dried from chloroform-methanol solution) in 0.3 M sucrose [7]. The composition of the lipid mixtures was chosen according to the required charge. These lipids chains were unsaturated. This provided membrane vesicles in a fluid state, which ensured good mixing of the lipids. Vesicles labeled #1 contained DOSPA and vesicles labeled #2 contained either DOSPA or DOST plus SOPS to set the surface charge density. All experiments were performed at a fixed *p*H of 5.5.

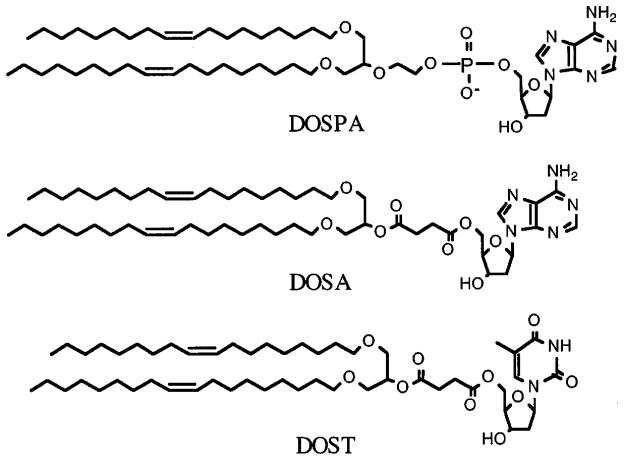

FIG. 1. Structures of the functionalized lipids. DOSPA is negatively charged while DOSA and DOST are neutral.

TABLE I. First two columns: composition of the vesicles; third column: range of ionic strengths that brackets the crossover C^* from bound to unbound vesicles; the limits on binding energy E_b that correspond to the bracket of ionic strengths as calculated with Eq. (1) (using an area per lipid [11] of 0.65 nm^2 and a single negative charge for DOSPA and SOPS with DOST, DOSA, and SOPC uncharged).

Vesicle #1	Vesicle #2	C^* (mM)	Binding energy (k_BT)
DOSPA/SOPC (5:95)	DOST/SOPS/SOPC (5:5:90)	$1 < C^* < 10$	$1.83 < E_b < 3.83$
DOSPA/SOPC (5:95)	DOST/SOPS/SOPC (5:10:85)	$1 < C^* < 10$	$2.97 < E_b < 5.18$
DOSPA/SOPC (5:95)	DOSA/SOPS/SOPC (5:5:90)	$5 < C^* < 50$	$0.90 < E_b < 2.37$

Using micropipets, single vesicles were selected from a dilute suspension in a chamber on the microscope stage and transferred to an adjacent chamber that contained NaCl (plus glucose needed for osmotic balance) and PEG 20000 polymer. To identify vesicles in the initial suspension, the two types of vesicles were prepared with different optical densities, which could be easily discriminated in the Hoffman modulation contrast microscopy. After transfer to the polymer solution, the vesicles were maneuvered to just touch in the second chamber where a macroscopic-size contact ($\approx 10\ \mu m^2$) was formed immediately, driven by the attractive depletion force (Fig. 2) [8]. Next, the adherent vesicle pair was released from the depletion stress by transfer into a third chamber that contained only NaCl (plus glucose). At this point, the only interaction left to sustain persistent adhesion of the vesicles in opposition to the electrostatic repulsion was the specific nucleoside attraction. If the internucleoside binding energy was smaller than the electrostatic double-layer energy, the vesicles separated; otherwise, they remained in contact. In accordance with Gouy-Chapman theory [9], the double-layer energy, scaled to one DOSPA molecule, E_{dl} depends on the charge density ρ_e (number of charges per nm^2) and ionic strength c_i (mol/l) : $\sinh(E_{\mathrm{dl}}/2k_BT) \approx 1.36\,\frac{\rho_e}{\sqrt{c_i}}$ (1). The double-layer energy E_{dl} was adjusted, by varying the salt concentration c_i (from 1 to 200 mM) and the SOPS density ρ_e in the #2 vesicles.

First, we titrated the nucleoside binding interaction against the ionic strength for fixed concentration of charged SOPS lipid. The ionic strength was reduced until the electrostatic repulsion was sufficient to unbind the adherent vesicles after transfer from the polymer solution. To stabilize initial contact in the polymer solution, the long-range electrostatic repulsion was overcome by a strong depletion stress in a 5% to 10% w/w concentration of PEG. As seen in Table I, the crossover between persistent adhesion and separation established bounds for the binding energy based on the discrete value of ionic strength used in the experiments. The binding energies deduced from the results in Table I for A/T and A/A interactions are in good agreement with values measured by other methods as seen in Table II.

Second, to test the range of the specific interaction, the concentration of PEG polymer was lowered until the persistent adhesion vanished, even though a stable depletion-driven contact existed before transfer from the initial PEG solution. For example, specific bonding of vesicles with 10 mol % charged SOPS lipid and 5 mol % reactant lipids (cf. second line of Table I) disappeared when the PEG concentration was reduced from 10%

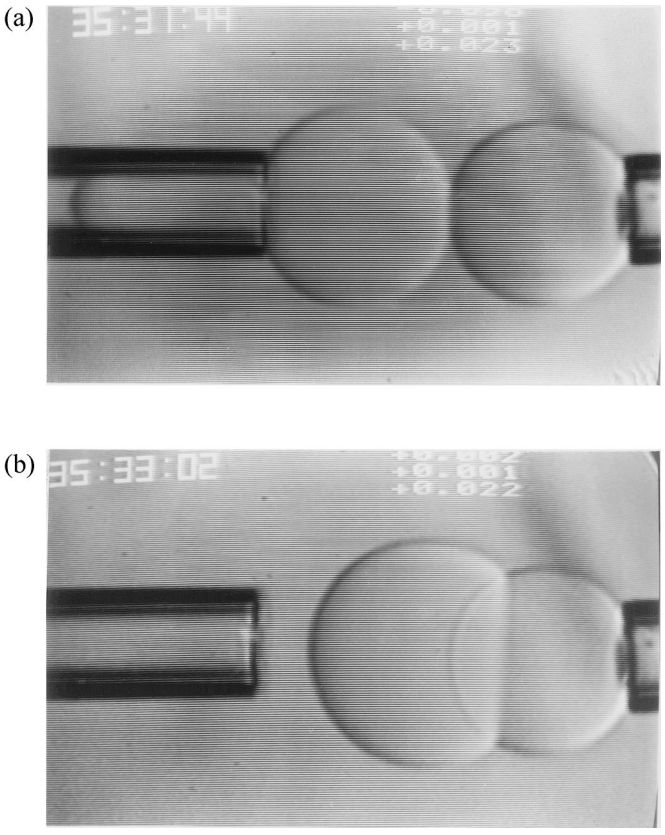

FIG. 2. Video micrographs of vesicle adhesion driven by polymer depletion in a 10% solution of PEG 20000. (a) The vesicles are maneuvered into point contact by micropipets. (b) The left hand vesicle is released from its holding pipet to enable adhesion to the right hand vesicle. The adherent vesicle pair was then transferred to a polymer-free solution to test persistent bonding after removal of depletion force.

TABLE II. Comparison of experimental values with published binding energies [10,11].

Binding energy (k_BT)	Experimental values	Literature values
A/T	$2.97 < E_b < 3.83$	3.5
A/A	$0.90 < E_b < 2.37$	1.7

to 5% (w/w) even in 20 mM salt. Analyzing the balance of osmotic depletion stress and electrostatic repulsion for these polymer concentrations shows that the initial separation between the surfaces increased from near molecular contact (stabilized by repulsive hydration and steric forces) in the 10% PEG solution to ≈2 nm in 5% PEG, which was the approach used to obtain a bound for the range of the specific interactions. The range of 2 nm for the A/T interaction is much smaller than the long-range attraction (≈38 nm) seen in SFA experiments with the functionalized lipids supported on mica substrates [10,11].

This new titration technique provides a simple approach to evaluate binding energies (from $2-25\ k_BT$) [12] and ranges of biochemical bonds under conditions most relevant for biology. In particular, the reactants are restricted to soft-flexible interfaces whose stiffness can be controlled, which provides an environment similar to bonding between semiflexible polymer chains. As such, the electrostatic and steric microenvironment around the binding sites can be preset by introducing membrane lipids with special charge and polymer moieties grafted to the headgroups. With this approach, it is possible to examine the nontrivial consequences of membrane conformational degrees of freedom and mobile-focal adhesion sites, which remain as unresolved issues between recent theoretical models [13].

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[1] G. Binnig, C. F. Quate, and C. H. Gerber, Phys. Rev. Lett. **56**, 930 (1986).

[2] J. N. Israelachvili and G. E. Adams, J. Chem. Soc. Faraday Trans. **174**, 975 (1978).

[3] A. Ashkin, Biophys. J. **61**, 569 (1992).

[4] E. Evans, K. Ritchie, and R. Merkel, Biophys. J. **68**, 2580 (1995).

[5] At equilibrium, the adhesion energy between surfaces with mobile reactants (ligands and receptors tethered to lipids) is simply the surface pressure produced by the crossbridged groups confined to the contact region (i.e., energy/area $\approx \rho_b k_B T$ where ρ_b = bound sites/area). The concentration of bound sites in the contact zone is set by the 2D equilibrium between bound and free sites [i.e., $\ln(\rho_b/\rho_f) \approx$ binding energy]. See Appendix of E. Evans, Biophys. J. **48**, 175 (1985); M. Dembo, Proc. R. Soc. London B **234**, 55 (1988). Rapid diffusion of the ligand/receptor groups is essential to the success of this measurement. The key question is what is the equilibration time ($t_D \sim 4\pi R^2/D$)? For 10 μm size vesicles and fluid lipids with diffusivity of order $D \sim 10\ \mu m^2/sec$, the equilibration time is of order 1 min, which is well within the waiting period at each step in the experiment.

[6] Functionalized lipids were synthesized by coupling the unprotected nucleosides with 2-(1,3-dioleyloxy) propyl hemisuccinate using a modified DCC/DMAP method. L. Lebeau, S. Olland, P. Oudet, and C. Mioskowski, Chem. Phys. Lipid **62**, 93 (1992).

[7] D. Needham and E. Evans, Biochem. **27**, 8261 (1988).

[8] Depletion-driven attraction arises as a subsequence of steric repulsion of the polymer chains from nonadsorbing surfaces. When separation between the surfaces reaches the scale of the correlation length of the polymer in solution, chains are excluded from the gap and the osmotic imbalance in the solvent pulls the surfaces together [J. F. Joanny, L. Leibler, and P. G. Gennes, J. Polym. Sci. **17**, 1073 (1979)]. See E. Evans and D. Needham, Macromol. **21**, 1822 (1988); E. Evans, Macromol. **22**, 2277 (1989); E. Evans, D. Klingenberg, and F. Szoka, Langmuir **12**, 3031 (1996) for details of depletion-driven attraction between lipid bilayer vesicles and methods used in testing bilayer vesicle adhesion in concentrated solutions of nonadsorbing PEG and Dextran polymers.

[9] R. M. Peitzsch and S. Mc Laughlin, Biochemistry **32**, 10 436 (1993).

[10] F. Pincet, E. Perez, G. Bryant, L. Lebeau, and C. Mioskowski, Phys. Rev. Lett. **73**, 2780 (1994).

[11] F. Pincet, E. Perez, G. Bryant, L. Lebeau, and C. Mioskowski, Mod. Phys. Lett. B **10**, 81 (1996).

[12] To determine a sensitivity limit for the novel technique presented in our manuscript, many subtle aspects must be taken into account. The most important to consider is steric repulsion and unbinding driven by confinement of collective bending excitations as originally worked out by Helfrich [W. Helfrich, Z. Naturforsch. **33A**, 305 (1978); W. Helfrich and R.-M. Servuss, Nuovo Cimento Soc. Ital. Fis. **D3**, 137 (1984); R. Lipowsky and S. Leibler, Phys. Rev. Lett. **56**, 2541 (1986); E. Evans and V. A. Parsegian, Proc. Natl. Acad. Sci. U.S.A. **83**, 7132 (1986)]. The most restrictive estimate is based on the critical level of short-range adhesion energy ($c_0 E_b^*$) below which the unbinding transition is expected in the absence of lateral tension [R. Lipowsky, Europhys. Lett. **7**, 255 (1988); E. Evans, Langmuir **7**, 1900 (1991)]. As shown by Lipowsky and coworkers [R. Lipowsky *et al.*, Phys. Rev. Lett. **77**, 1652 (1996), and references therein], this threshold is determined by the magnitude of the steric interaction in the range of the attraction l_a, i.e., $c_o E_b^* \sim 0.2\ (k_BT)^2/(k_c l_a^2)$. Given the 1 nm range of attraction found here and the membrane bending stiffness for these bilayers of $\sim 25\ k_BT$, the critical level of bare adhesion energy would be on the order of 10^{-2} mJ/m^2, which for ligand-receptor fractions in the membrane above one mole percent ($c_o > 0.014\ nm^{-2}$) predicts unbinding at an energy/bond of a small fraction of

$E_b^* < 0.2\ k_BT$. For weak bonds of order k_BT, the fraction of adhesive molecules is raised to 5 mol % or more so that fluctuations are not expected to significantly affect the sensitivity.

[13] D. Zuckerman and R. Bruinsma, Phys. Rev. Lett. **74**, 3900 (1995); R. Lipowsky, Phys. Rev. Lett. **77**, 1652 (1996).