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Subnanometer Actuation of a Tethered Lipid Bilayer Monitored with Fluorescence Resonance Energy Transfer

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Biofunctional solid surfaces incorporate characteristic features of both inorganic and biological chemistry[1−3] and are used for applications ranging from biosensing to drug screening and diagnostics. A topic of great interest in the field is the coupling of biomembranes to functionalized surfaces.[4−6] This can be achieved using lipid bilayer membranes of either spherical[7,8] (vesicular) or planar[9] geometry that are physisorbed or bound on a substrate. Use of lipids modified with different chemical groups, such as biotin[7] or cDNA strands,[8,10] provides a way to mediate specific binding of the membrane to the surface. The main motivation for working with such supported or tethered planar membrane systems is characterizing the properties and function (i) of membranes and (ii) of transmembrane proteins with the plethora of available surface-sensitive techniques.[11,12]

The main drawback of supported membranes is unspecific interactions with the surface that may lead to a nonphysiological environment by steric restriction of both lipid and protein movement.[6] One way to overcome this problem is the use of polymers as intermediate tethers between the membrane and the substrate.[13] Increasing the length of the polymer tethers increases the membrane−surface distance (D_{MS}); the efficiency, however, is rather low since the Flory radius (R_F) of a polymer in a mushroom regime increases only with the 3/5 exponent of the polymer's full length. Higher polymer surface densities can increase D_{MS} further (due to a phase transition of the tethers to the brush regime) but at the same time reduce (i) lipid/protein mobility[14,15] and (ii) the free aqueous space under the bilayer. To overcome these issues, we present here a method that employs electrostatic forces to actuate the membrane−surface separation, stretching reversibly the polymer tethers as a function of easily accessible experimental parameters as ionic strength (Figure 1A, B).

The separation of the bilayer membrane from the surface is determined by the equilibrium length and spring constant of the flexible poly(ethylene glycol) (PEG_{2000}) tethers. Charges on the membrane and the surface introduce an additional repulsive or attractive electrostatic term that we modulated by changing the ionic strength of the solution.

We tethered the lipid bilayer to the surface using the well-characterized binding pair of avidin/biotin. Glass surfaces were functionalized via adsorption of biotinylated bovine serum albumin (BSA−biotin) and consecutive association of either avidin or streptavidin. The surface coverage of both proteins (results not shown) corresponded to 50 ± 5% of a full monolayer. We prepared giant unilamellar vesicles (GUVs) containing 0.1 mol% biotinylated poly(ethylene glycol) lipid (PEG_{2000}−biotin) and 10 mol% negatively charged lipids (POPG).

Biotinylated GUVs were observed to recognize the protein-functionalized surface and undergo rupture, that is, formation of bilayers, upon change of solvent[16] from physiological buffer to MilliQ water. Tethering of PEG_{2000}−biotin on the surface prevented free lateral diffusion of the molecule in the bilayer and phase separation.[17] The tether density was kept to a minimum (0.1 mol%) on account of previous mobility studies[14,15,18] showing a significant decrease in diffusion rates of lipids with an increase of tether density.

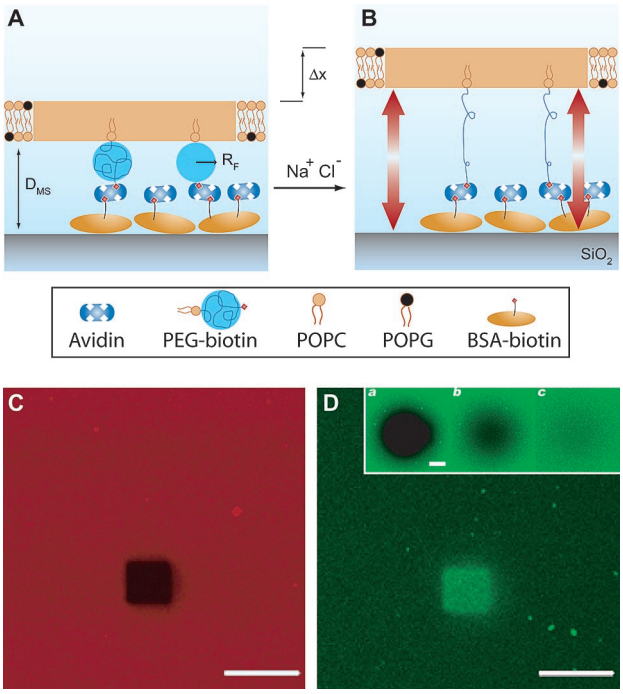

Figure 1. Ion-induced actuated displacement of a tethered bilayer. (A) Schematic illustration of a lipid bilayer supported by PEG_{2000} resting on a cushion of positively charged avidin, drawn to scale. The avidin is in turn bound to BSA−biotin adsorbed onto a glass substrate. The Flory radius of PEG_{2000} is indicated by the blue circles. (B) Upon addition of NaCl, the attractive electrostatic forces between the negatively charged bilayer and the positive avidin are shielded. Thus the new equilibrium conformation of PEG_{2000} is $D_{MS} + \Delta x$. (C) Confocal fluorescence microscopy (TCS SP2, Leica) of avidin conjugated to Texas Red. The dark square is photobleached. (D) PEG_{2000}-supported lipid bilayer labeled with the dye NBD. The bright square, an increase of the NBD fluorescence, is due to the bleaching of Texas Red, which is a FRET acceptor of NBD. Inset shows recovery of fluorescence from a site-selective photobleached spot on the bilayer membrane. This demonstrates the fluidity and homogeneity of the bilayer (see Supporting Information). All scale bars are 10 μm.

Fluorescence imaging was performed with a confocal microscope (TCS SP2, Leica). Fluorescence recovery after photobleaching (FRAP) experiments demonstrate convincingly the homogeneity and fluidity of the bilayers; see Figure 1D inset and Supporting Information. To monitor the variations of D_{MS}, we used fluorescence resonance energy transfer (FRET)[19] between the bilayer and avidin, labeled, respectively, with the donor/acceptor pair of NBD and Texas Red. Localized excitation with the 594 nm laser line allowed

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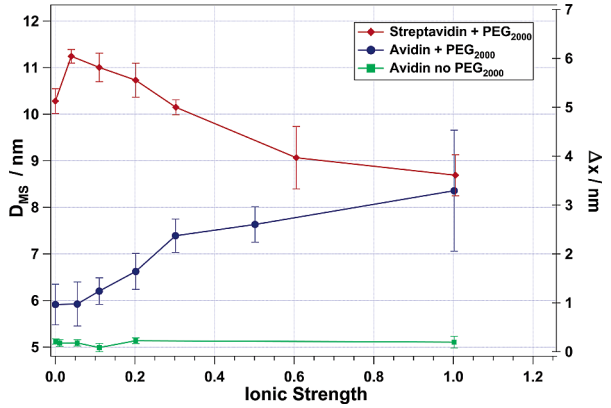

Figure 2. Controlled actuated displacement (D_{MS}) of a tethered lipid bilayer from the underlining substrate as a function of ionic strength. D_{MS} was measured using FRET between donors in the bilayer and acceptors on the surface. The control experiment shows that bilayers with no PEG_{2000} (green) exhibit practically no actuation with ionic strength. Surfaces functionalized with streptavidin (red) contract with increasing ionic strength; in contrast, systems with avidin (blue) stretch. D_{MS} is also shown relative to the control, *y*-axis on the right. Error bars are the standard deviation of >5 measurements.

the selective bleaching of the acceptor and a concomitant increase in the fluorescence of the donor (Figure 1C). FRET efficiency, E_F, was deduced from the ratio of donor fluorescence before and after acceptor bleaching. To calculate D_{MS}, we assumed a planar geometry of the assembled membrane and neglected FRET contributions from NBD fluorophores situated in the upper leaflet of the lipid bilayer.[20] This approximation in addition to the random positioning of Texas Red fluorophores in the protein tertiary structure prevents absolute values of D_{MS} to be inferred from the collected E_F; the data, however, may be used to compare different samples and extract accurately a relative displacement Δx. We looked at the dependence of D_{MS} on ionic strength at neutral pH for surfaces functionalized with two different proteins: avidin (positive, pI = 11) and streptavidin (negative, pI = 5−6).

We observed separations of up to 6 nm when employing proteins and bilayers with the same sign of charge (Figure 2). This corresponds to about 40% of the calculated full length of the tether (∼16 nm). Shielding of the repulsive electrostatic forces between streptavidin and POPG bilayers leads to a decrease in D_{MS} by 2.5 ± 0.2 nm. On the contrary, shielding of attractive forces between avidin and a POPG bilayer increases D_{MS} by a similar amount, from 5.9 to 8.4 nm.[21]

As a control system that should not be actuated, we used biotinylated lipids that did not have a PEG polymer extension but a short ∼4 Å carbon spacer. Indeed, within experimental errors (±0.1 nm), the control exhibited no actuation. We see that at the limit of high ionic strength both streptavidin- and avidin-tethered bilayers relax to a separation of ∼3.2 ± 0.2 nm above the control bilayer; this value corresponds well with the calculated R_F of PEG_{2000} that is 3.4 nm. The time constant of the actuation is related to the equilibration of the salt concentration above and below the bilayer and is hence diffusion limited. Under our conditions, the transition time was out of experimental access, that is, less than a minute.

FRAP measurements showed that a DOPE-conjugated Oregon Green 488 fluorophore in a silica-supported POPC/POPG bilayer has a diffusion coefficient of ∼0.51 ± 0.02 $\mu m^2/s$. Addition of 0.1% PEG lipid in such a bilayer increased lipid mobility by ∼40%. Tethering and stretching the system, however, gives an almost 80% increase (Figure 1D and Supporting Information). This last preliminary finding was rather surprising as we had expected that stretching the tethers would have an impact mainly on the mobility of transmembrane molecules with large extracellular parts and not so much on the mobility of lipids that anyway should be "shielded" by the PEGs from interactions with the solid substrate. A more systematic study of lipid mobility deserves further attention and most importantly should be complemented by mobility measurements of transmembrane peptides and proteins.

Here we demonstrate how to initiate and control in real time *nanoscopic* displacements of lipid bilayers by varying external *macroscopic* factors. These results highlight the dominant role long-range electrostatic forces play in the interaction of charged lipid bilayers with the underlining substrate; they should, therefore, be taken into consideration when designing the architecture of such systems in general.

Other actuation parameters that could be used apart from variation of ionic strength are changes in pH, affecting the overall charge of (strept)avidin, and temperature-induced changes of the polymer's spring constant. The experimental setup can be further optimized by choosing polymer tethers with a greater R_F. Controlled actuation combined with low tether densities and the resilience of PEG against dehydration[22] should allow the in vitro reconstitution of transmembrane molecules on surfaces under conditions that resemble more closely native membranes.

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Supporting Information Available: Materials and Methods, FRAP and FRET measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

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