Nanoparticle-induced surface reconstruction of phospholipid membranes

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The nonspecific adsorption of charged nanoparticles onto single-component phospholipid bilayers bearing phosphocholine headgroups is shown, from fluorescence and calorimetry experiments, to cause surface reconstruction at the points where nanoparticles adsorb. Nanoparticles of negative charge induce local gelation in otherwise fluid bilayers; nanoparticles of positive charge induce otherwise gelled membranes to fluidize locally. Through this mechanism, the phase state deviates from the nominal phase transition temperature by tens of degrees. This work generalizes the notions of environmentally induced surface reconstruction, prominent in metals and semiconductors. Bearing in mind that chemical composition in these single-component lipid bilayers is the same everywhere, this offers a mechanism to generate patchy functional properties in phospholipid membranes.

fluorescence  |  adsorption  |  phase transition

That phospholipid membranes possess patchy functional properties (different from spot to spot) is fundamental to their use as biomaterials and biosensors (1, 2) as well as abundant cellular activity (3–6). The extensive and sometimes contentious literature on the origins of spatial modulation supposes patchiness to arise from inhomogeneous distribution of the different lipids and other components within typical membranes and, in some cases, to specific binding (7–10). Here, phospholipid vesicles that do not satisfy the traditional requirements are stimulated to display spatial patchiness in response to nonspecific binding by charged nanoparticles. By using fluorescence and calorimetry methods to study membranes formed from single-component lipids with phosphocholine head groups, anionic nanoparticles are shown to induce local gelation in otherwise fluid bilayers and cationic nanoparticles to induce local fluidization of otherwise gelled bilayers. This work generalizes the notions of environmentally induced surface reconstruction, prominent in metals and semiconductors (11–13); however, unlike adsorption-induced surface restructuring of solids, the present systems are more strongly influenced by the high mobility of the lipid molecules that comprise phospholipid membranes. It also suggests origins of potential biological activity of nanoparticles that increasingly are exposed through the environment to living systems by accident and design (14, 15).

The hypothesis that motivates this study is summarized in Fig. 1: A phospholipid bilayer’s local phase state can be switched by binding of charged nanoparticles such that they alter the tilt angle of the phosphocholine (PC) head group, which is terminated by an electric dipole of phosphate and choline, P^−N^+. Negatively charged (anionic) nanoparticles interact preferentially with the N^+ terminus, raising the angle of the dipole above the average angle of 0° to ~3° characteristic of the fluid phase (16) and recruiting lipid tails to increase in density; conversely, positively charged (cationic) nanoparticles reduce the tilt angle below the angle of 30° to ~65° characteristic of the gel phase (16), stimulating a reduced lipid density. To exclude the traditional explanations of spatial patchiness based on specific binding, redistribution of membrane components, and phase separation between different lipids (7–10), these possibilities were eliminated by constructing phospholipid membranes comprised of a sole lipid type. The possibility of specific binding was eliminated by selecting lipids bearing phosphocholine head groups, which are unchanged under the buffer conditions of these experiments (17).

The phospholipids used here, DOPC (dioleoyl PC), DLPC (dilauryl PC), and DPPC (dipalmitoyl PC), have a gel-to-fluid phase transition temperature (T_m) of approximately −20 °C, approximately −1 °C, and approximately +40 °C, respectively. Large unilamellar lipid vesicles (liposomes) were prepared at 1 vol% concentration in PBS buffer (10 mM; pH 6.0) by the well-known extrusion method. The main nanoparticles used were carboxyl-modified (negatively charged; ≈0.91 e^-/nm^2) and

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The authors declare no conflict of interest.

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amidine-modified (positively charged; \(\approx 0.25 \text{ e}^-/\text{nm}^2\)) white polystyrene (PS) latex with a diameter of 20 nm; in control experiments, silicon dioxide nanoparticles (\(\approx 0.11 \text{ e}^-/\text{nm}^2\)) and supercoiled plasmid DNA were also used. Charged nanoparticles were mixed by vortex into the liposome suspension at the desired molar ratio (18). Measurements were performed at room temperature. It is known that charged nanoparticles, both anionic and cationic, adsorb to the PC group of phospholipids and that liposomes carrying adsorbed nanoparticles maintain their integrity as discrete liposomes (18, 19).

A simple initial test of our hypothesis was that adsorption by anionic nanoparticles should cause liposomes to shrink because the area per lipid head group is less in the gel than in the fluid phase. The anticipated shrinkage of initially fluid liposomes when anionic nanoparticles adsorb was confirmed, showing shrinkage by \(\approx 20\%\) [see supporting information (SI) Text]. A delicate point then became to decide whether phase-separated regions would clump together; the tendency to minimize line tension favors this, but electrostatic repulsion between charged nanoparticles resists it. Electron microscopy was not successful in imaging the spatial distribution of nanoparticles, their binding to liposomes being too weak to survive quench to the needed cryogenic temperatures. However, fluorescence imaging at room temperature revealed a highly dynamic spatial distribution and no aggregation of the nanoparticles on optical length scales when they adsorbed to giant unilamellar vesicles (GUVs). Förster resonance energy transfer (FRET) experiments described below set an even smaller upper bound on the size of phase-separated regions, 10–100 nm.

Moving to information on a molecular level, Fig. 2A illustrates the raw fluorescence data when anionic nanoparticles were allowed to bind to DLPC liposomes containing embedded Laurdan, which is an uncharged fluorescent dye whose emission is known to be diagnostic of a phospholipid membrane’s phase state (20). Normally, Laurdan assay is used to diagnose a membrane’s phase when temperature is varied; this work is considered an application to diagnose surface reconstruction. Fluorescence emission is plotted against wavelength, and one observes the progressive rise of blue emission and loss of red emission as nanoparticle concentration increased—suggestive of fluid–gel phase coexistence such that the proportion of fluid to gel phase varies. For quantification, emission intensity was compared at the wavelengths of peak emission intensity for bilayers in pure fluid and gel phases. Fig. 2B plots against normalized nanoparticle concentration the intensity fraction of these 2 peaks, and one sees that the changes are linear over a considerable span of nanoparticle concentration. Their normalized difference is the traditional definition of the net polarization, \(P = (I_B - I_R)/(I_B + I_R)\), where \(I_B\) and \(I_R\) are the emitted intensity at these wavelengths in the blue and red, respectively (20). From the data in Fig. 2B, \(P\) was calculated and found to vary smoothly between values characteristic of the membrane fluid phase (no added nanoparticles) and the gel phase (maximum concentration of added nanoparticles). The proportionality to nanoparticle concentration signifies that nanoparticles bound in proportion to their concentration in the environment and that lipid gelled in local spots where nanoparticles bound. Fig. 2C plots the implied lipid gel fraction against surface coverage.

The findings did not depend on choice of the lipid: The data are the same when the same anionic nanoparticles were used to bind to DOPC liposomes, whose \(T_{m}\) is approximately –20 °C. The findings did not depend on liposome size either, being indistinguishable for liposomes 200 and 80 nm in mean diameter. Silica particles had a similar but weaker effect (data not shown) but presented the advantage of allowing a range of particles of different size but similar chemical makeup. These experiments demonstrated that the nanoparticle size plays a minor role. The density of surface charge on the nanoparticles, nearly an order of magnitude larger for carboxyl-modified polystyrene latex than for silica, correlates with the stronger enhancement of the phase transition that was observed for carboxyl-modified latex. All these nanoparticle systems share the feature that charge on these objects was held rigidly in place. Adsorbed DNA, which also is anionic, did not produce this effect. We believe the reason to be that whereas DNA is flexible, the rigidity of charge placement on nanoparticles enables them to template the phase state of the phospholipids to which they bind. This null result for the case of flexible charged objects incidentally demonstrates that the photophysical response of the fluorescent dye was unmodified by charge, thus validating the data in Fig. 2.

The hypothesis of this article predicts the opposite effect for cationic nanoparticles. This was validated by allowing cationic nanoparticles to bind to a DPPC membrane (\(T_{m}\) \(\approx 40 \degree C\)), which at the experimental temperature (\(\approx 20 \degree C\)) displayed \(P \approx 0.6\) before nanoparticles were added. As shown in Fig. 2D, the net polarization then decreased, and detailed inspection of the emission raw data shows that the shift of emission wavelength was less pronounced than after adding carboxyl-modified nanoparticles to DLPC membrane as shown in Fig. 2B, consistent with the expected weaker binding of cationic particles (19). The decrease of \(P\) again suggests that the phase transition was localized to regions where nanoparticles had bound. In Fig. 2D, the final fraction of liquid phase can be estimated as 20%—although the experimental temperature was less than the literature value of this lipid’s \(T_{m}\). The addition of cationic nanoparticles to DLPC (\(T_{m}\) \(\approx -1 \degree C\)) liposomes induced negligible
change of net polarization, consistent with the anticipation that positively charged particles do not fluidize initially fluid lipids.

FRET experiments afforded an independent test of the hypothesis of nanoparticle-induced gelation, because the efficiency of energy transfer between 2 fluorescent dyes must decrease when they become spatially separated by partitioning into different phases. For this purpose, NBD and Rhodamine B (RhB) were selected because phospholipids bearing an NBD probe are known to partition into the gel phase of lipid membranes, but phospholipids bearing a RhB probe do not (21). Fig. 3 plots the logarithmic normalized emission against time on the nanosecond time scale under the conditions specified in the figure legend. The lifetime of NBD increased as anticipated after adding nanoparticles, consistent with its partitioning into the gel phase. In addition, the FRET efficiency in lifetime experiments decreased, indicating increased distances between the 2 dyes, as should be expected since the donor and acceptor dyes partitioned into different lipid phases. The magnitude of decrease is in the range expected from prior studies on microscopic phase separation involving 2 chemically different lipid components (21), corroborating the idea of local phase transition. The absolute value of the observed FRET efficiency shows that the domain size is in the range of 10–100 nm, according to theoretical estimates (22).

A final independent test of the hypothesis of nanoparticle-induced surface reconstruction consisted in using isothermal titration calorimetry (ITC) to measure the enthalpy (ΔH) of binding (23). Fig. 4 shows raw data when anionic nanoparticles were added to DLPC liposomes, and Fig. 4C shows the integrated heat change plotted against normalized nanoparticle concentration. Consistent with the hypothesis of nanoparticle-induced gelation, the initially exothermic binding was followed, at the highest NP concentrations, by an endothermic process, which is expected because binding-induced shrinkage of the liposome should be endothermic. Fitting gives the binding constant of ≈10^5 M^-1, corresponding to a modest free energy of binding, ≈20 k_BT per particle. The dramatic difference between enthalpy and free energy reveals a significant contribution from entropy, presumably partly from loss of entropy by gelation, partly from release of hydrated water from the PC head groups when nanoparticles bind. The deviation of the fitting in the range of high molar ratio may reflect cooperative gelation, which also reconciles the absence of plateau in heat change with linearity of the phase transition measured by fluorescence polarization. But in contrast to this, allowing positively charged nanoparticles to bind to gel-phase DPPC liposomes showed that this process was uniformly endothermic, as anticipated if nanoparticles caused the lipids to fluidize. The raw data (Fig. 4B) was integrated to give the integrated heat change plotted against normalized nanoparticle concentration in Fig. 4D. Thus, the sign of calorimetric heat changes is consistent with the conclusions that emerged from the independent fluorescence measurements. We speculate that cooperativity of this fluidization may be less than of the gelation transition, which may be facilitated by long-range correlations of lipid chain alignment, but no quantitative explanation is presented at this time.

The traditional notion of how to produce spatially patchy stiffness of a phospholipid membrane is by phase separation of lipids with different intrinsic stiffness (10); but when phospholipid membranes reconstruct locally in response to binding as described here, it is reasonable to infer that they will be locally stiffer in the gelled patches and less stiff in the fluidized patches, so nanoparticle-induced reconstruction of the phase state offers a new mechanism to modulate stiffness. Similarly, the traditional notion of how to produce a thermodynamic line tension in a phospholipid membrane is from the phase coexistence of different components (24); this article, showing that even the same lipid can coexist in 2 different phases according to what binds to it, presents an additional mechanism. Binding-induced structural reorganization also provides a potential mechanism to couple
particles were mixed by vortex into the liposome suspension at the desired phospholipid concentration, were related to D by using the Stokes–Einstein equation for a single-photon counting module. The translational diffusion coefficients sample through an objective lens. The excitation spot had a diffraction-limited diameter of 180 nm were purchased from Interfacial Dynamics. In control experiments, silicon dioxide nanoparticles with diameters of 4, 10, or 20 nm were obtained from Alfa Aesar. Plasminoids (4 kbp) with a radius gyration of ~10 nm were extracted from Escherichia coli by using standard protocols. Charged nanoparticles were incorporated into the liposome suspension at the desired molar ratio (18). From data given by the manufacturers, the surface charge density was calculated to be 1 charge per ~1.1 nm² for carboxyl-modified polystyrene nanoparticles, ~9 nm² for silica particles, and ~4.0 nm² for amine-modified nanoparticles. The fluorescent dye Laurdan, 6-dodecanoyl-2-dimethylaminonaphthalene, was purchased from Pierce. The FITC pair, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-dioleoylphosphatidylethanolamine (NBD-DPPE) and N-(lissamine-rhodamine B)-dioleoylphosphatidylethanolamine (RhB-DOPPE), were purchased from the same company.

**Materials and Methods**

**Materials.** The phospholipids D LPC (dilauryl PC), DOPC (dioleyl PC), and DPPC (dipalmitoyl PC) were obtained from Avanti Polar Lipids. Large unilamellar lipid vesicles were prepared at 1 vol% concentration in PBS buffer (10 mM pH, 6.0) by the well-known extrusion method, employing procedures described in detail in ref. 18. Carboxyl-modified (negatively charged) and amine-modified (positively charged) white polystyrene (PS) latex with a diameter of 20 nm were purchased from Interfacial Dynamics. In control experiments, silicon dioxide nanoparticles with diameters of 4, 10, or 20 nm were obtained from Alfa Aesar. Plasminoids (4 kbp) with a radius gyration of ~10 nm were extracted from Escherichia coli by using standard protocols. Charged nanoparticles were incorporated into the liposome suspension at the desired molar ratio (18). From data given by the manufacturers, the surface charge density was calculated to be 1 charge per ~1.1 nm² for carboxyl-modified polystyrene nanoparticles, ~9 nm² for silica particles, and ~4.0 nm² for amine-modified nanoparticles.

**Fluorescence Correlation Spectroscopy (FCS).** FCS measurements were carried out in the 2-photon excitation mode by using a home-built apparatus. Briefly, a near-infrared femtosecond-pulse laser was focused onto the sample through an objective lens. The excitation spot had a diffraction-limited diameter of ~0.35 μm. Fluorescence was collected and detected by a single-photon counting module. The translational diffusion coefficients (D) were obtained by fitting the fluorescence intensity–autocorrelation function model. The mean diameters of the liposomes, present at dilute concentration, were related to D by using the Stokes–Einstein equation (Fig. 1).

**Dye Orientation Within Liposomes.** The final concentrations of Laurdan and phospholipid were 0.8 μM and 0.4 mM, respectively (probe/phospholipid = 1:500). Steady-state emission spectra of Laurdan were measured by using a fluorometer (Photon Technologies) at magic angle condition with excitation at 340 nm. Emission spectra were corrected for instrument response. A fluorometer (Photon Technologies) at magic angle condition with excitation at 340 nm. Emission spectra were corrected for instrument response.

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**FRET Experiments.** The approach of measuring fluorescence lifetime was used to make time-resolved FRET measurements. The donor was NBD-DPPE (probe/phospholipid = 1:1000), the acceptor was RhB-DPPE (probe/phospholipid = 1:200), in liposomes with 200 nm mean diameter. Excitation, achieved by a frequency-doubled Ti:sapphire laser, was performed at 460 nm to minimize RhB excitation and emission was measured in the region 500–530 nm, which selectively characterize NBD emission. The time-resolved lifetimes were measured by using a data acquisition board purchased from Becker & Hickl. Because the fluorescence lifetimes did not follow single-exponential kinetics, the mean lifetime was defined, in the conventional way (21), as $\tau = \sum \alpha_i \tau_i$.

**Isothermal Titration Calorimetry (ITC).** Isothermal titration calorimetry was performed by using the VP-ITC high-sensitivity titration calorimeter (MicroCal) housed in the Post Genomics Institute at the University of Illinois. Typically, 58 consecutive aliquots of 5 μl each (for anionic PS particles), or 29 consecutive aliquots of 10 μl each (for cationic PS particles), containing nanoparticles at a concentration of 3 μM, were injected into the cell (1.43 ml) filled with 0.4 mM lipid solution (1 mM liposome). Both liposome and nanoparticle solutions were degassed under vacuum for 30 min immediately before use. Injections were made at 5-s intervals and had a 1 μl/s rate. A constant stirring speed of 300 rpm was maintained during the experiments to keep the suspension mixing after each injection. For calculation of the binding isotherm, the heat of dilution was measured in separate nanoparticle-buffer titrations and was subtracted. Surface coverage was estimated from the raw data as follows. First, the projected nanoparticle area showed complete surface coverage in hexagonal close packing to occur at $C_{\text{NP}} = 400$; this estimate gives an upper bound of surface coverage. Second, $C_{\text{NP}} = 300$ is the point up to which changes of ref. 25 polarization (P) were linear; therefore, the linear region in Fig. 2C ended at a fractional surface coverage of at least 0.75. Third, the calorimetry experiments showed that heat changes were completed approximately at $C_{\text{NP}} = 500$. Assuming the binding affinity is still constant in the range of molar ratio between 300–500, but weaker because of electrostatic repulsion, we were able to extrapolate the surface coverage, and found out the relation of P to surface coverage remained linear in the whole range. We should mention that the surface coverage is estimated on the strong assumption that no cooperativity of phase transition exists, which cannot strictly be true but provides meaningful physical insight into the situation and quickly affords comparison between different systems, which would be difficult to achieve otherwise.

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