Salt-induced lipid transfer between colloidal supported lipid bilayers†

Eric L. Kendall,Emily Mills,Juewen Liu,Xingmao Jiang,C. Jeffrey Brinker and Atul N. Parikh*‡

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We show that a coordinated interplay between mesoscale colloidal interactions and molecular-scale membrane perturbations affords a novel material platform to controllably recapitulate membrane interactions, such as during fusion and vesicle-based drug delivery. Specifically, a simple modulation of ionic strength is used to alter electrostatically determined colloidal interactions, producing conditions for pre-fusion contact between two independent colloidal and/or planar supported lipid bilayers. The same process also perturbs the membrane at the molecular level, reproducing conditions needed for subsequent fusion steps. We envisage that this platform will yield insights relevant to optimal design and implementation of lipid-coated inorganic constructs used for therapeutic drug delivery and sensing.

Interactions between closely apposed independent lipid bilayers are broadly relevant in many disparate lines of research. First, in living cells, lipid–lipid interactions represent some of the earliest defining events during membrane fusion—a universal mechanism for many vastly different biological processes.1–4 Examples span a broad range including exocytosis, organellar membrane trafficking, synaptic transmission, sperm–egg fertilization, enveloped viral infection, and biosynthetic vesicular transfection.5–9 Second, hybrid systems involving interactions between cells and synthetic lipid bilayers (vesicles and supported lipid bilayers) arise in the context of drug delivery10,11 as well as fundamental studies of cell signaling.12 Understanding how these delivery agents interact with native membranes of host cells is critical in optimally designing these vectors. Third, interactions between two synthetic membranes are also of interest in fundamental studies of interfacial interactions13 and can be exploited to create supported membrane systems that display compositional complexities.14

Much of our understanding of interactions between membranes has arisen from studies employing synthetic vesicles and planar unsupported lipid membranes, specifically in the context of fusion. Although catalyzed by different proteins, the energy barriers for biological membrane fusion in many cases (e.g., exocytosis, viral fusion, and membrane trafficking) appear comparable to those found for the fusion of protein-free phospholipid membranes.15 That lipid-based interactions play an important role during fusion is perhaps best exemplified by “lipid block” experiments.16 When biological membrane fusion is blocked by adding lipids of positive intrinsic curvature (e.g., lysolipids), fusogenic proteins still undergo their conformational transitions when triggered by appropriate ligands, (e.g., calcium or pH) but do not induce fusion. Lifting the lipid block, such as by removing those lysolipids or by adding lipids of opposite intrinsic curvature, reinitiates the fusion. The roles of membrane lipids and intermembrane interactions during fusion have been derived from experiments employing model systems17 often reconstituting putative fusion components.18 Specifically, vesicle–vesicle, vesicle–planar bilayer configurations, in conjunction with native or artificial triggers such as those provided by calcium, poly(ethylene glycol), or osmotic stress, have proved particularly valuable.19,20 Efforts employing supported membrane configurations, in conjunction with surface force apparatus (SFA) measurements, have yielded useful insights into modulations in intermembrane interactions and forces in the presence of fusion triggers.21–23 Lipid vesicle suspensions have also been used as model systems to investigate the effects of ionic strength on interactions between membranes.24,25 For like-charged and neutral membranes, these studies find that elevated ionic strength conditions, including high concentrations of mono- or divalent ions, foster vesicular aggregation and sometimes fusion.

Taken together, these studies reveal that membrane–membrane interactions are governed by membrane composition as well as the state of the intermembrane environment. Despite the tremendous advances made through such studies these unsupported synthetic membrane platforms have some fundamental limitations. Initiating contact between unsupported lipid bilayers is challenging due to their inherent fragility (in the case of black lipid membranes and giant vesicles) or small size (in the case of small vesicles). Any fusion or exchange of membrane components between these assemblies is a transient event often too rapid to allow characterization of the intermediate steps. Moreover, in vesicular systems substantial changes occur to the geometry of the system (e.g., vesicle flattening at the contact point) which in turn introduce structural perturbations complicating the analysis of physical forces involved during fusion. While inter-bilayer forces between closely apposed membranes have been studied in great detail using SFA,23 these measurements, however, do not readily shed light on the exchange of material between two fusing supported lipid bilayers. In this regard, single lipid bilayers supported on discrete, solid scaffolds, such as silica microspheres, provide a particularly attractive model configuration.

Here, we show that colloidal supported bilayers interacting with a planar supported lipid bilayer provide an alternative platform to controllably recapitulate membrane interactions, such as during membrane fusion. Our approach is enabled by a coordinated interplay between mesoscale colloidal interactions and molecular-level...
membrane perturbations induced by the addition of a monovalent salt. Manipulation of colloidal interactions by modulation of the ionic strength of the solution allows a controlled approach between the membranes, poising them for subsequent fusion (Fig. 1A). Simultaneous ion-mediated bilayer deformations allow the merger of the apposed membranes via hemi-fusion or possibly fusion-pore formation. Several features of our system are particularly noteworthy. Namely, the presence of the underlying solid support suppresses membrane surface undulations and the attendant repulsive forces which present a problem in unsupported systems. Moreover, the rigid geometry of our system restricts the progression of the fusion process by preventing the expansion of either the hemi-fused membrane area or the fusion-pore. Likewise, flattening, common in vesicle fusion due to osmotic stresses, cannot occur in colloid-supported membranes. Many studies of membrane fusion now establish that the stalk forming hemi-fusion state requires membranes to be tense. In unsupported systems these conditions can be met by fusion proteins or osmotic conditions. In supported systems such as the one described here moderate changes in ionic strength may be sufficient to achieve this effect. Because our approach allows juxtaposition of large membranes, visualization of single fusion events as well as arrays of simultaneous events is greatly simplified.

We begin with the synthesis of nanoporous silica microspheres by adapting a previously reported evaporation-induced silica self-assembly process. Briefly, ethanolic mixtures of monomeric silica precursor, namely tetraethyl orthosilicate or TEOS and cetyl trimethyl ammonium bromide (CTAB) are controllably evaporated via controlled aerosolization. The solvent evaporation prompts micellization of CTAB, which in turn templates the hydrolytic polycondensation of TEOS forming well-defined nanostructured silica microspheres. Subsequent thermal calcination removes the organic template yielding relatively monodisperse population of silica microspheres displaying high-density of nanoscale pores in an organized hexagonal lattice.

Silica microspheres so formed and planar coverslips of commercial borosilica glass [Corning] are then wrapped with single fluid supported phospholipid bilayers using the well-established vesicle fusion method. Briefly, freshly oxidized silica substrates are exposed to aqueous dispersions of small unilamellar vesicles (hydrodynamic dia., ~100 nm) consisting of single phospholipids, (1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPC or 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPC) [Avanti Polar Lipids] at temperatures well above the gel–fluid transition temperature. To enable fluorescence-based characterization the bilayers are doped with small amounts of fluorescent lipid analogs: Texas Red 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (TR-DHPE, 1 mol%) or N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-PE, 1 mol%) [Invitrogen].

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Fig. 1  (A) Schematic of three stages of lipid coated microsphere interaction with a planar bilayer. (B) Aggregation of lipid coated silica microsphere above a planar supported bilayer of identical composition. Visible on the right side of each frame is an area of bleached fluorophores which slowly recovers, indicating that the planar bilayer is in a fluid state. (C) Photobleaching and subsequent recovery of fluorescence on a single microsphere in contact with a patch of planar bilayer.
Bilayers on planar glass substrates are gently rinsed several times in de-ionized water without exposure to air. Bilayer coated silica microspheres are rinsed by means of several repetitions of centrifugation in water with removal of the supernatant solution, addition of fresh water, and resuspension by vigorous stirring each time. The uniformity of the fluorescence due to the bilayer around the bead confirms the expected formation of single homogeneous phospholipid bilayers.

Initial approach between bilayer coated microspheres and underlying planar bilayers is achieved by allowing controlled concentrations of the former to gravitationally settle from a drop of aqueous suspension onto planar bilayers positioned at the bottom of a water-filled Petri dish. Note that the weight of our nanoporous beads is still sufficient to ensure gravitational settling but insufficient to impede lateral Brownian motion. In order to isolate sphere–sphere interactions, the same steps were taken in an empty Petri dish with no planar bilayer present.

Fluorescence and bright-field images of planar supported bilayers and bilayer-coated silica microspheres were obtained using a Nikon TE200E inverted fluorescence microscope (Technical Instruments, San Francisco, CA). Additionally, confocal microscopy images of bilayer-coated microspheres were obtained using an Olympus FV1000 scanning confocal microscope.

Initially in pure water, membrane-wrapped microspheres gravitationally settle near a planar supported bilayer on a silica coverslip. The microspheres move freely in two dimensions via Brownian motion, and given sufficient time, form hexagonally ordered, two-dimensional aggregates as can be seen in Fig. 1B. Despite this apparent proximity, the bilayers wrapping the microspheres neither exchange lipids with the planar bilayer nor with each other. These observations are qualitatively similar to those reported previously where negatively charged colloids were seen to levitate and form 2D aggregates above a negatively charged surface at an equilibrium height determined by the surface potentials.38,39 Although the composition of the surface bilayer in our case is essentially neutral, the actual surface charge on the colloids, determined by a combination of lipid ionization, fluorophore ionization, and contribution from underlying microsphere, is also likely to be slightly negative. Indeed, previous studies of membrane functionalized silica microspheres have revealed that approximately 2–4% positive charge on the lipid bilayer is needed to produce a net zero surface charge.38,40 Gomez et al.38 reported an equilibrium lateral attractive minimum at approximately 1 micron separation for 6.5 micron silica particles (with a measured surface potential of –22.4 mV). The tendency of like-charged colloids to aggregate in low-ionic strength media (e.g., pure water) has also been examined previously and attributed either to heterogeneous (mosaic) distribution of surface charge41,42 or to oft-cited long-range like-charge electrostatic attraction.43,44

Upon addition of a small concentration (>2 mM) of sodium chloride, we find that the lateral Brownian motion of the microspheres is quickly arrested, and the microspheres develop an enduring physical contact with the underlying planar supported bilayer as demonstrated by fluorescence recovery after photobleaching (FRAP) experiments (Fig. 1C). Briefly, a small volume, encompassing an

![Image](https://via.placeholder.com/150)

**Fig. 2** Interaction between two populations of lipid coated microspheres in water. One group is coated in a bilayer of 99% POPC and 1% Texas Red-DHPE; the other is coated with 99% POPC and 1% NBD-PE. In the two image sequences shown we can follow the movement of the initially separate red (A–C) and green (D–F) fluorophores. In groupings of microspheres where two types of fluorophores are present we see a slow equilibration of fluorophore concentration between connected spheres. This sphere to sphere lipid transfer only occurs between directly contacting spheres as shown in confocal microscope images (G–I).
entire microsphere and some surrounding area of planar membrane, is photobleached using an intense beam of light. As the permanently darkened fluorophores in the membrane on the microsphere and on the planar surface diffuse away and are replaced by new, unbleached fluorophores from the contiguous surrounding bilayer, the fluorescence intensity in the bleached area is replenished. If this experiment is performed before continuity between the two membranes is established (ESI, Fig. S1†), a fluorescence recovery in the planar bilayer may be observed while the colloidal supported bilayers remain darkened. When lipid continuity is subsequently induced by addition of monovalent salt into the surrounding water, the concentrations of fluorophores in each membrane begin to equilibrate and a marked increase in fluorescence on the microspheres is observed. An alternate method of observing the formation of enduring membrane–membrane links is a fluorescent probe mixing assay (supplemental video†). In this case, two different fluorescent dyes are used and the establishment of a link between the two membranes allows the fluorescent probes to mix until concentrations of each are approximately equal in both bilayers. Enduring links formed between bilayers on two spheres are comparatively rare but may be visualized as well with dye mixing experiments (Fig. 2).

These observations may be understood in terms of a coordinated interplay between micro-scale colloidal interactions and nanoscale membrane perturbations both of which are modulated by the ionic strength of the ambient solution. Micro-scale colloidal behavior dictates the approach of the two membranes. Changing ionic strength simultaneously affects the supported bilayers on the molecular level, allowing formation of complex, fusion-intermediate-like structures with high local curvature.

It is well-known that the addition of electrolytes reduces the Debye screening length and allows for a closer approach between similarly charged surfaces. A thorough treatment of gravitationally settled spheres interacting with a plane is given by Wu et al. and Odiachi et al. Briefly, these studies establish that interaction energy of a charged spherical particle with a charged plane is dependent on the sum of the electrostatic repulsion, van der Waals attraction, and the force of gravity pulling the sphere downwards towards the plane. A sum of these energies is given as:

$$\Phi(h) = \Phi_{el}(h) + \Phi_{gr}(h) + \Phi_{vdw}(h) \quad (1)$$

where \( h \) is the separation between the sphere and the planar surface, \( \Phi_{el}(h) \) is the energy of interaction between the electrostatic double layers at the approaching surfaces, \( \Phi_{gr}(h) \) is the gravitational potential energy of the sphere, and \( \Phi_{vdw}(h) \) is the van der Waals attraction between the two. \( \Phi_{el} \) scales exponentially with \( e^{-\kappa h} \) where \( 1/\kappa \) is the Debye length which itself scales inversely with square root of the concentration of electrolytes in the solution. Neglecting van der Waals force, an increase in NaCl concentration reduces the Debye length thereby reducing the height \( h \) at which electrostatic repulsion can balance the force of gravity. For large silica colloids coated with nearly neutral bilayers, such as those used in this study, as little as 2 mM NaCl is sufficient to reduce \( h \) effectively to zero. Because gravity plays no role in the lateral sphere–sphere interactions, the addition of salt is not sufficient to reduce the equilibrium separation distance to zero. The sphere to sphere lipid exchange evident in our system is likely due to surface roughness and particle shape irregularities allowing the equilibrium separation distance to be reduced to effectively zero in a small number of cases.

The emergence of an enduring link between interacting membranes, brought into close proximity by reduced electrostatic and undulation-based repulsion, can be further explained in terms of ion-induced membrane deformations. The rate of lipid transfer across the aqueous gaps between two closely spaced bilayers is sufficiently small compared to the timescale of our experiments that we may assume any fluorescent probe moving into or out of the spherical supported bilayer must involve the creation of a bilayer–bilayer junction. The emergence of such a communicative link thus necessarily requires the formation of either a hemi-fusion state, where only the outermost leaflets of each membrane connect, or complete fusion. Our present experiments cannot conclusively differentiate between these two possibilities but we note that an examination of fluorescence intensity pattern in the vicinity of the junction is consistent with both leaflets of each interacting bilayer coming to compositional equilibrium over time.

Three types of molecular level membrane deformations induced by the presence of salt may play a role in the formation of the bilayer–bilayer link we observe. First, it has been previously shown that an elevated salt concentration has a condensing effect on fluid bilayers. Briefly, these authors show that increasing sodium chloride concentration both thickens a pure POPC membrane and reduces the lateral mobility of lipids therein. This may facilitate the formation of membrane defects needed for the formation of more complex fusion intermediate structures. Second, diminished electrostatic repulsion in the presence of salt should also induce local deformation of interacting membranes at the contact site. Indeed, Boulbitch has previously shown that the adherence of a gravitationally settled bead to a planar supported membrane can induce non-trivial local deformation and bending of the planar bilayer, which may also contribute to fusion intermediate formation. Estimating the specific nature of deformation by these inter-bilayer forces, estimated to be in nanonewton range, and its spatial extent is, however, difficult to ascertain from present experiments because of the expanded membrane–membrane contact area prior to fusion. Third, it is well known that elevated ionic strength or ionic strength shock can induce high curvature in once planar supported membranes. Cambrea and Hovis showed that sudden introduction of ionic strength asymmetry across a planar supported bilayer gives rise to the formation of highly curved, out of plane structures which may foster the formation of stable stalk-like structures.

Previous study of the exchange of membrane components between supported lipid bilayers and other lipid assemblies has been largely restricted to systems with vastly different net membrane charges. In these cases the transfer of lipids between two supported bilayers or between a vesicle and a supported bilayer is driven by large surface charge differences, which must be present for lipid mixing to be initiated, and connections established between these lipid assemblies may be transient. Other studies have focused on the rheological aspects of interactions between supported bilayers and have not directly addressed material exchange.

Taken together, the experiments above suggest that the interplay of colloidal interactions and salt-induced membrane deformations allows bead-supported lipid bilayers to serve as a powerful model system to probe events during membrane fusion. Tuning of colloidal interactions such as by modulating electrostatics allows for the close apposition of membranes and parallel membrane deformations produce long-lived fusion intermediates that can be conveniently assayed.
This work has implications in a variety of supported membrane applications. The successful design and implementation of engineered protocells, lipid-coated inorganic constructs used for drug delivery and sensing, demand an understanding of how supported lipid bilayers interact with each other and with native biological membranes. Additionally, the ability to modulate compositions of model membranes is critical if they are to serve as vehicles to determine how membrane composition, structure, and function are established and evolve. We have shown that silica microparticle supported membranes may be used to modulate the composition of existing planar supported membranes in a controllable and predictable manner.

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