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How to Stabilize Phospholipid Liposomes (Using Nanoparticles)

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ABSTRACT

The simple strategy of mixing phospholipid liposomes with charged nanoparticles and using sonication to mix them at low volume fraction produces particle-stabilized liposomes that repel one another and do not fuse. Subsequently, the volume fraction can be raised as high as ≈50%, reversibly, still without fusion. In studies of liposome longevity, we verified the stability of particle-stabilized liposome suspensions with volume fraction up to 16% for up to 50 days, the longest period investigated. Fluorescent dyes were encapsulated within the particle-stabilized liposomes, without leakage. Although these particle-stabilized liposomes were stable against fusion, ≈75% of the outer liposome surface remained unoccupied. This opens the door to using particle-stabilized liposomes in various applications.

"Liposomes" (the term refers to artificially constructed capsules of phospholipid bilayers) would be more useful if only they could be stabilized against fusion with one another. First, they are tremendously biofunctionalizable; antibodies, protein receptors, and other biosensor molecules can attach to them.[1,2] Second, they comprise compartments that can be used to encapsulate and store various cargoes, such as enzymes, proteins, DNA, and various drug molecules.[3−5] Their small and controllable size, diameter from tens to thousands of nanometers, signifies that individual liposomes comprise nanocontainers with volumes from zeptoliters (10^{-21} L) to femtoliters (10^{-15} L). When biomolecules or other chemical reactants are loaded into this biocompatible container, cellular processes and chemical reactions including protein expression, mRNA transcription, and enzyme-catalyzed reactions can be performed inside.[6−8] To release the final products, one can either change the temperature to below the lipid main phase transition temperature, beyond which lipid packing defects create transient pores in the membrane,[9] or use pulses of strong electric field to break the membrane apart.[10]

This paper is concerned with stabilizing liposomes against fusion with one another. When liposomes encounter one another in suspension, they are prone to adhere and fuse to form a larger one.[11,12] Consequently, the liposome size distribution becomes polydisperse. Moreover, as liposomes open during the fusion process, the "open-and-fuse" process results in inclusion leakage, unexpected mixing between chemicals that are nominally separated into different liposome capsules, and inefficient reactions.

How to avoid vesicle fusion? Tadros and co-workers employed steric stabilization using block copolymers.[13] Semple and co-workers incorporated cholesterol into membrane to reduce the mobility of phospholipid molecules.[14] Polymerization either of modified lipids that comprised vesicles or of molecules adsorbed onto vesicle surfaces was carried out by others.[15,16] While these important studies are effective, it is also desirable to develop a stabilization method that would leave untouched a substantial fraction of the liposome surface, leaving a substantial portion of phospholipid surface area free for functionality.

In this Letter, we show that particle stabilization meets this need. Charged nanoparticles, allowed to adsorb to the liposome surface to ≈25% surface coverage, enable one to stabilize liposome suspensions up to very high volume fraction. In the description that follows, we demonstrate stability with respect to liposome concentration, with respect to aging, and with respect to preventing leakage through the membrane wall.

Materials. For study, the phospholipid DLPC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine, was selected because its gel-to-fluid phase transition of −1 °C was far below the experimental temperature, 23 °C. This was mixed with small quantities of fluorescent-labeled lipid such that one fluorescent molecule was incorporated, on average, in each liposome. The fluorescent lipid was DMPE, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine, with polar headgroup labeled by rhodamine B (DMPE-RhB). Both lipids were obtained from Avanti Polar Lipids, Inc. Carboxyl-modified white polystyrene (PS) latex with a diameter of 20 nm, whose surface was hydrophilic and negatively charged, was purchased from Interfacial Dynamics Corp. (Eugene, OR). Small unilamellar lipid vesicles (liposomes) were prepared in PBS buffer (10 mM, pH = 6.0) using the well-known extrusion

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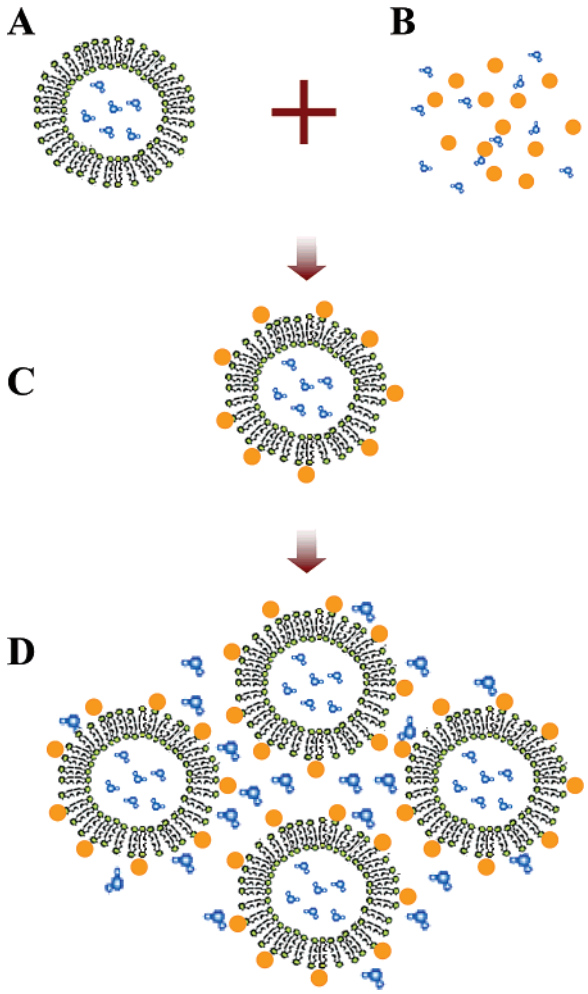

Figure 1. Schematic illustration of the experimental strategy to produce particle-stabilized liposomes. (A) DLPC liposomes with a diameter of 200 nm were made using the extrusion method. (B) Carboxyl-modified polystyrene nanoparticles with a diameter of 20 nm were prepared. (C) Nanoparticle-stabilized liposomes were formed by mixing A and B at the molar concentration ratio of 1:100, with 10 min of sonication. (D) To condense the dilute liposome suspension C, nitrogen gas was blown gently over the suspension until the desired volume fraction was attained.

method, employing procedures described in detail elsewhere.[17] Figure 1 shows our strategy to stabilize liposomes with adsorbed nanoparticles. Liposomes of DLPC, diameter 200 nm, were prepared at 1% concentration using the extrusion method; note that at this low volume fraction, appreciable vesicle fusion occurred when the sample was older than a few days. Charged PS nanoparticles were mixed with sonication (see Figure 1) into the liposome suspension at the molar ratio 100:1, which is approximately 1:1 by weight. The nanoparticles adsorbed to the phospholipid membrane and formed a nanoparticle−liposome hybrid structure as shown in Figure 1C. The driving force for adsorption is believed to be charge-dipole attraction between the charged nanoparticle and the P−N dipole of the phospholipid headgroups, which is known to be oriented preferentially nearly parallel to the local bilayer membrane surface.[18,19] Also favoring adsorption may be the entropy increase when water molecules at the hydrated membrane surfaces are displaced by adsorbed nanoparticles.

Methods. Vesicle stability was monitored using fluorescence correlation spectroscopy when the volume fraction was relatively low and epifluorescence microscopy when it was higher (see below). The volume fraction of liposomes was estimated from the difference between initial concentration and final suspension volume. In all cases, the liposomes were first prepared at volume fraction ≈1%, then their concentration was raised by bubbling nitrogen gas gently over the suspension. This was found to accelerate the process of water evaporation while not strongly disturbing the liposomes.

Fluorescence correlation spectroscopy (FCS) measurements were carried out in the two photon excitation mode using a home-built apparatus described elsewhere.[20] Basically, a near-infrared femtosecond pulse laser was focused onto the sample through an objective lens, giving an excitation spot whose diffraction-limited diameter was ∼0.35 μm. Fluorescence was collected by the same objective and detected by a single photon counting module. The fluorescence intensity−intensity autocorrelation function provided the translational diffusion coefficient (D). The FCS method was very sensitive to vesicle fusion because the vesicle diameter was so close to that of the FCS excitation spot. In the case of severe vesicle fusion, the resulting large vesicles and polydisperse vesicle size distribution broadened the FCS autocorrelation function in ways that were obvious in the raw data. We found the FCS technique to be not just a quantitative diagnostic of translational diffusion coefficient but also a qualitative diagnostic of the incidence of vesicle fusion. However, for volume fractions >16%, diffusion measured by the FCS technique became unreliable because the fluorescence signals were strongly scattered.

For imaging, epifluorescence microscopy was used. To improve the signal-noise ratio, one hundred DMPE-RhB probes were doped (on average) into each initial DLPC liposome, then the fluorescence was reduced by diluting the fluorescent-labeled liposomes by a factor of 100 using unlabeled liposomes.

Results. The first hint of exceptional improvement of liposome stability came from measurements (using FCS) of translational diffusion, summarized in Figure 2. Here the measurements were made 1 day after the vesicles were prepared. The liposome translational diffusion coefficient (D) is plotted against its volume fraction (Φ), and it is obvious that particle-stabilized liposomes generated meaningful measurements up to volume fraction of 16%. The observed decrease of D with increasing volume faction is attributed to increasing solution viscosity. In contrast, for naked liposomes (no nanoparticles added), D was nearly constant, 0.80 ± 0.09 μm^2/s provided that $\Phi < 2\%$, but for $\Phi > 3\%$, vesicle fusion and particle scattering precluded FCS measurements already 1 day after the vesicles were prepared. Using epifluorescence microscopy, vesicle suspensions of higher volume fraction were studied 1 day after preparation. First, consider the naked liposomes (no nanoparticles added). Direct observation at $\Phi = 2\%$ showed discrete objects with nearly homogeneous size, displaying free Brownian motion

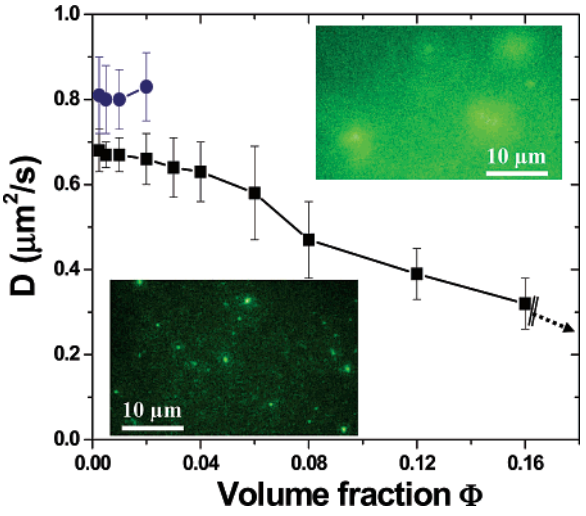

Figure 2. Translational diffusion coefficient (D) of unstabilized liposomes (solid circles) and nanoparticle-stabilized liposomes (solid squares) plotted against their volume fraction (Φ). When the time scale of comparison is 1 day, the former became unstable for $\Phi >$ 2% but the latter were stable up to the highest Φ investigated. The lower D of the particle-stabilized liposomes follows from their larger diameter owing to the presence of nanoparticles. Epifluorescence images visualized the occurrence of vesicle fusion in unstabilized liposome suspensions at $\Phi = 3\%$ (upper photographic inset) and showed that particle-stabilized liposome suspensions remained stable up to $\Phi = 50\%$, at which point an immobilized glassy state of discrete liposomes appeared to be reached (lower photographic inset). In the experimental setup, the CCD camera used to record the fluorescence images had a resolution of 150 nm pixel^{-1}. In the photographic images, the image area is 40 μm × 25 μm.

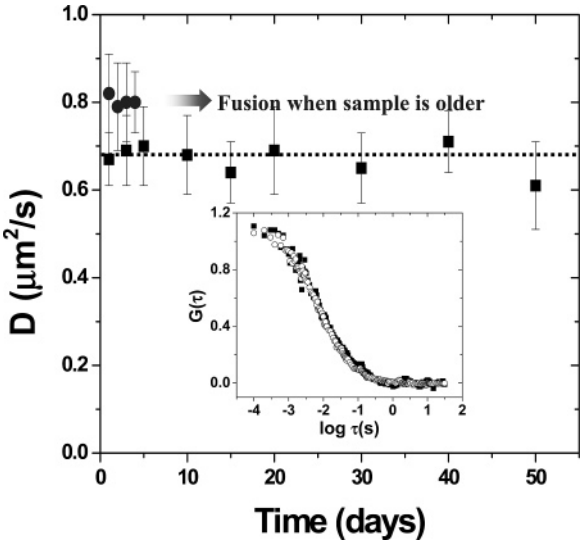

Figure 3. Translational diffusion coefficient (D) of unstabilized liposomes (solid circles) and particle-stabilized liposomes (solid squares), both with $\Phi = 1\%$, plotted against time elapsed after their preparation. The former was characterized by $D = 0.80$ μm^2/s (up to an age of 4 days) but was unstable when older. The translational mobility of the latter revealed no time dependence up to the longest age investigated, 50 days. The inset illustrates FCS autocorrelation functions of nanoparticle-stabilized liposomes at volume fraction $\Phi = 1\%$ measured 4 days and 40 days after the sample was prepared. They overlap within the experimental uncertainty.

(data not shown), but for $\Phi = 3\%$, giant liposomes with diameters of micrometers were observed with heterogeneous sizes, as illustrated in Figure 2 (upper photographic image). In strong contrast, when nanoparticles were added, discrete objects remained observable up to the highest volume fractions. To overcome the scattering problem that prevented FCS measurements at $\Phi >$ 16%, the laser beam was focused onto the bottom of the glass sample chamber with a 2 μm depth of view. Individual liposomes can be identified clearly in the resulting epifluorescence images. Figure 2 (lower photographic image) shows a typical image at the very high volume fraction of $\Phi = 50\%$. At this point, the liposomes failed to move at all over the experimental time scale of 10 s. The system appeared to have reached a glassy state in which particle motion was quenched; yet the particles persisted as discrete objects.

Stability is relative to observation time, of course. The stability of unstabilized suspensions at low volume fraction ($\Phi \approx 1\%$) concerns times up to 4 days because the probability of particle encounter with ensuing vesicle fusion was low, during this time period. In contrast, we have not yet found a limit to the lifetime of particle-stabilized liposomes; they remain stable up to the longest time (50 days) that we investigated at the time this communication was submitted. For example, the inset in Figure 3 illustrates FCS autocorrelation functions of the same particle-stabilized liposome sample measured 4 days and 40 days, respectively, after its preparation; there was no change. That the stabilized liposomes have at least a 50-day-long lifetime held not just for the dilute situation ($\Phi \approx 1\%$) but also for higher volume fractions, up to at least 16%, which is the highest volume fraction whose longevity we investigated. Furthermore, reversibility was observed when younger suspensions with Φ as high as 50% were rediluted to the dilute state.

As it is not permissible that vesicles leak if they are to be useful as carriers of "cargo", this aspect also was investigated. While it is known that native liposomes encapsulate fluorescent probe molecules efficiently,[9] one might reasonably worry that the presence of nanoparticles could induce leakage. As a test of this possibility, a solution of fluorescent dye (rhodamine B) was loaded into the liposomes during their preparation, then free dye was removed by dialysis. The dye-laden liposomes were then mixed with nanoparticles to stabilize them. In Figure 4, epifluoresence images show that the fluorescent dyes remained encapsulated without leakage; no leakage occurred up to the longest periods investigated, samples up to 4 days old. This broadens prospective applications of this new liposome stabilization method.

We also investigated the generality of these findings. The findings described in this communication held over the range of liposome diameter investigated, 100−400 nm. Furthermore, the same findings held when cationic nanoparticles were employed for stabilization, rather than the anionic nanoparticles described here.

Testing the Proposed Model. All of the above results were extracted from nanoparticle stabilized liposomes with a molar ratio of 100:1 between particle and liposome,

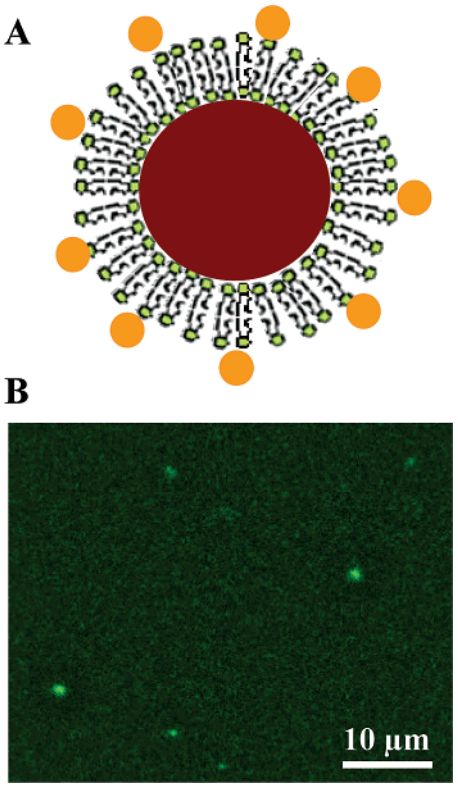

Figure 4. Checking the permeability of particle-stabilized liposomes. (A) Schematically, this shows that unstabilized liposome was first loaded with fluorescent molecules (rhodamine B), then nanoparticles were added as described in the text, to impart dimensional stability. (B) This epifluorescence image shows that from the particle-stabilized liposome no leakage occurred; bright and isolated liposomes are easily identified, for particle-stabilized liposomes up to the longest times investigated, 4 days old. The image area is 50 μm × 40 μm.

amounting to ≈25% surface coverage assuming that all nanoparticles present in the system were adsorbed. What would happen at lower surface coverage? Figure 5 shows an epifluorescence image of a liposome suspension with ≈5% surface coverage ($\Phi \approx 1\%$). In this image, it is obvious that the liposomes aggregated. The most plausible interpretation is that nanoparticles stuck neighboring liposomes together, bridging between them, as was previously observed in nanoparticle−microsphere systems.[21] This observation supports our model that when the surface coverage is higher, the stabilization mechanism stems from mutual repulsion between nanoparticles adsorbed onto the outer surfaces of liposomes that encounter one another. This approach can also be used to form gels.

The interactions and spacings between particles adsorbed on the same liposome are a more subtle question, currently under investigation.

We emphasize that the estimated free membrane outer surface, not covered with nanoparticles, was large, ≈75%. In this respect, the particle stabilization demonstrated here, while reminiscent of particle stabilization of Pickering emulsions,[22−24] differs in the sense that all of the emulsion surface is usually coated with particles,[22−24] although in at least one study stable emulsions were obtained even using very low surface coverage.[25] The relation to the study

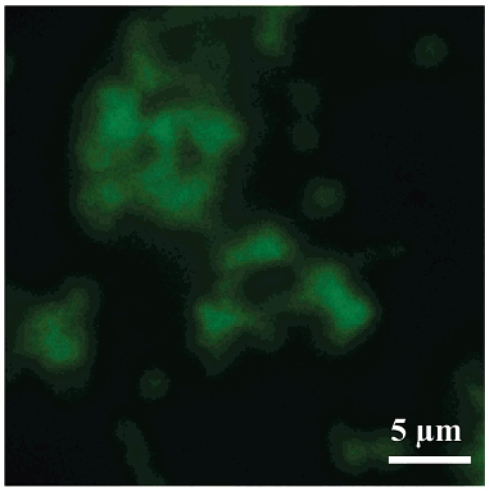

Figure 5. Epifluorescence image illustrating attraction between liposomes when the nanoparticle surface coverage was low. The resulting bridging between liposomes caused the liposomes to aggregate. In this experiment, the molar ratio of particle to liposome was 20:1, an estimate surface coverage of 5% assuming that all nanoparticles adsorbed, and the liposome volume fraction was 1%. The image area is 30 μm × 30 μm. The objects in this image greatly exceed the size of individual liposomes (200 nm).

presented here is not clearly understood at the present time, but Weitz has made the intriguing suggestion (private communication) that this may signify that vesicle fusion is disallowed if the local radius of liposome curvature, near the nanoparticles, is prohibitively reduced.

Outlook. The particle-stabilized liposomes introduced in this communication may be interesting from several functional perspectives. First, particle-stabilized liposomes comprise potentially a novel kind of colloidal-sized sensor. Antigens and membrane proteins can be embedded within the large free area contained within them; yet because they resist fusion with one another, they can be concentrated to exceptionally high volume fractions and consequently to exceptionally high functionality when the ratio of surface-to-volume is large. Second, we imagine that chemical reactions can be performed within these objects at the individual liposome level. Because each liposome is impermeable, this signifies that elementary reactions, each of them performed at dilute concentration, can be studied in parallel among many such objects. Third, these particle-stabilized liposome objects afford a new type of colloidal particle: hollow, deformable, and biofunctionalizable. Their rheology, diffusion, and potential crystallization may contribute to the study of structure and dynamics of colloidal-sized objects, at very high volume fractions where particle−particle interactions dominate, in the case where the particle contour is more “raspberry” than spherical.

It is obvious that by functionalizing these nanoparticles so that their wettability is altered by optical stimulus, one can envision that stimulus-responsive adsorbed nanoparticles can be used to switch liposomes from being impermeable (the case demonstrated in this communication) to permeable, thus releasing cargo contained within them. Studies in this direction are in progress and will be reported presently.

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