Properties of a Self-Assembled Phospholipid Membrane Supported on Lipobeads

Charlene C. Ng,* Yu-Ling Cheng,† and Peter S. Pennefather*
*Department of Pharmaceutical Sciences, †Department of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, Ontario, Canada

ABSTRACT The overall objective of our work was to make a hydrogel-supported phospholipid bilayer that models a cytoskeleton-supported cell membrane and provides a platform for studying membrane biology. Previously, we demonstrated that a pre-Lipobead, consisting of phospholipids covalently attached to the surface of a hydrogel, could give rise to a Lipobead when incubated with liposomes because the attached phospholipids promote self-assembly of a phospholipid membrane on the pre-Lipobead. We now report the properties of that Lipobead membrane. The lateral diffusion coefficient of fluorescently labeled phosphatidylcholine analogs in the membrane was measured by fluorescence recovery after photobleaching and was found to decrease as the surface anchor density and hydrogel crosslinking density increased. Results from the quenching of phosphatidylcholine analogs suggest that the phospholipid membrane of the Lipobead was composed mostly of a semipermeable lipid bilayer. However, the diffusional barrier properties of the Lipobead membrane were demonstrated by the entrapment of 1.5–3.0 K dextran molecules in the hydrogel core after liposome fusion. This hydrogel-supported bilayer membrane preparation shows promise as a new platform for studying membrane biology and for high throughput drug screening.

INTRODUCTION

Hydrogel-supported phospholipid membranes may be useful tools in both fundamental and applied research in protein biology, membrane biophysics, and biosensor technology. A proposed mechanism for phospholipid bilayer deposition on the support substrate surface includes the sequential steps of liposome adsorption, rupture, and then spreading (Seifert and Lipowsky, 1990; Lipowsky and Seifert, 1991; Nissen et al., 1999; Keller et al., 2000). However, unless some input of energy is applied, such as by homogenization or Langmuir-Blodgett type processes, the lipid membrane formed on substrate surfaces may be heterogeneous, and their properties hard to predict. For instance, Gao and Huang (1987) showed that membrane formation depends critically on the lipid composition and the size and shape of polymer cores. Major et al. (1997), Kiser et al. (1998, 2000), and De Miguel et al. (2000) needed to cohomogenize phospholipids and hydrogel beads to form a stable encapsulating membrane. Reviakine and Brisson (2000) demonstrated that the ability of liposomes to fuse on mica surfaces depends on vesicle size, surface potential, lipid concentration, and the presence or absence of calcium ions.

In a previous article (Ng et al., 2001), we reported the synthesis of pre-Lipobeads, hydrogel beads with covalently attached phospholipid anchors on the surface. We showed that upon incubation with liposome those hydrophobic anchors drove the formation of a phospholipid membrane on the surface of the pre-Lipobeads (Fig. 1) to form Lipobeads. The manner in which anchors attract liposomes to the hydrogel may be similar to the way that fatty anchors on solid substrates, such as silica, drive the self-assembly of a supported phospholipid membrane by the hydrophobic effect (Plant, 1999).

In contrast to other supported membrane systems in which hydrophilic oligomer spacers are grafted to a solid surface (Naumann et al., 2002; Wagner and Tamm, 2000, 2001; Theato and Zentel, 1999, 2000), the mechanically stable hydrophilic support platform for the lipid membrane in Lipobeads is provided by the crosslinked hydrogel core. Thus, the volume of the hydrophilic compartment accessible to the ''inner'' leaflet of the membrane is greatly increased.

In this article, we examine the mobility and barrier properties of the Lipobead membrane. The transport of different sized solutes through the membrane is measured to probe the barrier properties of the membrane. Cobalt quenching of fluorescent phospholipids (Lakowicz, 1999) is used to determine the homogeneity of the bilayer. Lateral mobility of lipids within the membrane is studied by fluorescence recovery after photobleaching (FRAP) and compared to other membrane systems. In addition, since the membrane is tethered to the surface of the hydrogel, we also explore how the nature of the support can influence membrane properties. Our long-term goal is to demonstrate that the hydrogel-supported and lipid-anchored Lipobeads can be useful in the study of membrane biology and transmembrane protein properties.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine (ePC), bovine serum phosphatidylserine (PS), cholesterol, dimethylacrylamide, *n,n′*-ethylene-bis(acrylamide), cobalt chloride hexahydrate, (*n*-[2-hydroxyethyl]piperazine-*n′*-[2-ethane-sulfonic

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Address reprint requests to Peter S. Pennefather, E-mail: p.pennefather@utoronto.ca.

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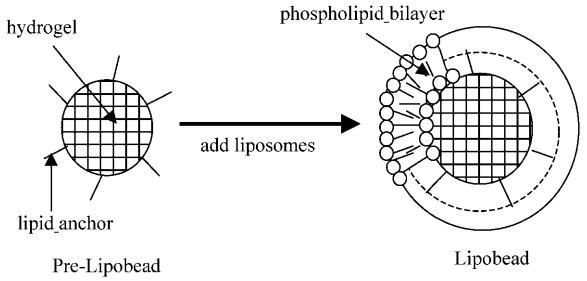

FIGURE 1 Schematic illustration of the self-assembly of a phospholipid membrane around a lipid anchor-hydrogel conjugate, called pre-Lipobead, forming the Lipobead. The picture is not drawn to scale. In reality, the lipid anchors likely collapse on the pre-Lipobead surface instead of adopting an extended conformation in an aqueous environment (from Ng et al., 2002).

acid]) (HEPES), ethylene-diamine-tetra-acetic acid (EDTA), and acrylic acid succinamide ester were obtained from Sigma-Aldrich Canada (Mississauga, ON). The fluorescent probes dextran-tetramethylrhodamine conjugate (1.5–3 kDa, 0.3–0.7 dye per dextran molecule, anionic, lysine-fixable) and Fluo-3 were obtained from Molecular Probes (Eugene, OR). The fluorescent phospholipid 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]-caproyl]-*sn*-glycero-3-phosphocholine (PC-NBD) and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE) were purchased from Avanti Polar Lipids (Alabaster, AL). The phospholipid anchors used for the preparation of the Lipobeads were obtained from the reaction of acrylic acid succinamide ester and DMPE as described in Ng et al. (2001). Spectroscopic grade chloroform was obtained from Fisher Scientific Canada (Nepean, ON). All other reagents used were analytical grade.

Unilamellar liposome preparation

The selected phospholipids and cholesterol were dissolved in chloroform at known concentrations in the range of 1–5 mg/mL. To evaporate chloroform, air was blown over glass vials containing the solution, which then results in a thin lipid film of the desired composition at the bottom of the vial. HEN buffer (20 mM HEPES, 1 mM EDTA, 160 mM sodium chloride, pH 7.4) was added to the dried film to make the final concentration of lipid 1 mg/mL. The suspension was subjected to three freeze/thaw cycles to promote the formation of larger lipid aggregates. The lipid aggregates were then extruded at 40°C through a polycarbonate membrane, with an etched pore size of 100 nm, using the Avanti Mini-Extruder apparatus (Avanti Polar Lipids). Extruded liposomes, checked by dynamic light scattering (90Plus Particle Size Analyzer, Brookhaven Instruments, Holtsville, NY) before use, had a unimodal distribution with an average particle size of 120 nm and a polydispersity (standard deviation over mean of the distribution) of <0.05.

Lipobead synthesis

The pre-Lipobead was synthesized according to a previously described one-step method in which the phospholipid anchors are covalently attached to the surface of the hydrogel at the same time as the polymerization and crosslinking of the hydrogel (Ng et al., 2001). An aqueous solution of the monomer dimethylacrylamide (DMAA) (1.9 mL $\approx$ 1.9 g), the crosslinker *n*,*n*′-ethylene-bis(acrylamide) (E-BIS) (150 mg), and ammonium persulfate (50 mg) was placed in a round bottom reaction flask along with a hexane:carbon tetrachloride (50 mL:29 mL) organic phase mixture. A 90-mg mixture of surfactant composed of sorbitan monostearate and the specially synthesized anchor (in various ratios ranging from 0 to 100 wt % of the anchors) was then added to the flask and stirred to form a water-in-oil emulsion. *N*,*n*,*n*′,*n*′-tetramethylethylene-diamine was then added to react with ammonium persulfate to generate free radicals and to initiate polymerization. The phospholipid anchors localize at the oil/water interface and become covalently attached to the hydrogel surface upon polymerization. The ratio of the anchor mass to the total mass of the surfactant mixture provided an estimate for the expected surface anchor density on the pre-Lipobead. Assuming that the crosslinker is completely consumed by the polymerization process, the molar ratio of E-BIS to the sum of DMAA and E-BIS was taken as the hydrogel crosslinking density.

Approximately 7 mg of dried pre-Lipobeads were prehydrated with 50 μL of HEN buffer for 15 min, then incubated with 300 μL of a 1 mg/mL suspension of unilamellar liposomes in HEN buffer for 2 h at room temperature. Unbound liposomes were removed by rinsing with HEN buffer and by decanting the supernatant four times. Fusion experiments with fluorescently labeled liposomes showed that the liposomes fuse, upon mixing, with the hydrogel beads and form a uniform phospholipid membrane on the bead surface (Ng et al., 2001).

Fluorescent probe encapsulation and imaging by laser scanning confocal microscopy

Pre-Lipobeads were first immersed in buffer then incubated in a 0.5 mg/mL dextran-tetramethylrhodamine conjugate solution until hydration equilibrium was achieved. At that point, a 1 mg/mL egg phosphatidylcholine (ePC) liposome suspension containing 3.2 wt % of PC-NBD was added and equilibrated with the loaded beads for 2 h at room temperature. Before imaging, the beads were thoroughly washed with buffer to remove unbound liposomes and fluorescent dextrans that were not encapsulated. Crosslinked poly(dimethylacrylamide) beads with no surface anchors were hydrated in the same dextran solution as controls.

Laser scanning confocal microscopy images were obtained using a Model 5.10 Carl Zeiss Axiovert 100-M laser scanning confocal microscope equipped with a C-Apochromat 63×/1.2 NA water immersion lens; a 10×/0.5 NA Fluar lens; an argon laser (488-nm line); a helium/neon laser (543-nm line); a beam splitter, NFT 488/543; and two emission filters, BP505-530 and LP 560. A 120-μm pinhole was used along with the 10× and 63× lens, which generated optical sections of 10 and 1 μm, respectively.

Fluorescence quenching by cobalt ions

A 0.5 M cobalt chloride hexahydrate stock solution in HEN buffer was prepared. A 1-mg/mL liposome suspension containing PC-NBD was allowed to fuse with pre-Lipobeads for 2 h at room temperature. Free liposomes were removed by washing with buffer. The fluorescent intensity of the liposome and Lipobead samples were determined by a Delta Scan spectrofluorometer (Photon Technology International, South Brunswick, NJ), with the excitation monochrometer set at 460 nm. The cobalt solution was added incrementally while stirring, and the average emission from 535 to 545 nm was measured after each cobalt addition at 90° angle of the incident excitation beam. The background fluorescence was determined using a sample containing pre-Lipobeads in buffer and subtracted from the fluorescence intensity of the Lipobead sample. The collected data were fitted to a modified Stern-Volmer model which assumes that a fraction of membrane phospholipids is not accessible to quenching by the externally added cobalt ions (Lakowicz, 1999),

$$\frac{F_0}{(F_0 - F)} = \frac{1}{f \times K[Q]} + \frac{1}{f}, \tag{1}$$

where F_0 is the fluorescence intensity of the sample before the addition of quencher; F is the fluorescence intensity at a specific concentration of the quencher, Q; f is the fraction of phospholipids accessible to the quencher ($f \leq 1$); and K is the quenching constant. The parameters K and f were determined by obtaining the best fit of Eq. 1 by linear regression through the data points.

Diffusion coefficients and mobile fractions determined by fluorescence recovery after photobleaching (FRAP)

Lipobeads were prepared from the fusion of ePC/PS/cholesterol/PC-NBD (11:12:1:1) liposomes, and the diffusion coefficient of the fluorescently labeled phospholipids in the Lipobead membrane at 20°C was determined by FRAP. The same laser scanning confocal microscope described above was used to measure the fluorescence intensity of the samples. A 15-mW argon laser was used to bleach the membrane. The bleached membrane width varied among experiments, but was between 3 and 10 μm in all cases. Fluorescence recovery in the bleached area was measured using the same laser at 10% of the maximum transmission level, and there was <10% photobleaching in other parts of the membrane during recovery. A beamsplitter, HFT 488, and a long-pass filter, LP505, were used to isolate the emission.

The diffusion coefficient of the fluorescently labeled phospholipid was determined by finding the values that gave the best fit between the experimental fluorescence intensity time profile and the solution of the transient one-dimensional diffusion model (Baker and Lonsdale, 1974),

$$\frac{I_m(t) - I_m(AP)}{I_m(i) - I_m(AP)} = \frac{2}{L}\sqrt{\frac{Dt}{\pi}}, \quad (2)$$

where $I_m(t)$ is the average fluorescence intensity contributed by the mobile fraction in the bleached area at time t after photobleaching; $I_m(AP)$ is the fluorescence intensity contributed by the mobile fraction in the bleached area immediately after photobleaching; $I_m(i)$ is the initial average fluorescence intensity contributed by the mobile fraction in the bleached area before photobleaching; L is the length of the bleached area; and D is the diffusion coefficient of the mobile and fluorescently labeled phospholipids.

Since the total intensity recovered was the sum of fluorescence contributed by the mobile and immobile fractions and assuming only the fluorescence of the mobile fraction recovered (see Fig. 5),

$$I_m(t) = I(t) - \phi I_{im}(i), \quad (3)$$

$$I_m(0) = I - \phi I_{im}(i), \quad (4)$$

where $I(t)$ is the total intensity of the bleached area at time t after photobleaching; ϕ is the fraction unbleached and is equal to I_{AP}/I_i; $I_{im}(i)$ is the average fluorescence intensity contributed by the immobile fraction in the bleached area before photobleaching.

The mobile fraction of the phospholipid was determined by the ratio of fluorescence estimated to recover during data collection to the amount reduced by photobleaching. Mobile fraction is equal to

$$1 - \frac{I_{im}(i)}{I_i} = \frac{I_\infty - I_{AP}}{I_i - I_{AP}}, \quad (5)$$

where I_∞ is the fluorescence intensity recovered at infinite time after photobleaching.

RESULTS

Functional encapsulation

Fig. 2 shows confocal images of Lipobeads loaded with 1–3 kDa dextran-tetramethylrhodamine conjugates incubated with liposomes containing the fluorescently labeled phospholipids, PC-NBD, on the day of preparation. Using a band-pass (BP505-530) and a long-pass (LP560) filter allowed independent imaging of the two fluorescent probes. Fig. 2 *A* shows a lipid membrane encapsulating the beads as indicated by the rings of fluorescence from PC-NBD markers. Fig. 2 *B* shows the encapsulation of dextran-tetramethylrhodamine molecules within the Lipobeads, as indicated by the red emission from the hydrogel core. Peaks in green fluorescence intensity coincide with sharp drops in red fluorescence intensity, and both occur at Lipobead surfaces. The sample was reimaged 10 days later, and no detectable drop in core red fluorescence was found, indicating that little diffusional release of dextran-tetramethylrhodamine had occurred and that the membrane acted as a barrier to dextran-tetramethylrhodamine transport.

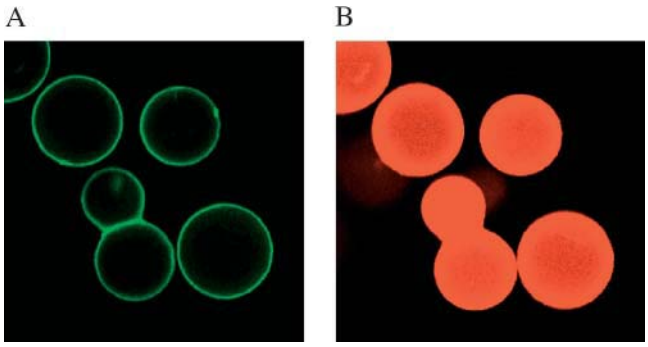

FIGURE 2 The encapsulation of dextran-tetramethylrhodamine conjugates (molecular weight 1.5–3 kDa) by Lipobeads, the membrane of which is composed of egg phosphatidylcholine and 3 mol % PC-NBD. The samples were dually excited by argon and helium/neon lasers, and the emitted fluorescence was collected by passing through (*A*) a band-pass filter of 505–530 nm, or (*B*) a long-pass filter of 560 nm. Beads were imaged within a 0.1-ml droplet suspension deposited on a coverslip after they settled to the coverslip surface.

A control experiment was performed using polydimethylacrylamide hydrogel beads without any anchors on the surface but incubated by the same fluorescent lipid mixture. There was no green fluorescence on the bead surface (Fig. 3 *A*), confirming that minimal amounts of fluorescent liposomes fused on the unmodified hydrogel surface. A slight red background was observed because dye leakage and imaging happened concurrently. Shown in Fig. 3 *B* is a plot of the intensity of the loaded hydrogel bead along the white line drawn in Fig. 3 *A* at different times after washing. The amount of loaded dextran decreased significantly over 20 min, and at 20 min, there was a concentration gradient of dextran-tetramethylrhodamine at the hydrogel bead surface indicating release of the marker from the bead (Fig. 3 *B*). Clearly, the transport resistance seen in Fig. 2 corresponds to the presence of a phospholipid membrane.

Lipobeads were also prepared with fluorescent calcium indicator dye Fluo-3 (855 D) encapsulated in the hydrogel core with or without calcium. Upon washing, the contrast intensity between the Lipobeads and the background was greatly reduced (images not shown), indicating that these

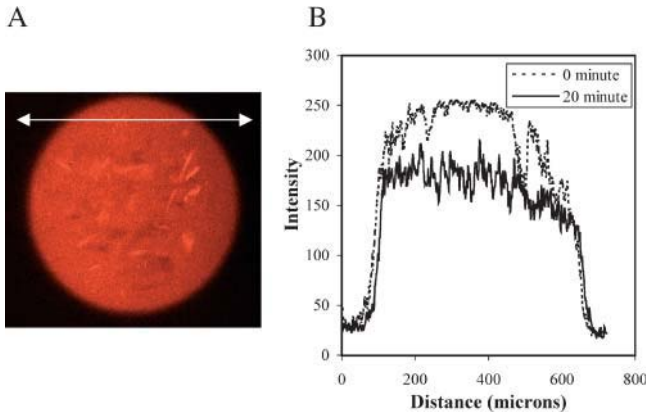

FIGURE 3 Polydimethylacrylamide (*pDMAA*) hydrogel beads without any anchors were hydrated with dextran-tetramethylrhodamine conjugates until equilibrium and then were incubated with liposomes composed of egg phosphatidylcholine. Emitted sample fluorescence passed through a long-pass 560-nm emission filter and was recorded (*A*). Fluorescence intensity through the midplane of the hydrogel (*white line* drawn in *A*) was collected at two different times and plotted in *B*.

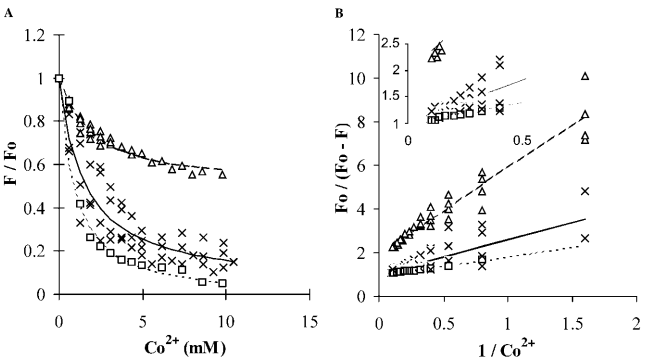

FIGURE 4 The quenching of liposomes and Lipobeads surface fluorescence by cobalt ions. The Lipobead membrane, ×, was composed of ePC/PC-NBD (30:1); ePC liposome, △, was composed of ePC/PC-NBD (30:1); PS liposome, □, was composed of ePC/PS/cholesterol/PC-NBD (11:12:1:1). (*A*) A plot of the fraction of fluorescence remaining versus cobalt ion concentration. (*B*) The same set of data fitted with the modified Stern-Volmer model. The inset is an expanded view of the plot near the *y*-intercept region.

two smaller solutes diffused rapidly out of the Lipobeads to the suspending buffer. Thus, the barrier properties of the Lipobead membrane may be dependent on the size of the solute. These results are in contrast with those obtained by Jin et al. (1996) with a different Lipobead formulation made of a polyvinyl alcohol hydrogel core and phosphatidic acid anchors. In that case, the Lipobead membrane restricted flux of both calcium and the indicator dye Fluo-3.

Cobalt ion quenching

Fluorescence quenching experiments by cobalt ions were conducted to reveal the nature of the phospholipid membrane on the Lipobeads. The access of cobalt ions to fluorescently labeled lipids is through hydrophilic pathways in bilayer membranes. Since cobalt ions do not penetrate an intact lipid membrane readily and are known to quench the fluorescence of NBD molecules (Lakowicz, 1999), the addition of excess cobalt to an NBD-labeled, wholly formed liposome will quench fluorescence in the outer membrane leaflet, thereby reducing the fluorescence intensity to ~50% of the initial value.

Fig. 4 *A* shows fluorescence intensity, normalized as the fraction of initial fluorescence, plotted against the concentration of added cobalt ions for Lipobeads and two control liposome preparations. The same data are replotted according to a modified Stern-Volmer model (Fig. 4 *B*) which accounts for quencher-accessible and quencher-inaccessible populations of fluorescent probes. The inverse of the *y*-intercept in Fig. 4 *B* is indicative of the quenchable fraction of fluorophores. Liposomes made from ePC prepared by extrusion are known to have a bilayer membrane (Macdonald et al., 1991). Fig. 4 *A* shows that the fluorescence of ePC liposomes asymptotes toward 50% in the presence of excess cobalt ions, whereas the *y*-intercept of the corresponding line in Fig. 4 *B* is 1.96 ± 0.15, indicating that two distinct membrane leaflets are present and that, as expected, ~50% of all fluorescently labeled phospholipid can be quenched by CO^{2+}. Quenchable fluorophores are located exclusively on the outer membrane leaflet and the quencher-inaccessible fluorophores face the inner liposome core.

Phosphatidylserine-containing membranes are known to be leaky to divalent cations, thus allowing CO^{2+} access to the liposome core (Düzgünes and Papahadjopoulos, 1983). PS liposomes, consisting of ePC/PS/cholesterol/PC-NBD (11:12:1:1), shows quenching curves that are consistent with 100% of the fluorophores being accessible to CO^{2+}. The curve in Fig. 4 *A* asymptotes toward $F/F_0 = 0$, whereas the line in Fig. 4 *B* gives a *y*-intercept of 0.97 ± 0.02.

Lipobeads made by incubating pre-Lipobeads with ePC/PC-NBD (30:1) liposomes show a quenching curve that asymptotes toward $F/F_0 = 0$ (Fig. 4 *A*), and a Stern-Volmer plot with a *y*-intercept of 0.86 ± 0.08 (Fig. 4 *B*), indicating that nearly 100% of the phospholipid fluorophore are accessible to the quencher. Since Lipobead membranes allow transmembrane transport of calcium ions (see above), it is expected that the divalent cobalt ions can also transport through Lipobead membranes and quench the fluorophores in both leaflets of a phospholipid bilayer. The nearly complete quenching observed does not exclude the possibility of multiple bilayers; however, multiple bilayers are unlikely to form since steric repulsion would prevent the close approach of two bilayers, and without additional attractive forces, liposomes are unlikely to fuse with a bilayer-covered Liposome surface. The complete quenching also suggests that lipids in the membrane are not arranged in aggregated or globular constructs that may prevent CO^{2+} access.

Phospholipid mobility in the Lipobead membrane and the influence of substrate

The time course of fluorescence intensity for a typical FRAP experiment is shown in Fig. 5 *A*; the asymptote of the curve is then used to determine the fraction of mobile phospholipids. The fluorescence recovery of the mobile fraction is then replotted in Fig. 5 *B* as suggested by the form of Eq. 2, and the diffusion coefficient of the mobile fraction is extracted from the slope of the line. Fig. 6 *A* shows that as the anchor density on the pre-Lipobead surface increases, both the lipid diffusivity and the fraction of mobile phospholipid of the Lipobead membrane decrease slightly. In contrast, Fig. 6 *B* shows that increasing the hydrogel crosslinking density only has a marginal effect on diffusivity and mobile fraction. The diffusivity of PC-NBD in the Lipobead membrane at 20°C ranges from 0.30 to 5.0 $\times$ 10^{-10} cm^2/s over the range of experimental variables examined.

DISCUSSION

Encapsulation experiments using dextran, and the Fluo-3/calcium mixture showed that Lipobead membranes significantly retarded dextran transport while freely allowing the transport of smaller solutes. A comparison of Figs. 2 and 3 shows that the diffusional barrier formed to encapsulate dextran is due to the Lipobead membrane. However, small hydrophilic pathways are presented as indicated by the fast permeation of small solutes such as calcium ions and the fluorescent calcium indicator dye Fluo 3 (960 Da). The hydrodynamic radius of a 1.5–3.0 kDa dextran molecule is between 1.03 and 1.46 nm (Hagel, 1988); therefore any defect in the membrane may be <6.7 nm^2 in area, assuming a circular hydrodynamic cross section. The presence of hydrophobic anchors on the hydrogel seems to promote lipid fusion and a certain degree of bilayer healing that facilitates the formation of a coherent bilayer, whereas bilayers supported on unmodified hydrophilic polymers were reported to be patchy and defective (Kühner et al., 1994; Reviakine and Brisson, 2000). The ability of Lipobeads to encapsulate preloaded content and either prevent or modulate their release needs further development but points to potential drug delivery, antigen presentation, and diagnostic applications.

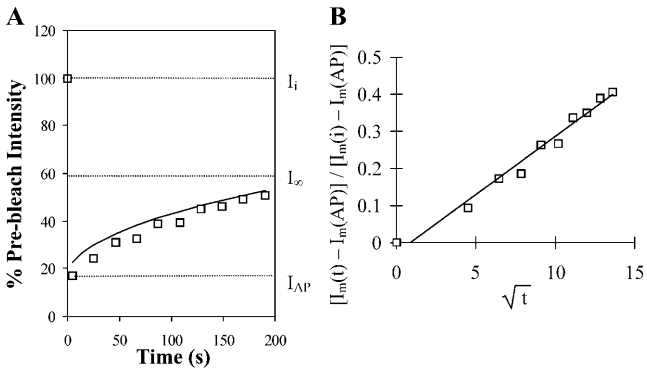

FIGURE 5 (*A*) The results of a typical FRAP experiment to measure the labeled phospholipid diffusivity. (*B*) The fitting of data to the one-dimension transport diffusion model ($[I_m(t) - I_m(AP)]/[I_m(i) - I_m(AP)]$ vs. $\sqrt{t}$).

The quenching experiments showed that the fluorescent phospholipids in the Lipobead membrane are all accessible to cobalt quenching. The membrane is likely composed of two leaflets of phospholipids in a bilayer configuration that permit cobalt transport, as multiple bilayers stacked on top of one another would lead to protection of some of the fluorescent phospholipid from quenching by cobalt. Previous attempts to determine the number of phospholipid leaflets on polyvinyl alcohol bead surfaces by dipping experiment using Liposheets also suggested that the formed Lipobead membrane was a bilayer (Jin et al., 1996). Maltodextrin encapsulated liposomes (SMBV) prepared by the homogenization of crosslinked maltodextrin cores with liposomes, characterized by cryoelectron microscopy, demonstrated a 5-nm-thick membrane around the hydrogel and suggested the membrane was also a monobilayer (De Miguel et al., 2000). In contrast to the SMBVs, however, the membrane of Lipobead self-assembles without any homogenization, probably by using the anchors as pinning centers.

The extent of polymerization (yield) achieved during pre-Lipobead synthesis was $>90\%$ as determined by performing a mass balance calculation of the reagent added and product produced. This indicates that nearly all reactants, including the anchors, were incorporated into the pre-Lipobeads. Assuming that the reactivity of anchors does not change significantly with changing anchor concentration in the reaction mixture, then the surface density of anchors on pre-Lipobead surfaces can be controlled by varying the fraction of anchors relative to surfactants during bead synthesis. By maintaining the same level of hydrogel crosslinking, while using a larger fraction of anchors in the anchor-surfactant mixture during polymerization, the anchor density on the hydrogel surface increases. As shown in Fig. 6 *A*, the lateral diffusivity of membrane phospholipid decreases slightly as the fraction of anchors in the surfactant mixture of the reaction medium increases, presumably because a larger fraction of covalently coupled anchor on the Lipobead surface retards phospholipid mobility. The mobile membrane fraction also decreases as the surface anchor density increases.

At a constant anchor/surfactant ratio 1:1 (i.e., 50% anchor in the surfactant mixture) during pre-Lipobead synthesis we expect that $\sim 50\%$ of the inner leaflet phospholipid of the Lipobead membrane could be covalently attached to the hydrogel core. With such Lipobead membranes, phospholipid diffusivity decreases as hydrogel crosslinking increases from 2% to 5%, but showed no further decrease as hydrogel crosslinking density increased to up to 15% (Fig. 6 *B*). The mobile fraction of phospholipid also showed a similar trend,

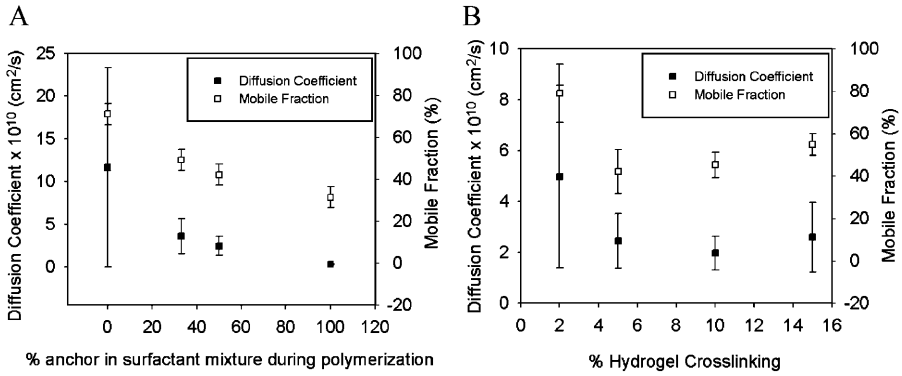

FIGURE 6 The correlation of PC-NBD lateral diffusion coefficients in the Lipobead membrane (composed of ePC/PS/cholesterol/PC-NBD (11:12:1:1)) to (*A*) the fraction of anchor in total surfactants added during pre-Lipobead polymerization in 5% crosslinked beads and (*B*) the percent crosslinking in the hydrogel with 50% surface anchor in the surfactant mixture during polymerization.

dropping from 80% to 40% as crosslink density increased from 2% to 5%, remained approximately constant with higher crosslink density. One reason for the higher phospholipid mobility at lower crosslink densities may be that the hydrogel core is less rigid and more hydrated when crosslink density is low. Perhaps there are also fewer interactions between the bilayer and more widely spaced polymer chains. Qualitatively, a similar trend was observed by Naumann et al. (2001) who found that lateral diffusivity varied inversely with the amount of lipid-polymer conjugate used in creating a polymer cushion with anchor lipids for supporting a lipid bilayer. However, they showed that at lower concentrations of surface lipid-polymer conjugate anchor (5–30%; the absolute value cannot be compared with our experiment due to different setup), the diffusivities measured were independent of conjugate concentration. They argued that diffusion slows significantly as the polymer density in the cushion layer increases above a certain level, presumably reflecting the possibility of direct interactions between adjacent polymer chains, forming a more rigid network. Our results may indicate that at crosslinking densities above a critical value, further increase in network rigidity has little effect on the already low diffusivity values. It is clear that future development of the Lipobead platform will require more experimentation with anchor structures. In particular, introducing hydrophilic spacers between the polymer core matrix and the hydrophobic anchor molecule may reduce coupling between the bilayer and the core. In addition, use of more hydrophilic polymer cores may increase hydrogel swelling and reduce interactions with the lipid bilayer.

Comparisons of the diffusion coefficients of PC-NBD in the Lipobead membrane determined in this study with values measured by FRAP in other similar supported systems are summarized in Table 1. Differences in diffusivity value may be due to 1), the nature of the underlying support substrate; 2), the fluorescent probe used; 3), the lipid composition of the membrane under study; and 4), the gel-fluid phase transition of the membrane.

The phospholipid PC-NBD has similar diffusivity in human red blood cells, skin fibroblast membranes, and the Lipobead membrane. This agreement may suggest that the hydrogel limits the mobility of the supported phospholipids to a similar extent as the cytoskeleton of a cell. The amount of phospholipid tethered to the underlying elements can affect the measured diffusivity of a phospholipid in the supported

TABLE 1 A comparison of the labeled phospholipid lateral diffusion coefficients in membranes measured by FRAP in various systems

Diffusion species	Media	Diffusion coefficients (cm^2/s, 20–25°C)
NBD-PE	Egg PC monolayer at air/water interfaces*	1×10^{-7}
NBD-PE	Outer leaflet of a 80% PC and 20% PG membrane supported on quartz†	3.6×10^{-8}
NBD-PE	POPC membrane bilayer supported on glass with a layer of water in between‡	1.3×10^{-8}
NBD-PE	A DMPC monolayer supported by 0–30% DMPE-PEO_{45} conjugate at air-water interfaces§	$4–75 \times 10^{-9}$
NBD-PE	Outer leaflet of a POPC membrane supported by 1–10% DMPE-PEO_{77} conjugate on silicate substrate‡	$5–9 \times 10^{-9}$
NBD-PE	A monolayer of DLPC with the some lipids attached to silica¶	1.9×10^{-9}
PC-NBD	Human intact red blood cell‖	1.1×10^{-9}
PC-NBD	Human skin fibroblast‖	9×10^{-10}
PC-NBD	Egg PC/PS/cholesterol (10:10:1) membrane supported by Lipobead (this study)	$0.3–5 \times 10^{-10}$

NBD, nitrobenz-2-oxa-1,3-diazol-4-yl (NBD is attached to the headgroup of the phospholipid if the short form is placed before the phospholipid, otherwise is attached to the acyl chain); PE, phosphoethanolamine; PC, phosphocholine; PG, phosphoglycerol; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-PC; DMPC, 1,2-dimyristoyl-3-glycero-PC; PEO_n, *polyethylene oxide*$_{\text{number of repeat units}}$; DLPC, 1,2-dilauroyl-3-glycero-PC; PS, phosphatidylserine.

*Lalchev and Mackie (1999).

†Kalb et al. (1992).

‡Wagner and Tamm (2000).

§Ke and Naumann (2001).

¶Yang and Yu (1999).

‖Lee and Jacobson (1994).

membrane. Wagner and Tamm (2000) reported that the measured diffusivity of a phospholipid in a polyethylene oxide (PEO) supported membrane was reduced by the presence of lipid-PEO conjugate deposited on the silica surface, despite claims that PEO is a polymer which has near ideal behavior in water and negligible interaction with proteins. It is likely that substantial interaction arises between the headgroups of the phospholipid and the free ends of the polymer.

For many of the studies of polymer-supported membrane discussed above, the fluorescent probe used was NBD-PE in which the probe NBD is conjugated to the headgroup of the phospholipid. The probe we used was PC-NBD in which NBD is attached to one of the acyl chains. We attempted to measure the diffusivity of NBD-PE in a 3 mol % ePC membrane and found the fluorescence emission of NBD in water to be weak and insufficient in intensity for FRAP measurement using our instrumentation.

Lipid composition affects the fluid-to-gel transition of a membrane, and thus at temperatures below the transition, the diffusivity of a probe is typically 100-fold lower than at temperatures above the transition. Our FRAP experiments were conducted at 20°C, and the low diffusivity of the PC-NBD probe may indicate a gel phase ordering of the Lipobead bilayer. Differential scanning calorimetry experiments did not show a clear phase transition in the Lipobeads; however, this is not surprising given the presence of cholesterol in the membrane. It is notable that in studying a poly(ethyloxazaline)-supported phospholipid membrane in which the polymer was photocrosslinked to the substrate, Naumann et al. (2002) reported that the membrane remained in the gel phase even when heated to 52°C; their membrane seems to have been stiffened by the polymer support. When the polymer support was not conjugated to the substrate surface, the membrane gel-to-fluid transition was between 27°C and 30°C, and the measured phospholipid diffusivity in the sol state varied between 1 and 18 $\times$ 10^{-8} cm^2/s depending on the amount of lipid-polymer conjugate used in the support.

The addition of an ionic phospholipid also affects mobility. The PEO-supported membrane described by Wagner and Tamm (2001) had a measured NBD-PE diffusivity of 6 $\times$ 10^{-9} cm^2/s. However, the value dropped to 4 $\times$ 10^{-9} cm^2/s when 15% of dioleoylphosphoserine, a common phospholipid found in the cytoplasmic leaflet of cell membrane, was added to the system. Our Lipobead membrane lipid composition was 48% ePC, 48% PS, and 4% cholesterol. This composition was selected in anticipation of experiments with transmembrane receptor constituted proteoliposomes (see Park et al., 2004). Thus, the high fraction of phosphatidylserine used here may have further restricted probe diffusivity.

Another interesting observation from the FRAP measurements was that fluorescence intensity of the bleached area did not recover completely to the prebleached value. The apparent diffusivity measured by FRAP is likely dominated by the fastest diffusing species in the membrane. The fraction of fluorescence not recovered is considered to reflect the immobile or slower diffusing phospholipids in the membrane. In artificial membranes supported at air/water interfaces, only a limited amount of immobile phospholipid is present. In a real cell membrane, on the other hand, membrane phospholipids and proteins show anomalous diffusion, which generally is attributed to the interaction with the underlying cytoskeleton elements (Feder et al., 1996; Schutz et al., 1997; Cherry et al., 1998). Single-particle tracking (Elliott et al., 2003) or image correlation spectroscopy (Rocheleau et al., 2003) may be a better technique to measure diffusion coefficients in such complex, multicomponent, and multicompartment systems.

A three-step mechanism to liposome fusion on a substrate surface has been proposed and partly validated (Lipowsky and Seifert, 1991; Keller and Kasemo, 1998; Nissen et al., 1999). To form a membrane on a substrate surface via liposome fusion, it was shown that liposomes first have to adhere to the substrate surface. By using hydrophobic anchors, the hydrogel surface likely attracts a large amount of liposomes to minimize anchor exposure to water. As the concentration of liposome rises on the hydrogel surface, the liposomes may fuse and rupture. Because the underlying substrate is in proximity, yet yielding and flexible, defects may be healed by a combination of bilayer rolling and spreading. Since the hydrophobic surface is the main driving force for liposome fusion, the self-assembly of a membrane on Lipobead is likely to be relatively unaffected by the size of the beads and the composition of the lipid mixture. The minimum anchor density to effect a complete, self-assembled bilayer needs still to be determined.

A brush-like, noncrosslinked polymer without any hydrophobic anchors on the surface may actually prevent vesicle adhesion and thus lower the effective vesicle concentration on that substrate surface. The flexible polymer chain may also trap intact vesicles and prevent the vesicles from fusing to form a complete bilayer. This may explain why membranes could only form with specific polymer or specific lipid composition on unmodified hydrophilic polymer surfaces (Kiser et al., 1998). Because of the presence of anchors on the surface of pre-Lipobeads, the adhesion wall potential of the anchor-rich hydrogel surface is so strong that the shape, size, and surface charges of the hydrogel surface do not seem to be critical in promoting formation of the supported membrane. In addition, using pre-Lipobeads to form a supported membrane seems to be a more robust strategy, as lipid composition, ionic strength, and the size of liposomes are no longer critical.

CONCLUSIONS

A phospholipid membrane can self-assemble on the pre-Lipobead surface through spontaneous fusion of liposomes with that anchor-modified hydrogel surface. The membrane

formed is likely a bilayer of phospholipids and is fairly complete so that it forms a diffusion barrier to dextran molecules of 1500–3000 Da. The diffusivity of PC-NBD, determined by FRAP, in a Lipobead membrane composed of egg phosphatidylcholines, phosphatidylserine, and cholesterol is in the range of 0.3 to 5 $\times$ 10^{-10} cm^2/s, depending on the anchor density and hydrogel crosslinking. Increasing the anchor density on the Lipobead surface or the hydrogel crosslinking density in the core have a slowing effect on phospholipid mobility in the membrane. The value of phospholipid diffusivity in the Lipobead membrane is comparatively smaller than those measured in other polymer-supported membrane systems, an effect that may be related to the rigid three-dimensional polymer network as the underlying membrane support. Compared to other hydrogel-supported membrane structures (De Miguel et al., 1995, 2000; Kiser et al., 1998; Wong et al., 1999) which require homogenization to form a membrane, our self-assembly system allows easy and robust incorporation of a phospholipid membrane. The nature of the hydrogel as well as the amount and identity of anchors can be varied because of the unique synthesis scheme. As a result, the ability to make a variety of Lipobead with distinct functionality allows one to examine different membrane-protein properties. Furthermore, the micron size of pre-Lipobeads facilitates the ease of handling and possibly its use as a new platform in high throughput drug screening. We are currently investigating the use of Lipobeads to support and study transmembrane protein binding properties (Park et al., 2004).

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