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Lipobeads: a hydrogel anchored lipid vesicle system

T. Jin[a], P. Pennefather[a,*], P.I. Lee[b]

[a]*Faculty of Pharmacy, University of Toronto, 19 Russell St., Toronto, Ont., M5S 2S2, Canada*
[b]*Schering-Plough Research Institute, 2015 Galloping Hill Rd., Kenilworth, NJ 07703-0539, USA*

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Abstract A new vesicle system is described that combines complementary properties of liposomes and polymeric beads. 'Lipobeads' consist of a lipid bilayer shell anchored on the surface of a hydrogel polymer cores which acts like a cytoskeleton. Anchoring is provided by fatty acids covalently attached to the surface of the hydrogel. These hydrophobic chains drive spontaneous assembly of a lipid bilayer shell around the modified hydrogel bead when exposed to a suspension of liposomes. The bilayer is stable and acts as a permeability barrier to compound loaded by prior absorption into the polymer core. Lipid mobility in the shell is similar to that found in other unanchored lipid bilayers. The system has potential application in drug delivery and for functional reconstitution of membrane proteins.

Key words: Supported lipid bilayer; Hydrogel microsphere; Polyvinyl alcohol; Liposome; Model membrane; Artificial cell

1. Introduction

Artificial particulate systems such as polymeric beads and liposomes are finding a variety of biomedical applications in drug delivery, drug targeting, protein separation, enzyme immobilization and blood cell substitution [1–6]. Liposomes have a flexible, cell-like lipid bilayer surface which acts as a permeability barrier such that compounds can be entrapped in their aqueous interior. However, liposomes can be mechanically unstable and their loading capacity limited by the water solubility of the material to be loaded [7]. Polymeric beads, although mechanically more stable and having a larger loading capacity then liposomes, lack many of the useful surface properties of a lipid bilayer shell. Here, we describe the preparation and characterization a new hybrid vesicle system with structural similarity to natural cells that combines complementary advantages of liposomes and polymeric beads. This system, which we have called 'Lipobeads', consists of a lipid bilayer shell that is anchored on the surface of a hydrogel polymer core.

Lipid bilayers supported on various solid surfaces, such as glass [8], plastic [5], and metal [9] as well as modified polymers [10] have previously been shown to provide a stable and well defined cell membrane-like environment that has found a number of basic and applied uses [11,12]. It was, therefore reasonable to expect that assembly of lipid bilayers on spherical hydrogel surfaces could be a useful approach for preparing an artificial cell analogue. Indeed, in 1987, Gao and Huang [13] reported that encapsulation of hydrogel particles into liposomes enhanced the loading capacity and overall mechanical strength of the liposomal structure. However, in that system the unanchored bilayer is still somewhat unstable and the system could only be formed with specific lipid mixtures and only with polymer cores of certain sizes and shapes. We have developed a hydrogel anchored liposome in which these limitations have been overcome.

Our approach (see Fig. 1) has been to create hydrophobic anchors for the bilayer by attaching lipid molecules to the surface of pre-formed polyvinyl alcohol (PVA) xerogel (dry hydrogel) beads. When the surface modified xerogel is then hydrated and treated with a liposome suspension the hydrophobic lipid molecules and other intrinsic membrane components of the liposomes associate spontaneously with the hydrophobic fatty acid anchors on the surface of the hydrated polymer and self-organize into a distributed membrane over that surface through hydrophobic interactions. Thus, not only do these anchors add mechanical stability to the bilayer shell but they also promote its self-assembly. In a sense, these acyl anchors and the polymer to which they are attached act as a 'cytoskeleton'. This self-assembly property allows the bilayer coating to be established on cores ranging in shape from sheets to spheres.

2. Materials and methods

PVA was chosen as the hydrogel support because of its proven biocompatibility [14,15]. In addition, PVA can be physically cross-linked [16] so that drugs of large molecular weight can be incorporated into the hydrogel matrix via an absorptive loading process after the beads are made, avoiding exposure to other chemical reagents which might alter the properties of the incorporated drug. Furthermore, the high content of hydroxyl groups of PVA enables the hydrophobic anchor molecules to be covalently attached at a sufficiently high surface density to drive spontaneous assembly of a surface lipid bilayer.

PVA was formed into beads by dispersal in paraffin, freeze-thaw solidification and in situ drying facilitated by bubbling dry air through the suspension. Fully hydrolyzed polyvinyl alcohol (PVA) (Elvanol 71-30, from Dupont) with M_w 116000 and M_n 39500 [17] was dissolved in hot water at a concentration of 22% w/v and then dispersed in paraffin oil (Aldrich) containing 1.5% w/v surfactant (Span 80, Sigma) to form beads. The beads were then cross-linked by slowly freezing the suspension to −20°C for 12 h followed by a slow thaw at room temperature. The beads were then dried by bubbling dry air into the suspension for 1–2 weeks, followed by removal of the oil through re-suspension in hexane, ethyl acetate and alcohol. This procedure produced Polyvinyl alcohol (PVA) xerogel spheres ranging in size from a few micrometers to over 100 μm in diameter. Spherical beads ranging in size from 70 to 90 μm were selected by sieving for further modification.

Surface acylation with fatty acid chains was accomplished by treating the PVA beads with 1 M palmitoyl chloride (Aldrich) in hexane at room temperature for 2–3 days. The palmitic species became anchored on the bead via esterification with surface hydroxyls. The completion of this reaction was characterized using attenuated total reflectance (ATR) FTIR and X-ray photoelectron spectroscopy (XPS). With ATR-FITR, the formation of an ester bond was determined, and

*Corresponding author. Fax: (1) (416) 978-8511.
E-mail: pspen@phm.utoronto.ca

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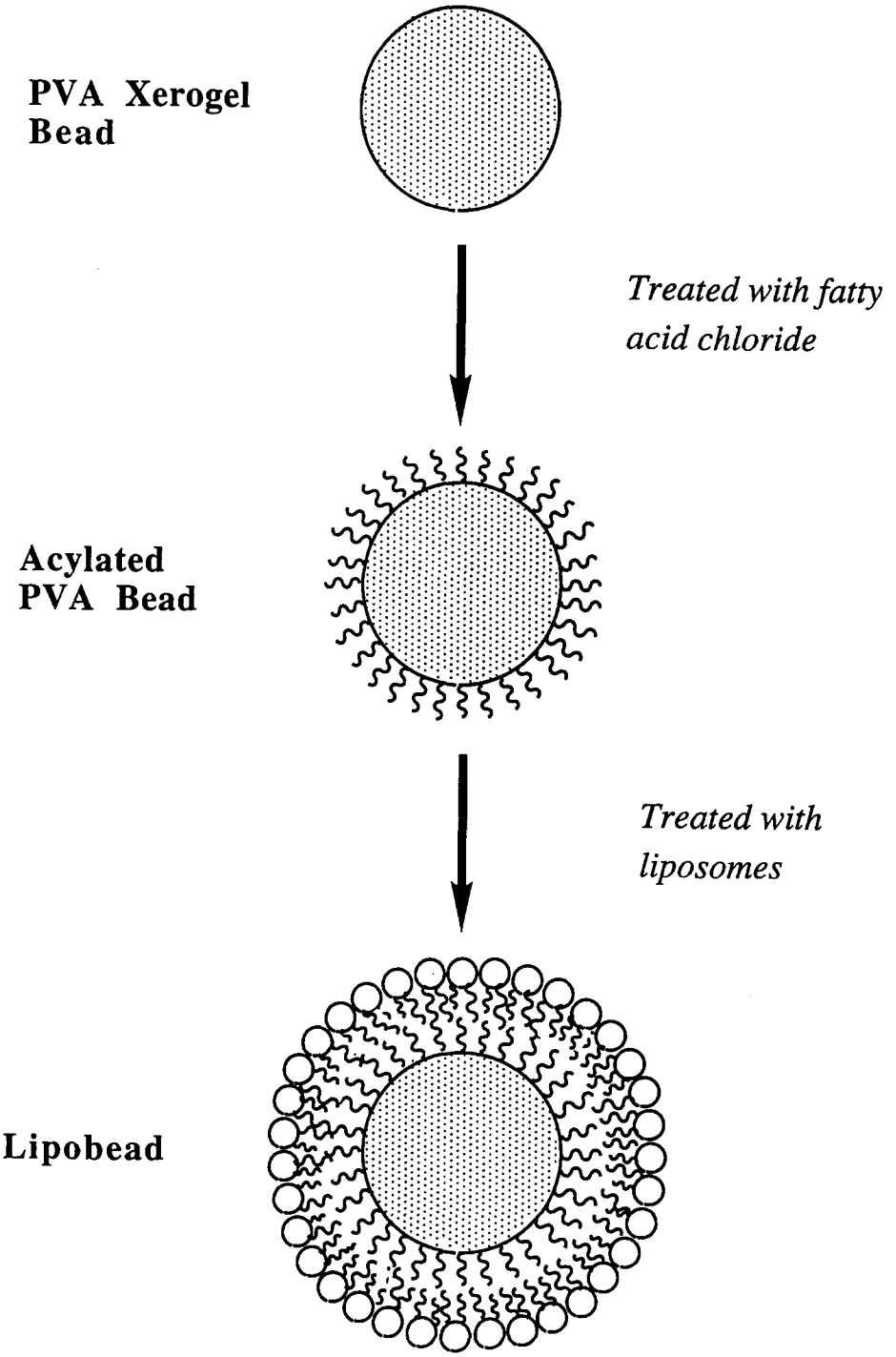

Fig. 1. Outline of lipobead preparation. The surface of pre-formed polyvinyl alcohol (PVA) xerogel (dry hydrogel) beads is exposed to chloro fatty acids dissolved in hexane which form ester linkages with surface hydroxyls on the xerogel and provide hydrophobic anchors for the bilayer coating. Final formation of lipobeads was accomplished by adding the surface modified beads to a liposome suspension which leads to transfer of lipid onto the acylated surface of the bead and self-assembly of an anchored bilayer shell around the hydrogel core.

with XPS, changes in atomic ratio at the surface was measured. For sampling the surfaces with these analytical tools, PVA films were subjected to identical chemical treatment as described above and summarized in Fig. 1. ATR-FITR spectra were recorded on a Bio-Rad Digilab FTS-7 system with a custom designed sample holder with which the polymer films were pressed against a Ge prism (25×5×1 mm with 45° slope of the edge) and XPS spectra recorded on a Leybold MX-200 system with a Mg source, beam energy of 12 kV and beam current of 25 mA. Further details of the surface modification reactions and characterization procedures will be published elsewhere but indicate complete derivatisation of surface hydroxyls with little evidence of penetration beyond 10 nm (Jin and Lee, in preparation).

Final formation of lipobeads was accomplished by combining equal parts of a suspension of surface modified beads to a liposome suspension (made by sonicating a 5 mg/ml suspension of phosphatidylcholine until transparent). The hydrophobicity of the acylated bead surface leads to transfer of lipid onto this surface and self-assembly of a bilayer shell around the hydrogel core.

The uniformity, lateral mobility and permeability of the supported bilayer to ions and model drugs were examined using an MRC-600 Laser Scanning Confocal Microscope (LSCM).

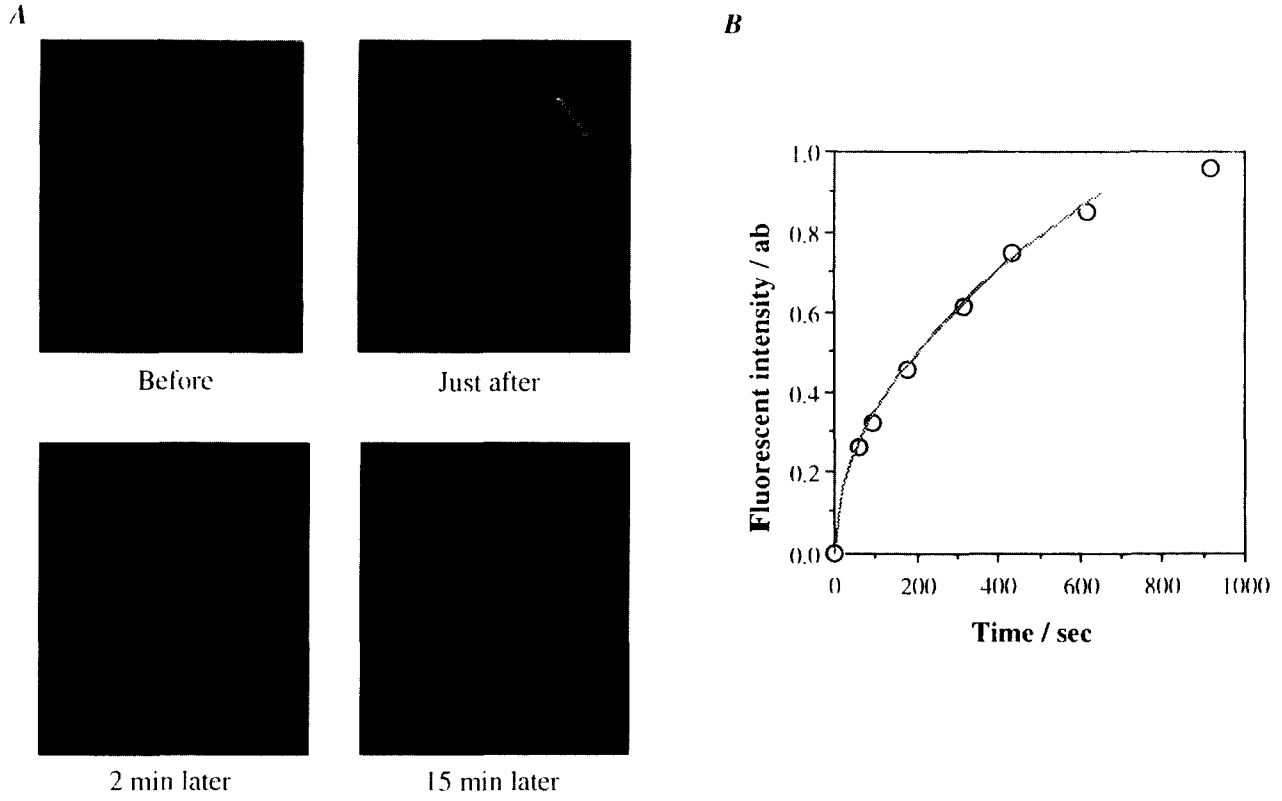

Fig. 2. Lateral mobility in hydrogel supported lipid bilayer labeled with fluorescent phospholipid. (A) Confocal images taken before and after a portion of a lipobead surface was photobleached. Time of capture is indicated below each image. (B) Time course of recovery of fluorescence in the photo-bleached area. The solid line describes the expected recovery based on an earlier time approximation [21] assuming a lipid diffusion coefficient of 1.55×10^{-9} cm^2/s.

3. Results

When dried acylated beads are placed in water, their diameter increases about 1.4-times at swelling equilibrium. However, the beads aggregate and float on the water surface due to their surface hydrophobicity. Adding a liposome suspension to the solution with floating beads causes the beads to separate and sink to the bottom. These observations are consistent with contact angle measurements. For acylated PVA surfaces, the observed angle was 103° indicating that the surface is hydrophobic. The contact angle of the surface was reduced to less than 15° after the film was treated with liposomes. When beads are separated from water by filtration and exposed to air, the bilayer is disrupted and the beads again become hydrophobic. This process of bilayer formation and destruction can be easily repeated with the same sample demonstrating the capability of lipobeads for self-repair.

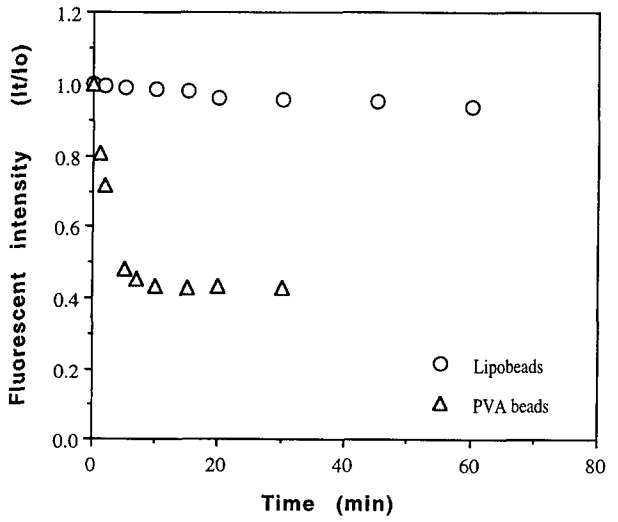

Fig. 3. Release of Lucifer yellow from lipobeads and bare PVA beads. Lucifer yellow was loaded by impregnating acylated and bare PVA beads with a solution of 50 μM Lucifer yellow in 100 mM KCl. Loaded lipobeads, were then formed by liposome treatment. The graph depicts the time course of release of Lucifer yellow from the beads, measured in terms of the decay of fluorescence intensity after a dilution to 10 μM Lucifer yellow.

Measurement of Langmuir-Blodget (LB) film deposition on PVA disks subjected to the same surface modification as lipobeads suggests that a single well-packed lipid layer is formed on the modified surface. For LB deposition, dioleoylphosphatidylcholine (from Avanti Polar Lipids, and diluted with chloroform to 5 mM prior to use) was spread over the water surface of an LB trough (Mgw Lauda Filmwaage) to form a monolayer with membrane pressure of 30 dyn/cm. When the hydrophobic film is gradually dipped into of the water through the monolayer, a layer of lipid is deposited on the film as indicated by the decrease in monolayer surface area. Another layer is deposited when the film is withdrawn but, this second layer is easily stripped from the film surface when the sample is passed back through the monolayer surface, indicating that lipid multilayers are not formed. This result suggests to us that the lipobead coating is a bilayer with the interior leaflet anchored by the surface fatty acid residues. The

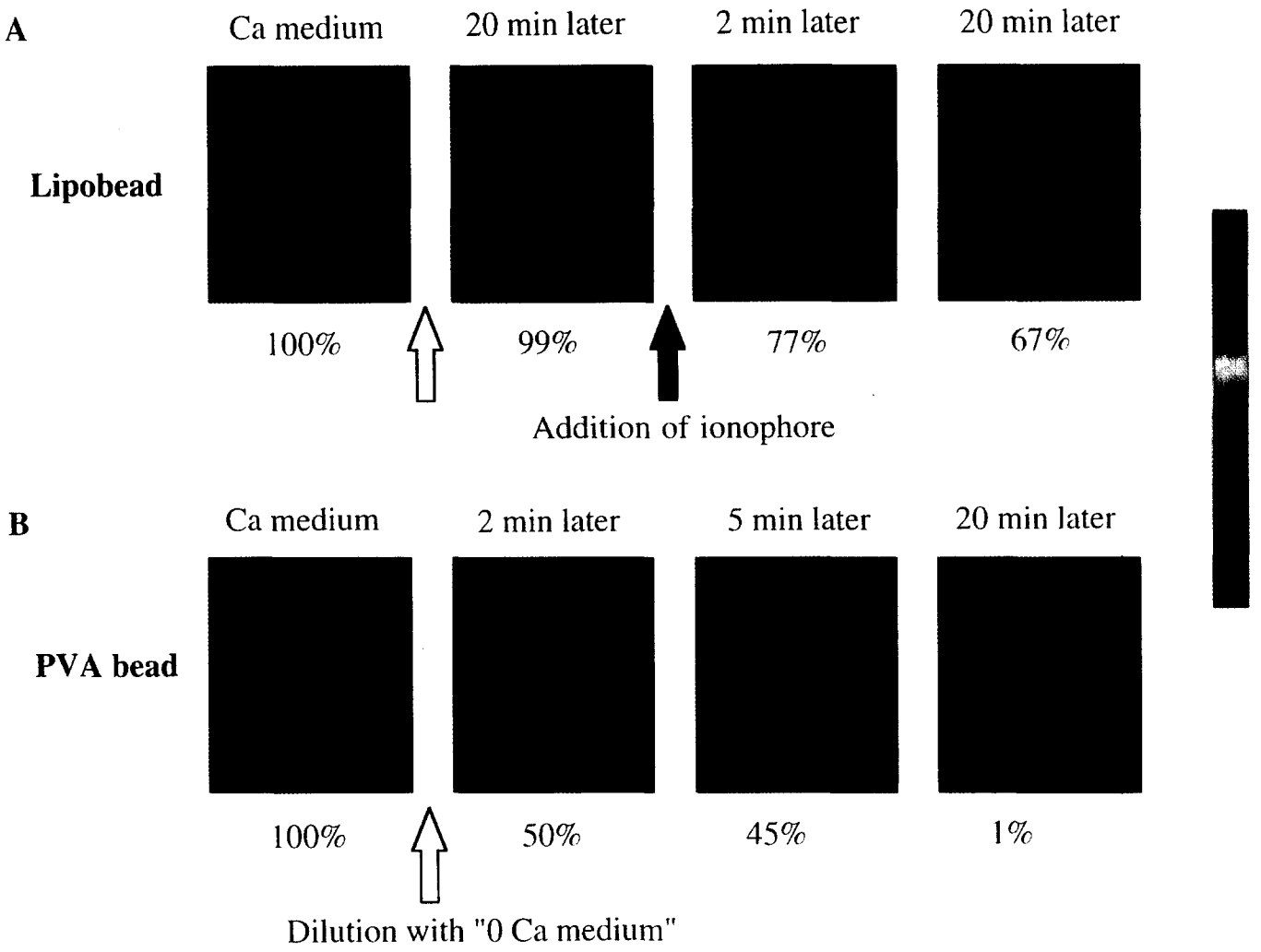

Fig. 4. Release of Ca^{2+} from lipobeads and bare PVA beads loaded with fluo-3. Lipobeads and bare PVA beads loaded with 50 μM fluo-3 in a 'Ca^{2+}' medium subjected to a 5-fold dilution with '0 Ca^{2+} medium' (see Section 2) which should reduce free extracellular Ca^{2+} to 60 nM. Numbers below the images indicate relative fluorescence intensity. (A) Lipid layer on lipobeads prevents Ca^{2+} efflux until an ionophore is added. Images of the same bead before dilution and 20 min after dilution, and 2 and 20 min after addition of the Ca^{2+} ionophore, 4-bromo-A23187 (5 mM in methanol to reach a final concentration of 50 μM). (B) Bare PVA beads do not trap Ca^{2+}. Images of a bare bead before dilution and at the times indicated after dilution. Color scale is from 0 to 250%.

result also illustrates that the bilayer can be anchored on any shape of surface from planar to spherical.

Fig. 2A show LSCM images of lipobeads treated with liposomes which contained 5% of the fluorescent membrane probe, 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBA) labeled dioleoylphosphatidylethanolamine. The continuous ring of fluorescence suggests that following deposition, liposome phospholipid becomes evenly distributed over the lipobead surface. This observation is in keeping the considerable lateral mobility expected for lipid bilayers [18,19]. The fluidity of the supported bilayer was tested using a photo-bleaching protocol. The LSCM laser beam was parked over a portion of the bead to induce local photo-bleaching of the fluorescence probe causing an opening in the ring of fluorescence (Fig. 2A). This opening gradually closed over 15 min. Since photo-bleaching of NBA is irreversible [19,20], this recovery is attributable to the diffusion of labeled lipid molecules through the bilayer into the bleached region. Fig. 2B shows the time course of fluorescence recovery in the bleached area. The solid line is that expected for one-dimensional (slab) diffusion of labeled phospholipid from non-bleached area assuming a diffusion coefficient of 1.55×10^{-9} cm^2/s. This value is of the same order of magnitude as that estimated for normal lipid bilayers [18,19]. One-dimensional diffusion was considered because the bleached region produced by the laser is expected to be several fold broader along the z-axis than in the confocal plane.

For examining the permeability of the supported bilayer a fluorescent dye, Lucifer yellow, was used as a model compound in that its physical-chemical properties are similar to those of many small, hydrophilic drugs. Both lipobeads and bare PVA beads without hydrophobic surface anchors loaded with Lucifer yellow were added to dye free solution and dye efflux was followed. The fluorescence intensity changes for both lipobeads and bare beads after a 5-fold dilution are plotted versus time in Fig. 3. For the bare beads, the loaded Lucifer yellow was rapidly released and reached an equilibrium in a few minutes. With lipobeads, however, the fluorescence intensity decreased only slightly over 60 min. It is clear that small hydrophilic molecules can be entrapped in these lipobeads by the lipid coating.

Fig. 4 illustrates the ability of lipobeads to trap ions. We

used the fluorescent indicator dye fluo-3 (Molecular Probes) to monitor levels of Ca^{2+} [22] trapped in the core of lipobeads by prior loading. Fig. 4A shows the fluorescence confocal images taken of lipobeads and bare PVA beads loaded with 50 μM fluo-3 in a 'Ca^{2+}' medium which contained (in mM): 2 $CaCl_2$, 1 EGTA, 5 HEPES, 140 KCl with pH adjusted to 7.4. The bathing solution was diluted 5-fold with a '0 Ca^{2+} medium' identical to the Ca^{2+} medium except that the $CaCl_2$ was omitted. This 5-fold dilution changes the ratio of Ca^{2+} to EGTA such that extracellular free Ca^{2+} levels should be reduced from 1 mM to 60 nM. With the Lipobeads, the fluorescence intensity of fluo-3 remained constant during the initial 20 min of incubation in the low Ca^{2+} medium but quickly decreased after adding a Ca^{2+} ionophore, 4-bromo-A23187 [22] (1:100 dilution (v/v) of 5 mM ionophore dissolved in methanol) to bypass the permeability barrier created by the bilayer coating. Adding the ionophore vehicle (methanol) alone to the same sample caused no change in fluorescence intensity (not shown), excluding possibility that the bilayer membrane was simply disrupted by the solvent. We conclude that the decrease in fluorescence is due to facilitated Ca^{2+} diffusion across the bilayer membrane mediated by the ionophore. Fig. 4B shows confocal images of bare PVA beads loaded with Ca^{2+} and fluo-3. The same 5-fold dilution with zero Ca^{2+} medium, caused the fluorescence intensity to decrease rapidly in the initial 2 min, followed by a more gradual decay (Fig. 4B). These two components of fluorescence decrement may be attributed to the loss of Ca^{2+} and fluo-3, respectively. These results indicate that the lipid coating on the beads provides a permeability barrier to hydrophilic ions such as Ca^{2+}.

4. Discussion

Lipobeads may potentially be useful in several novel biomedical applications. As a drug carrier, for example, lipobeads should be superior to liposomes in the sense of enhanced mechanical stability, controllable size and increased drug loading capability. Long-term storage is possible since acylated beads loaded with drug can be stored in a dry form before lipid coating. Also, many of the techniques developed for liposomes such as stimulus-sensitive drug release [23], steric stabilization [24] and receptor or antibody mediated drug targeting [7] should still be applicable with lipobeads. Another potential application of lipobeads is red cell substitution. For hemoglobin (Hb) to perform a conformational change so that oxygen can be released readily at sites of low pO_2, encapsulation of Hb with the cofactor, 2,3-DPG is required [6]. Combined loading with multiple compounds can be achieved before adding the lipid membrane coating and much less lipid will be needed to entrap a given amount of Hb. By combining the structural features of liposomes and hydrogels, lipobeads may provide a more realistic red cell replacement while at the same time having a much higher encapsulating efficiency than liposomes. Since supported lipid bilayers have been found to create a favorable environment for receptor and enzyme functions [25], lipobeads may also provide a model environment for studying and simulating various biological functions such as cell-cell interactions and the operation of purified and reconstituted trans-membrane proteins such as ion channels and transporters.

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