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Lipid–alginate interactions render changes in phospholipid bilayer permeability

Smadar Cohen [1], M. Carmen Bañó [1], Marie Chow [2] and Robert Langer [1]

[1] *Department of Chemical Engineering, M.I.T., Cambridge, MA (U.S.A.), and* [2] *Department of Biology, Massachusetts Institute of Technology, Cambridge, MA (U.S.A.)*

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Lipid vesicles, e.g. liposomes, generally release their contents in a continuous manner. However, when these vesicles are entrapped in Ca-alginate and coated with poly(L-lysine), they release their contents in an unusual fashion, in 'bursts'. Molecular-level studies indicated that lipid–alginate interactions are responsible for changes in the barrier properties of lipid vesicles. Differential scanning calorimetry revealed that exposure of liposomes to alginate resulted in a 4-fold reduction in the phase transition enthalpy, with no change in the melting temperature. Size-exclusion chromatography of liposomes-in-alginate gave an additional liposomal peak with a smaller elution volume. These studies suggested that alginate is inserted into the lipid bilayer of vesicles. Lipid-alginate interactions were highly dependent on phospholipid head group charge and the phase transition temperature of the phospholipid. Based on these interactions, a mechanism to explain the 'burst' from these entrapped liposomes is suggested.

Introduction

Lipid vesicles, known as liposomes, have been used as a model for cell membranes [1–3] and they are one of the most studied systems for drug delivery applications [4–6]. They generally release their contents in a continuous fashion. However, we have discovered that when these vesicles are entrapped in Ca-alginate gel spheres that are further coated with poly(L-lysine), they release their contents in an unusual manner-at two predetermined, distinct 'bursts'.

To understand this phenomenon, four possible factors that can affect lipid bilayer barrier properties were investigated, such as the effect of lysophosphatidylcholine, calcium ions, alginate, and poly(L-lysine). These factors, except for alginate, were previously studied for their affect on biological and model membranes. Lysophosphatidylcholine, derived from enzymatic deacylation of phosphatidylcholine, was shown to fuse two red cells, eventually causing their lysis [7]. Calcium ions can also fuse membranes, particularly those containing acidic phospholipids [8,9]. On the other hand, the positively-charged polyelectrolyte, poly(L-lysine), interacts with membranes, particularly those composed of acidic phospholipids, without changing their permeability [10,11].

Our studies identified alginate, a water-soluble polysaccharide composed of alternating guluronic and mannuronic acids, as the major cause for the changes in liposome permeability. The interaction of alginate with the phospholipid bilayers was lipid composition-dependent. Differential scanning calorimetry and size-exclusion gel chromatography suggested that alginate–lipid interactions involve the insertion of alginate into the liposomal bilayer.

Abbreviations: PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PG, phosphatidylglycerol; CH, cholesterol; SA, stearylamine; PLL, poly(L-lysine); CF, carboxyfluorescein; FITC-BSA, fluorescein isothiocyanate-labeled bovine serum albumin; PBS, phosphate-buffered saline; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DSC, differential scanning calorimetry; MELs, microencapsulated liposome systems.

Correspondence: R. Langer, Department of Chemical Engineering, M.I.T. Bldg E25, Rm 342, 77 Massachusetts Avenue, Cambridge, MA 02139, U.S.A.

Materials and Methods

Materials

Egg phosphatidylcholine (egg PC), egg hydrogenated PC, dipalmitoyl PC (DPPC), and phosphatidylglycerol

(PG) were from Avanti Polar Lipids, AL. Cholesterol (CH), stearylamine (SA), poly(L-lysine)-HBr (PLL, 39.5 kDa), 6-carboxyfluorescein (CF) and fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) were from Sigma Chemical Co. MO. Sodium alginate, Kelco-LV, was from Kelco. All chemicals were reagent grade.

Liposome preparation and characterization

Liposomes were prepared by the reverse-phase evaporation technique [12] with slight modifications. Briefly, 264 μmol lipid were dissolved in 12 ml of diethyl ether or chloroform/isopropyl ether (1 : 3, v/v) and 4 ml of dye in phosphate-buffered saline (PBS, pH 7.4) were added. The two phase system was bath-sonicated for 1 min until the mixture became a one-phase dispersion. The organic phase was removed under reduced pressure, using a rotary evaporator, until a homogenous suspension of liposomes was achieved. The liposomes were extruded through a 0.6 μm pore size polycarbonate membrane, and the unentrapped dye was removed by repeated centrifugation cycles (10000 × g; 15 min) and buffer replacements, or by gel filtration (Sepharose 6B, Pharmacia). The amount of encapsulated dye in liposomes was determined by dissolving the lipid vesicles with 1% (v/v) Triton X-100, and monitoring the absorbance of CF and FITC-BSA at 490 and 495 nm, respectively.

Vesicle size distributions were determined by quasi-elastic light scattering analysis, performed utilizing a Brookhaven model BI-90 particle sizer with a 5 mW Helium-Neon Laser at an exciting wavelength of 488.0 nm. Quasi-elastic light scattering employs digital autocorrelation to analyze the fluctuations in scattered light intensity generated by the diffusion of vesicles in solution. The measured diffusion coefficient is used to obtain the average hydrodynamic radius and hence the mean diameter of the vesicles.

Liposome encapsulation in Ca-alginate matrices

Liposome pellets were suspended with 3 ml sodium alginate-in-saline (1.5% w/v), to a final ratio of 30 μmole lipid/ml. This suspension was sprayed as microdroplets, using an air jet-head droplet-forming apparatus [13]. With this system the liposome/alginate suspension was extruded (at 200 ml/h) through a 22G needle located inside a tube through which air flows at 5 l/h. Droplets forming at the needle tip were forced off by the coaxial air stream and were collected in Hepes-buffered calcium chloride (13 mM Hepes, 1.3% (w/v) $CaCl_2$, pH 7.4), where they gelled and hardened for 30 min. The resultant beads were drained and further coated for 10 min by contact with 45 ml of 0.06% (w/v) poly(L-lysine) (39.5 kDa)-in-saline, with gentle agitation. Unreacted poly(L-lysine) was removed by washing the beads three times with 50 ml of Hepes-buffered saline. The amount of entrapped dye in microencapsulated liposome systems was determined by treating the microspheres with 10% (w/v) EDTA solution containing 1% (v/v) Triton x-100, followed by a bath-sonication for 10 min. EDTA dissolved the internal gel core while Triton X-100 dissolves the lipid vesicles, thus releasing the dye to the medium. Microspheres and liposome debris were removed by centrifugation and the amount of dye in supernatants was determined by its absorbance at 490 and 495 nm for CF and FITC-BSA, respectively.

Permeability studies

Lipid bilayer permeability was followed by measuring the release with time of dye/protein from liposomes and microencapsulated liposomes. Microencapsulated liposomes were incubated in a 10 ml filter-ended Econo-column (Bio-Rad) containing 8 ml PBS (pH 7.4), with 0.01% (w/v) gentamicin sulfate as a preservative. The studies were performed at 37°C, in an air gravity incubator, with gentle agitation on a rotating shaker. The buffer solution was changed at particular time points by filtering the releasing media through the Econo-column. With liposomes, the experiments were performed in polypropylene tubes, and the releasing media was collected by pelleting the liposomes (10000 × g; 15 min) and collecting the releasing media. Release of CF and FITC-BSA were followed by monitoring the absorbance at 490 and 495 nm, respectively.

Differential scanning calorimetry (DSC)

Calorimetric measurements were performed with a Perkin-Elmer DSC-7 calorimeter equipped with a PE 7500 Professional Computer for data collection and analysis. Vesicles composed of DPPC were used as a model since DPPC forms relatively stable liposomes, and its main phase transition temperature is at a distinct position away from that of water. The sample preparation for the DSC experiments was as follows: 200 mg of DPPC, in chloroform, was deposited on the side of a 250 ml round-bottom flask by removal of the organic solvent by rotary evaporation and flushing with N_2. The lipid film was dissolved in 3 ml PBS, and further hydrated for 30 min at 50°C. To study lipid–polymer interactions, liposomes suspensions were centrifuged (5000 × g; 30 min) and the pellets were resuspended in a saline solution of alginate (1.5% w/v). Fifteen μl of liposome sample (total of 1 mg lipid) were placed in an aluminum pan and sealed, using a volatile sample sealer. Thermograms of samples were recorded and compared to a reference sample of PBS, at a rate of 4 C°/min.

Size-exclusion gel chromatography

Multilamellar vesicles composed of DPPC, at a concentration of 66 mg lipid/ml, were prepared as above

and sonicated for 10 min in a bath-type sonicator. This suspension was divided into two samples and centrifuged at 10000 × g for 30 min. The pellets were resuspended with equal volumes of either PBS, or 1.5% (w/v) sodium alginate-in-saline. One hundred μl of 1 mg lipid/ml vesicles were injected into a size-exclusion column, Ultrahydrogel 2000 (Waters), and eluted with PBS, at a flow rate of 0.8 ml/min. Sample elution was monitored by optical density at 280 nm (Waters model 490).

Atomic absorption spectroscopy

Ca^{2+} concentrations were measured by atomic absorption spectroscopy (Perkin-Elmer, Model 360/360G), at 422.4 nm, using calcium chloride standard solutions for calibration.

Lysophosphatidylcholine analysis by thin-layer chromatography

Liposomes and microencapsulated liposomes, after various incubation times, were analyzed for lysolecithin formation. Phospholipid extraction was performed according to Folch et al. [14]. One hundred μl of liposomes were extracted with 2.5 ml of 2:1 (v/v) chloroform:methanol and 0.5 ml of 0.2% H_2SO_4 [14]. The mixture was vortexed for a few minutes, cooled to 4°C for 10–15 minutes, and then spun at 1000 rpm for 5 min to enhance phase separation. The organic phase with the extracted lipids was collected, and dried by blowing the solvent with nitrogen. The lipid film was redissolved with 25–30 μl 2:1 (v/v) chloroform/methanol and the samples were applied to the plates (Silica gel 60, Merck) against standards of phospholipids and lysophosphatidylcholine. The plates were eluted with chloroform/methanol/water/acetic acid (200:120:32:12, v/v) [15]. After separation was completed, the plates were dried and sprayed with molybdenum blue (molybdenum oxide in 4.2 M sulfuric acid; Sigma M-3389). The plates were put in the oven (80–100°C; 30 min) to char all nonspecific lipids. Phospholipids and lysolecithin were detected by the blue bands on the plate.

With the microencapsulated liposome systems, microspheres were first probe-sonicated in the presence of 10% (w/v) EDTA. This chelator dissolved the internal Ca-alginate gel; while the sonication disrupted the complex alginate-PLL, resulting in liposome release. Lipids were extracted from the crushed microspheres and analyzed for lysophosphatidylcholine as described above.

Results and Discussion

Liposome and microencapsulated liposome system characterization

Liposomes prepared by the reverse evaporation technique are unilamellar and oligolamellar vesicles [12,16] and entrapped 20–40% of the dye. Extruding the liposome preparation through a 0.6 μm polycarbonate membrane produced vesicles with unimodal size distribution with a mean diameter of 716.1 nm as determined by quasi-elastic light scattering analysis.

Encapsulation of liposomes in the Ca-alginate gel matrices using the droplet-forming apparatus, produced vesicles with a spherical shape and diameters of about 500 μm (Fig. 1A). The encapsulation of liposomes did not interfere with the formation of a cross-linked hydrogel matrix. When placed in releasing media (PBS), Ca-alginate microspheres disintegrated with time and medium changes resulting in liposome release. To retain the liposomes, Ca-alginate microspheres were further interacted with the positively-charged polyelectrolyte,

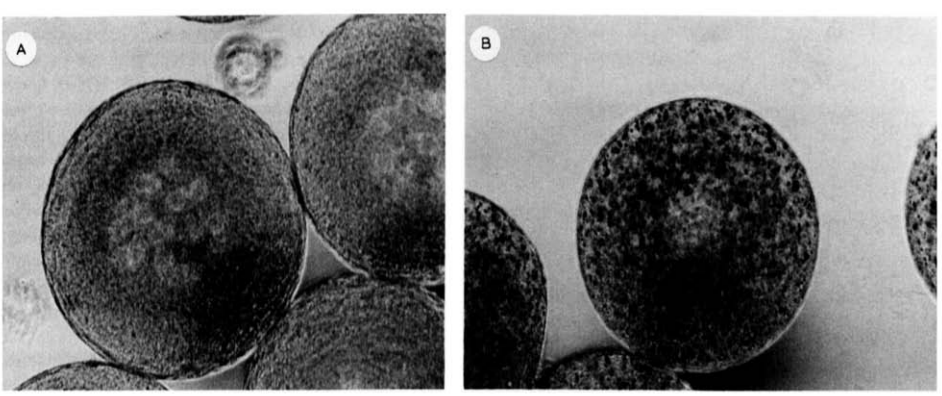

Fig. 1. Phase-contrast micrograph of PLL-(39 kDa)-coated Ca-alginate microsphere. (A) immediately after preparation (B) after 70 days of incubation in media.

poly(L-lysine) (PLL). The complex alginate-PLL produced a membrane with permeability properties that highly depend on the molecular mass of PLL [17]. It was found that coating with PLL of molecular mass of 40 kDa or less, and a reaction time of 10 min, produced membranes that retain the liposomes inside the capsules but allow the outward diffusion of proteins such as FITC-BSA, once they were released from the liposomes. Due to the stable complex alginate-PLL, the devices maintained their spherical shape throughout the release studies. A typical picture of PLL-coated devices after 70 days is presented in Fig. 1B.

Permeability characteristics of liposomes and microencapsulated liposomes

Liposomes composed of egg PC and cholesterol (1 : 1, molar ratio) released their contents in a continuous manner as demonstrated for FITC-BSA in Fig. 2 (open circles). The cumulative amount of FITC-BSA released from liposomes after 45 days was less than 20%. This result is in agreement with the permeability properties of lipid bilayers, particularly those containing cholesterol [18].

The encapsulation of liposomes, of the same lipid composition, within the PLL-coated Ca-alginate spheres resulted in a system (denoted MELs) that released its contents at two distinct 'bursts'. A typical FITC-BSA release profile from MELs is shown in Fig. 2 (dark circles). The first protein burst occurred at the beginning of the study, and 10% of FITC-BSA was released during this burst. After approx. 18 days, during which a small amount of FITC-BSA was continuously released, the system released a second, much more pronounced, protein 'burst'. More than 60% of the protein was released during the second 'burst'. The cumulative amount of FITC-BSA released throughout the experiment was more than 80% of the initially entrapped protein. Thus, the burst-type release from MELs implies a change in liposome permeability due to their entrapment within the PLL-coated Ca-alginate spheres.

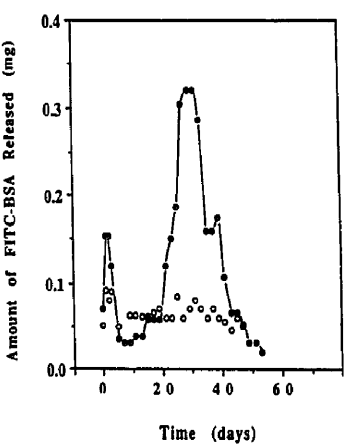

Fig. 2. Release of FITC-BSA from liposomes (○) and from microencapsulated liposomes (●), composed of egg PC/CH (1:1, molar ratio).

Liposome permeability: effects of lysophosphatidylcholine, calcium ions, poly(L-lysine) and alginate

Four factors that can change phospholipid bilayer permeability in MELs were considered: (1) Formation of lysolecithin within the phospholipid membranes upon prolonged incubation, and as soon as a critical concentration is reached it destabilizes the bilayers, producing a burst-type release [7,19,20]; (2) Destabilization effects of calcium ions that initially cross-linked alginate but with time and medium changes leached out into the medium and are free to interact with the phospholipid membranes; (3) Poly(L-lysine) (PLL) effects on liposomes. Direct contact between PLL and liposomes is possible only during the preparation of MELs, when Ca-alginate microspheres are interacted with the cationic polymer to form the complex alginate-PLL. After removing the unreacted PLL, the complexed PLL can not interact with the embedded liposomes. This complex appears to be very stable and does not dissociate with time or medium dilutions, as suggested by the maintenance of the well-kept spherical shape of MELs after prolonged incubation (Fig. 1B) (see also Refs. 17 and 21). (4) Interactions of soluble alginate with liposomes. Direct contacts between soluble alginate and liposomes are possible when liposomes are initially suspended in sodium alginate, and again when resolubilization of the internal alginate matrix occurs as soon as a critical amount of calcium ions leaches out from the system. When cross-linked by calcium ions, alginate is fixed and can not interact freely with liposomes.

The search for lysolecithin in liposomes, composed of egg PC and cholesterol (1 : 1, molar ratio), at different time points, revealed that no lysophosphatidylcholine is formed upon the prolonged incubation of these vesicles. With MELs, the results were inconclusive. For example, there were times where a band, that correlates with lysolecithin, was seen on the plate. A few days later, the same MELs did not show such a band. This inconsistency is attributed to the complex nature of the MELs and the harsh methods needed for the disruption of this system in order to extract the lipids. Because of its amphophilic nature, lysophosphatidylcholine absorbs to the liposomal membrane and is not released into the medium. To release it from MELs, the Ca-alginate gels and the complex alginate-poly(L-lysine) had to be disrupted by methods such as probe-sonication that can cause lysolecithin formation. However, if one considers that lysophosphatidylcholine was not formed in liposomes of the same lipid composition, and under the

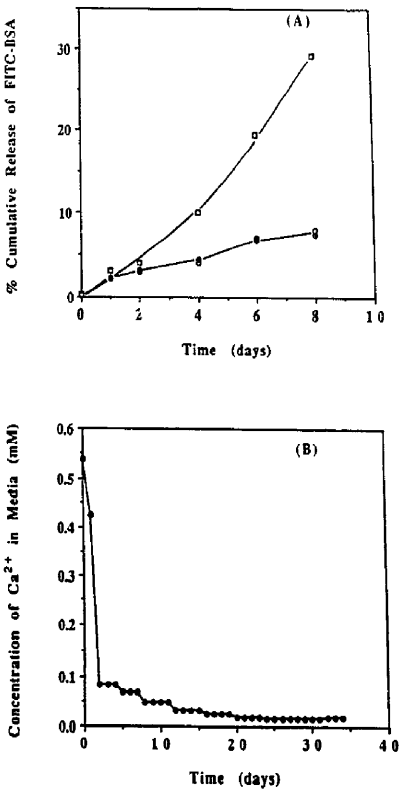

Fig. 3. (A) Effect of calcium ion concentrations on lipid bilayer permeability. Liposomes were composed of egg PC/CH (1:1, molar ratio); medium containing (○) 0 Ca^{2+} (●) 0.75 mM Ca^{2+}; (□) 1 mM Ca^{2+}. (B) Concentrations of calcium ion in releasing media of MELs. Media were replaced everyday and analysed for Ca^{2+} by atomic absorption spectroscopy.

same incubation conditions, and that these liposomes were stable upon prolonged incubation (Fig. 2, open circles) then the possibility that lysophosphatidylcholine is formed in MELs is unlikely. Thus it was concluded that lysophosphatidylcholine is not the factor responsible for the burst release from MELs.

Ca^{2+} destabilized liposomes composed of egg PC and cholesterol (1:1, molar ratio) only at concentrations of 1 mM and greater, resulting in greater release rates of FITC-BSA from the vesicles (Fig. 3A). It required almost 4 days of constant exposure to Ca^{2+} in order to change the permeability of the vesicles. This was also observed when liposomes were reacted with calcium ion concentrations of more than 50 mM (data not shown). These results eliminate the possibility that the short exposure (30 min and less) to calcium ions during the cross-linking reaction is the cause for the burst-type release that was seen immediately at the beginning of the experiment. The possibility that free calcium ions, that leached from MELs during the prolonged incubation, is the cause for liposome destabilization and for the second burst was examined by measuring their concentration in media at various times. Assuming steady state, these concentrations should reflect the concentrations of free calcium ions inside the microcapsules. By atomic absorption spectroscopy, the concentration of Ca^{2+} in releasing media from MELs, at all times, never exceeded 0.6 mM and decreased over time (Fig. 3B). Therefore, it is unlikely that Ca^{2+} is responsible for the changes in lipid bilayer permeabilities in MELs.

The effect of poly(L-lysine) on lipid bilayer permeability was studied by directly interacting a dilute solution of the polymer, for 10 min, with preformed egg PC and cholesterol (1:1, molar ratio) liposomes, containing FITC-BSA. The final ratio between the lysine groups and lipid molecules was about 1:22. The results are presented in Fig. 4, showing no differences in the release rates of FITC-BSA from PLL-treated liposomes and untreated liposomes. This result suggests that PLL is not the destabilizing factor in the microencapsulated liposome systems. This is in agreement with previous studies which showed that poly(L-lysine), up to a ratio of one lysine group per lipid molecule, does not change the permeability of dipalmitoyl PG and dipalmitoyl PC bilayers [10,11].

To study the effect of soluble alginate on bilayer permeabilities, liposomes, composed of egg PC and cholesterol (1:1, molar ratio), were incubated with alginate (1.5% w/v, in saline), for 24 h at 37°C, before the polymer was cross-linked with calcium ions. As seen in Fig. 5 (dark circle), the prolonged incubation of liposomes with soluble alginate, at 37°C, resulted in MELs that released more than 60% of the encapsulated FITC-BSA during the first few days after encapsula-

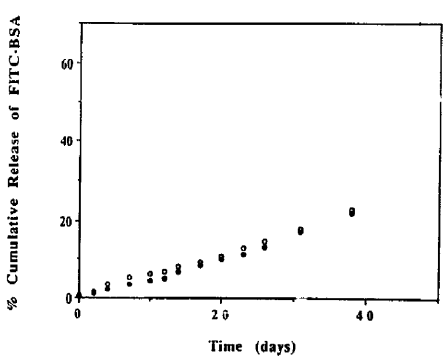

Fig. 4. Poly(L-lysine) effects on lipid bilayer permeability. FITC-BSA release rates from egg PC/CH (1:1, molar ratio) liposomes (○) coated with 0.06% (w/v) poly(L-lysine) 39.5 kDa); (●) without coating.

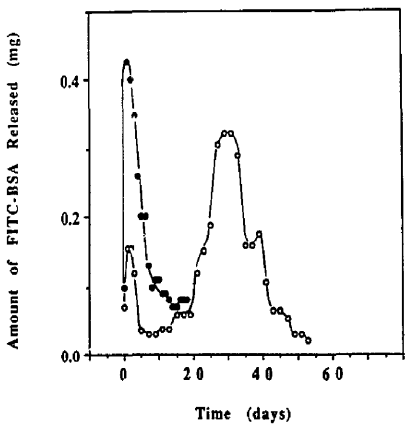

Fig. 5. Alginate effects on lipid bilayer permeability. Liposomes were suspended in alginate and were sprayed immediately into calcium chloride solution (1.3% (w/v)) (○); liposomes were incubated for 24 h at 37°C with alginate 1.5% (w/v) before they were sprayed into calcium chloride solution (●).

tion. However, liposomes that were exposed to soluble alginate for short times, during the preparation of MELs (less than 5 min, at room temperature) produced MELs with a small initial burst of protein (less than 10%). This result suggests that alginate interacts with liposomes in a time-dependent manner, causing a change in their permeability properties.

Based on the above studies, two sets of experiments were done to further substantiate the interactions of alginate with liposomes and to characterize their nature. These were done by differential scanning calorimetry and size-exclusion gel chromatography.

Interactions of alginate with phospholipid bilayers: differential scanning calorimetry

Effects of alginate on phospholipid bilayers composed of DPPC were investigated by differential scanning calorimetry (DSC). Hydration of DPPC with phosphate-buffered saline (PBS, pH 7.4), produced vesicles that gave a sharp endothermic peak at $T_m = 44.58°C$ with a transition enthalpy (ΔH_m) of 22.93 J/g (Fig. 6, curve a). T_m is defined as the temperature at which the maximum peak occurs. This parameter is in good agreement with the values 44°C and 43°C previously reported by Ikeda and co-workers [22] and Boggs and co-workers [23], respectively, but is slightly higher than that reported by Seki and Tirrell, 40.5°C [24], or Papahadjopoulos and co-workers, 41.5°C [10]. Differences in T_m values may arise from variations in the scan rates employed, faster scan rates giving higher T_m values [22]. However, the scan rate used in our studies (4 C°/min) was slightly slower than that employed by Papahadjopoulos and co-workers (5 C°/min) [10], and still they found a value of 41.5°C. The same researchers reported that sonication of phospholipid dispersions produced a shift of the mid-point from 41 to 38°C. Thus, it is possible that the higher T_m is due to variations in the phospholipid dispersions. Exposure of DPPC bilayers to alginate (1.5% w/v, sodium salt) resulted in preparations that melted at essentially the same temperature, but with a 4-fold reduction in the transition enthalpy ($\Delta H_m = 6.51$ J/g) (Fig. 6, curve b). This reduction suggests interactions between liposomes and alginate.

Studies have been made on the interaction between phospholipid bilayers and proteins which are useful to consider when examining alginate-liposome interactions. Seki and Tirrell [24] and Papahadjopoulos and co-workers [10,23] classified such interactions into three major classes: (1) surface adsorption only; (2) insertion into the lipid bilayer (by analogy to insertion of membrane proteins); (3) complete disruption of the bilayer structure. Processes of the first class involve a shift in phase transition temperature to lower temperatures, while processes of the second and third class resulted in reduction in the transition enthalpy. Since it is expected that a complete disruption of bilayer structure would reduce ΔH_m to zero, we suggest that DPPC–alginate interacitons are essentially of the second class, i.e. insertion of the polymer into the lipid membrane. A similar interpretation was made by Ohno and co-workers [25] who studied the interaction of a negatively-charged synthetic polymer, poly(L-glutamic acid), with dipalmitoyl PC liposomes by ^{1}H-NMR. They found that this polymer decreased the signal intensity of the choline methyl group, and also those of the hydrophobic methylene and terminal methyl groups, suggesting that this polymer might be incorporated into the hydrophobic region of the vesicle membrane.

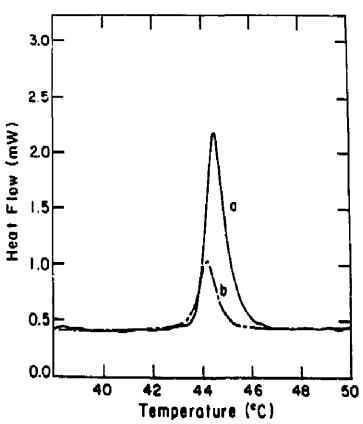

Fig. 6. Differential scanning calorimetry thermograms of DPPC liposomes without alginate (a) and with 1.5% (w/v) alginate (b).

Interactions of alginate with phospholipid bilayers: size-exclusion gel chromatography

Insertion of polymers, such as alginate, into liposomes should affect their overall size. To confirm this, size-exlcusion gel chromatography of bath-sonicated DPPC liposomes, prior to and after interacting them with alginate (1.5% w/v) was performed (Fig. 7). Bath-sonicated DPPC liposomes gave a single peak with an elution volume of 8.3 ml, eluting at a distinct position away from that of alginate. Interacting liposomes with alginate resulted in the appearance of an additional peak, with a smaller elution volume (6.8 ml). The new peak may represent the fraction of liposomes that inserted alginate into their bilayer, thus increasing their overall size (Stokes radius). The area of the new peak correlated with a reduction in the area of the original liposomal peak, an indication that lipid–alginate interactions do not cause complete destruction of liposome structure. Complete destruction of liposomes should result in disappearance of the original liposome peak. The possibility that the changes in media viscosity, due to alginate, are responsible for the changes in the chromatogram is unlikely since no effect on the location of original liposomal peak is observed.

Interactions of alginate with liposomes. Effect of phospholipid bilayer composition

Interactions of alginate with liposomes can be influenced by the phospholipid bilayer composition. For ease of analysis, release of dyes from MELs were used to study these interactions.

When introducing negative charges to the bilayer surface by incorporating phosphatidylglycerol into liposomes (PC/CH/PG (4:5:1, molar ratio)), no 'bursts' of carboxyfluorescein from MELs were observed (Fig. 8, dark circles). Inclusion of a positively-charged lipid, stearylamine, into liposomes (PC/CH/SA (4:5:1, molar ratio)) produced MELs that released carboxyfluorescein in a burst-type manner (Fig. 8, open circle). Thus, it appears that lipid-alginate interactions are sensitive to lipid head group charge. At physiologic pH, alginate is negatively charged, thus interactions between the polysaccharide and the negatively-charged liposomes are inhibited.

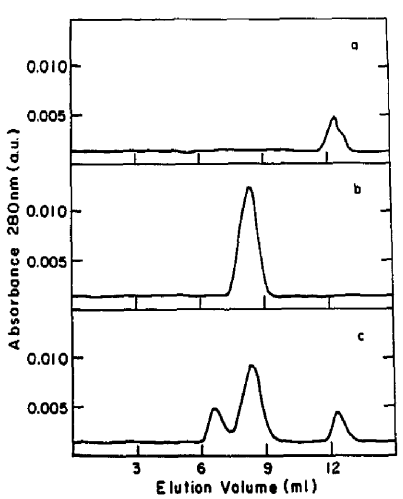

Fig. 7. Size-exclusion gel chromatography of 1.5% (w/v) sodium alginate (a); bath-sonicated DPPC liposomes-in-PBS (b) and liposomes with 1.5% (w/v) alginate (c).

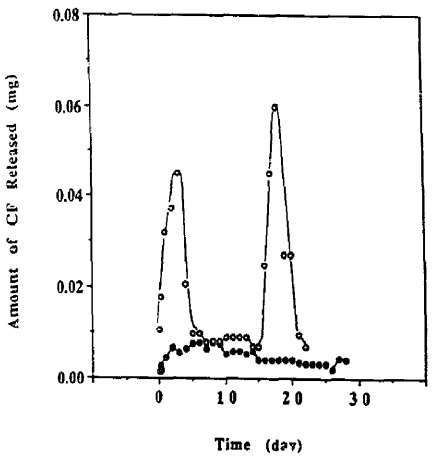

Fig. 8. Effects of phospholipid polar head group charge on alginate–lipid interactions. CF release from MELs composed of (●) negatively-charged liposomes (PC/CH/PG, 4:5:1 molar ratio); (○) positively-charged liposomes (PC/CH/SA, 4:5:1 molar ratio).

When liposomes were composed of egg hydrogenated PC and cholesterol, the resultant MELs released FITC-BSA in 'bursts', but on a different time scale (Fig. 9). As with unsaturated MELs, an initial 'burst' was observed, however, with saturated MELs, the second 'burst' of protein release occurred much later (approximately after 95 days). The delay in the second 'burst' from MELs suggests different kinetics of alginate–lipid interactions, kinetics that reflect, for example, difficulties in penetration of alginate into the liposome bilayer composed of this type of phospholipid. Due to its high phase transition temperature (approx. 45°C), egg hydrogenated PC forms bilayers in the 'gel' state in which the acyl chain motion is strongly restricted, thus making it difficult for the polymer to penetrate into the lipid bilayer.

Taking the above results together a mechanism to explain the initial and the delayed 'burst' of release from MELs is suggested. The initial protein 'burst' may be due to changes in bilayer permeability that occur when liposome pellets are suspended in sodium alginate. In the soluble form alginate is free to move and to

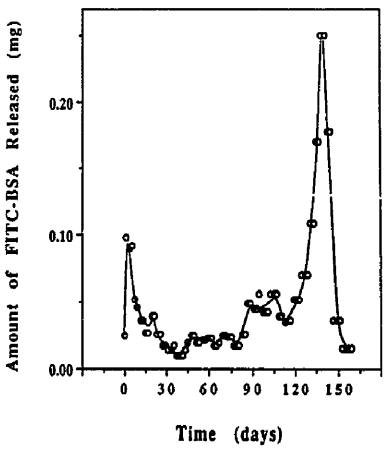

Fig. 9. Effect of phospholipid phase transition temperature on alginate–lipid interactions. FITC-BSA release from MELs composed of egg hydrogenated PC and CH (1 : 1, molar ratio).

be inserted into the lipid bilayer. Cross-linking of alginate molecules with calcium ions inhibits further interactions between polysaccharide and liposomes. With the frequent replacements of medium, Ca^{2+} leaches out of the capsules. When the amount of calcium ions in the matrix decreases to a level in which it can not hold the hydrogel network a resolubilization of the internal alginate matrix occurs. The soluble alginate can interact again with liposomes, leading to the second protein 'burst'.

Several reports showed that certain types of liposomes can undergo changes in membrane permeability that are triggered by environmental changes such as pH [23,26–28], or intentionally triggered by known lipid bilayer destructive methods such as Triton X-100 or sonication [29]. In contrast, the lipid–polymer interactions in the current study are such that the contents of the liposomes are released at predetermined times, regulated by the lipid composition and possibly other factors (e.g., alginate concentrations and the rate of Ca^{2+} leakage).

Lipid–alginate interactions suggest a number of future studies. Considering that a liposome is basically a cell model, this system may provide physicochemical information on interactions of polysaccharides with membranes. For example, polysaccharides are important cell surface components of bacteria and thus studying these interactions might contribute to a better understanding of bacteria-cell interactions. In addition, the ability to achieve 'burst' type release at preset times may extend the use of liposomes, particularly given the reported biocompatibility of poly(L-lysine)-alginate coatings [20,30,31], to situations where pulsatile rather than continuous delivery of substances is desired (e.g., vaccine delivery).

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