

Application of Alginate Gel as a Vehicle for Liposomes. I. Factors Affecting the Loading of Drug-Containing Liposomes and Drug Release

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To explore the feasibility of alginate gel as a vehicle for liposomes, we investigated the effects of various factors associated with the loading of drug-containing liposomes into the gel beads. The loading process includes (1) mixing of liposomes and alginate solution, (2) calcium induced gelation of alginates, (3) the time-dependent contraction of a gel body squeezing out interior water, and (4) possible leakage or release of a drug entrapped in liposomes in a series of each of these processes. These effects were examined in terms of the leakage of a marker 5(6)-carboxyfluorescein (CF) from liposomes of egg phosphatidylcholine (EPC) and EPC/cholesterol (EPC/Cho) and liposome (phosphorus) release from curing and fully-cured gel beads whose initial polymer concentrations were 4 and 2%. Major findings were: (1) Alginate induced the leakage of a water-soluble drug incorporated in the liposomes as a function of the polymer concentration and the mixing time. (2) Calcium ions also stimulated the leakage of the drug. EPC/Cho liposomes were several times more resistant to the leakage of CF than were EPC liposomes. (3) The liposomes were well loaded without any loss in the gel bead despite the squeezing outflow of water and the bead contraction during gel curing. (4) Such curing caused leakage of the drug from the EPC liposomes in the very early stage while no effect was observed in the EPC/Cho liposomes. (5) In the gel-eroding medium (pH 7.4 Tris-HCl, 37°C), the total drug release was controlled by the erosion rate of the bead body. Immediately after the bead erosion, EPC liposomes retained about 60% of the drug in the 2% bead and only about 20% in the 4% bead, whereas EPC/Cho liposomes retained more than 85% regardless of the initial alginate concentration. The results provide valuable information for the design and applicability of the gel-loaded liposome delivery system.

Key words alginate; gel matrix; liposome; 5(6)-carboxyfluorescein; gel erosion; sustained release

Liposomes have been extensively explored for use as drug carriers aimed at controlled release and localization of various drugs.^{1,2)} The route of administration is not always of concern or is implicitly assumed to be systemic administration. However, systemic application entails some disadvantages such as rapid clearance by reticulo-endothelial systems and poor targeting of tissues and sites. To avoid these drawbacks, strategies of more specific targeting to the desired spots have been explored based on a variety of physicochemical and biochemical concepts: lipid composition and hydrodynamic diameter, surface charge modifications of the vesicle, surface manipulation of the vesicles with the attachment of ligands like monoclonal, polyethylene glycol and oligosaccharides, and combinations of these.^{3–5)}

Alternatively, a direct local delivery of liposomal drugs to target sites or in the close proximity could be one possible means to maintain appropriate local drug levels and thus obtain a desirable therapeutic outcome.¹⁾ Such a substantial short circuit strategy would make it possible to use lower dose and would reduce adverse effects with the help of possible sustained release and localizing drugs over a prolonged period. However, local retention of liposome-entrapped drugs, which is likely to be longer than that of their free form, is not always long enough to maintain the therapeutic drug levels in a site, in part due to rapid clearance by macrophages and high density lipoproteins against lipid vesicles.^{3,6)} One possible solution to the problem may be to load drug-containing liposomes within a vehicle consisting of moderately disintegrated and inert materials and to let them stay in the local area for sustained periods.

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Alginic acid is a polysaccharide found originally in brown seaweed. The polysaccharide consists of (1–4)-linked residues of β -D-mannuronic acid (M) and α -L-guluronic acid (G) arranged in a block fashion.^{7,8)} It is well known that alginic acid can form translucent or transparent gels in association with calcium ions for which GG blocks are mainly responsible.⁹⁾ Calcium-induced alginate gel matrices have been proposed as oral delivery vehicles^{10–12)} and used as a long-term holding bed for fragile cells in bio-reactor technology^{13–16)} because of their low toxicity and almost instantaneous gel formation.¹⁰⁾ Also, a gel matrix loaded with drug-containing liposomes could be useful as a local delivery vehicle for retention, if it were formed by mixing alginate solution and the liposomes at an injection site, for example, in a solid tumor or its proximity.

The purpose of this study was to learn the effects of a series of processes associated with the loading of drug-containing liposomes into gel beads: mixing of liposomes and alginates in a solution, calcium-induced gelation and contraction of the gel body. The effects of gel erosion on the release of a drug incorporated in the liposomes was also examined in terms of liberation of the liposomes loaded in gel beads. 5(6)-Carboxyfluorescein (CF) was used as a water-soluble model drug.

Experimental

Materials Sodium alginate was purchased from Tokyo Kasei Kogyo, Tokyo and used after dialysis against distilled water using Visking cellulose tubing (36/32) for 3 d (three water displacements/d) followed by lyophilization. Viscosity average molecular weight, M_w , of alginates was determined by the following equation,¹⁷⁾

$$[\eta] = 2.0 \times 10^{-5} M_w$$

where $[\eta]$ means the intrinsic viscosity in 0.1 M NaCl at 20°C in 100 ml/g units. The M_w was 2.03×10^5 and M/G ratio was 1.26. MM, MG, and GG-block proportions were 37.9, 37.3, and 24.8 %, respectively.

Purified egg phosphatidylcholine (EPC, COATSOME NC-10, Nichiyu Liposome Co., Tokyo) and cholesterol (Cho, Wako Pure Chem., Osaka) were used. Molecular weight of EPC was assumed to be 780. CF (approx. 99% pure by HPLC) was purchased from Sigma (St. Louis, MO). All other chemicals were of reagent grade.

Preparation of Liposomes Liposomes were prepared using EPC according to the method of Bangham *et al.*¹⁸⁾ Briefly, EPC alone or EPC/Cho (2:1 by molar ratio) in stock solution (hexane/ethanol, 98/2) was placed in a round bottom flask and the solvent was removed to dryness. The lipid film was then hydrated with Tris-HCl buffer (10 mM, pH 7.4) containing 0 or 75 mM CF with intermittent vortexing at room temperature. The suspension was then subjected to ultrasonic irradiation under N_2 gas and centrifuged to remove titanium fragments. The resulting sonicated liposomes were allowed to equilibrate at room temperature for several hours and harvested through a 0.1 μ m polycarbonate filter (Nucleopore Co., Pleasanton, CA). Mean diameter of the liposomes was determined by dynamic light scattering equipment (DLS-700, Otsuka Electronics, Osaka) and was 28 ± 5 and 35 ± 7 nm (mean \pm S.D., $n=3$) for EPC liposomes and EPC/Cho liposomes, respectively. Free CF was removed by passing the suspension through a column (30 cm \times 2.2 cm) of Sephadex G-25. Tris-HCl buffer (10 mM, pH 7.4) containing 0.15 M NaCl was used as eluent to avoid CF leakage due to osmotic gradient between the liposome interior and the external media.¹⁹⁾

Preparation of Calcium-Induced Gel Beads Calcium-induced beads were prepared by the slightly modified method described previously.²⁰⁾ Briefly, sodium alginate solution (2 or 4%, w/w) or its liposome-containing solution was allowed to fall by drops into 0.12 M $CaCl_2$ (10 mM Tris-HCl, pH 7.4) at 25°C using a peristaltic pump (MP-3, Tokyo Rikakikai Co., Tokyo) with a polyethylene-tubing nozzle (0.50 mm i.d. and 0.8 mm o.d.). The pumping rate was 6 beads/min and the falling distance was 3.5 cm. Alginate solution was mixed with liposome suspensions at various EPC concentrations. The beads were allowed to cure in the $CaCl_2$ solution for various times. Instant curing was induced on the surface of the droplet and the physical strength to hold the bead body was achieved within minutes. The resulting beads cured for 3 d were referred to as fully-cured beads.¹⁰⁾

Size of Gel Beads The size of alginate beads was determined by taking a photograph of each bead ($\times 20$, 5 beads) and measuring its diameter at three different positions.

Water Contents in Gel Beads Beads were heated to dryness in an oven at 110°C for 2 h. The weight difference before and after drying was assumed to be the amount of water held in the bead, and represented as the volume of water assuming $d=1.0$. The average value of 15 beads was taken.

Determination of Alginic Acid Uronic acids were determined by the phenol-sulfuric acid method.²¹⁾ An aliquot (0.5 ml) of the bulk solution was well mixed with 0.5 ml of phenol (5%) to which 2.5 ml of conc. sulfuric acid was quickly added. After 30 min standing at 25°C, the absorbance at 480 nm was measured. A calibration curve of purified alginate (up to 100 mg/ml) was constructed.

Determination of Liposomes (Phosphorus) Loaded in Gel Beads Liposomes were assayed for their phosphorus content. Liposome-loaded beads were dissolved in 5 mM EDTA-50 mM Tris-HCl buffer (pH 7.4). After complete erosion of the gel body by incubation for several hours, the solution was digested by perchloric acid (70%) and hydrogen peroxide (30%) at 130°C, and the phosphorus content was measured by the Msrny method.²²⁾

Leakage of CF from Liposomes in Various Media CF leakage from liposomes was measured with a spectrofluorometer (FP-777, Jasco, Tokyo) (excitation at 495 nm and emission at 517 nm). Since the fluorescence of CF is quenched during its encapsulation in liposomes, the leakage of CF into the surrounding medium can be monitored as an increase in fluorescence due to its dilution in the external media.²³⁾ The fluorescence intensity of a diluted liposome dispersion was thus measured at 25°C before and after the addition of 10% Triton X-100, where 100% CF release was assumed. The effect of alginate and calcium ions on the leakage of CF from liposomes was examined in Tris-HCl buffer (pH 7.4) at 25 and 37°C. Liposome concentration (phosphorus) was 0.2 mM.

Leakage of CF from Curing Beads and from Fully-Cured Beads Ten droplets of alginate solution or fully-cured beads (cured for 3 d at

25°C) containing CF and "empty" liposomes were dropped or placed in 0.12 M $CaCl_2$ solution (pH 7.4, 10 mM Tris-HCl) in a thermostatted double-jacketed beaker at 25°C and the solution was gently stirred by a magnetic stirrer. The loaded liposome varied in content (A, 0; B, 1.6; C, 8.8×10^{-7} mol/droplet). The beads, which were still in the curing process immediately after the droplets were introduced in the calcium solution, were referred to as curing beads in contrast with fully-cured beads. An aliquot of the bulk solution (0.5 ml) was withdrawn for a fluorescence assay at appropriate time intervals. The diffusion coefficient of CF in the beads was estimated from the release rate of the drug from the fully-cured beads into a well-stirred solution (Tris-HCl buffered 0.12 M $CaCl_2$, pH 7.4), based on the mathematical treatments of Crank applicable to diffusion in a sphere.^{24,25)}

Leakage of CF from Liposomes-Loaded Curing Beads Ten droplets of alginate solution containing CF-entrapped liposomes were dropped in 0.12 M $CaCl_2$ solution (pH 7.4, 10 mM Tris-HCl) in a thermostatted double-jacketed beaker and the solution was gently stirred by a magnetic stirrer at 25°C. An aliquot of the bulk solution (0.5 ml) was withdrawn for a fluorescence assay at appropriate time intervals. The same amount of the solution sampled was added to the bulk solution, and the amount of CF was measured as described above.

Release Behavior of CF and Phosphorus (Liposomes) from Eroding Beads Ten beads were placed in 50 ml of Tris-HCl buffer solution (50 mM, pH 7.4) in a double-jacketed beaker. The solution was gently stirred by a magnetic stirrer and the temperature was maintained at 37°C by a thermostat. The buffer solution contained 0.15 M NaCl. The amount of CF released was measured after the addition of Triton X-100 and a phosphorus assay was also performed. In addition, after complete release of CF and bead erosion, liposomal and free CF was separated by passing an aliquot of the solution through a column (14 cm \times 1 cm) of Sephadex G-75 and measuring it by phosphorus assay and a spectrofluorometer, respectively.

Erosion Behavior of Alginate Gel Beads The erosion of a gel body was measured by an uronic acid assay as described above.

Results and Discussion

Characterization of Liposomes-Loaded Gel Beads

Table 1 shows the physical parameters of the droplets of alginate solutions containing EPC or EPC/Cho liposomes in which CF is loaded. These droplets were prepared so as to contain almost the equivalent amounts of liposomes and CF. The initial alginate concentrations little affected droplet weight. Droplets were allowed to cure in a Tris-HCl buffered calcium solution (pH 7.4) for 15 min at 25°C, and the physical dimensions of the cured beads is shown in Table 2.

The gelation of alginate occurred instantly on the surface of the droplets which formed an almost spherical bead, followed by the contraction of the gel body. This process is usually accompanied with massive squeezing out of the interior water.¹⁰⁾ However, the lipid content in the resulting beads remained unchanged, indicating that the liposomes were well reserved within them. While the mean diameter of the loaded liposomes was about 25–40 nm, the gel network formed seemed to be delicate enough to hold them in the beads. The leakage of CF from the beads during curing was about 10–15% and was 3% for EPC and EPC/Cho liposomes, respectively. However, the initial alginate concentrations little affected the leakage of the drug.

In the process of the incorporation of drug-containing liposomes into the alginate gel beads described above, there are various factors which must be considered to achieve the loading of both the vesicles and the drug as efficiently as possible: (1) effect of mixing of liposomes with alginate solution on drug leakage, (2) effect of bead contact with calcium ions used to induce gelation, and (3)

effect of bead contraction as interior water is squeezed out during curing. We will therefore describe each effect pertinent to the rational development of the liposome-containing bead.

Effect of Alginate on Drug Leakage from Liposomes

The first step is to disperse drug-containing liposomes in an alginate solution. To study the effect of alginate on the leakage of CF entrapped in EPC or EPC/Cho liposomes, the vesicles were incubated with alginate in Tris-HCl buffer (10 mM, pH 7.4) containing 0.15 M NaCl. As seen in Fig. 1, the more prolonged the incubation and the higher the alginate concentration, the greater was leakage of the drug. Also, the alginate-induced leakage was enhanced by increasing temperature while little temperature effect was observed without the polymer. These results indicate that alginate interacts with liposomes, causing a change of the permeability properties of the membrane. Cohen *et al.* suggested insertion of alginate molecules into DPPC (dipalmitoylphosphatidylcholine) liposomes from thermochemical studies by differential scanning calorimetry, and the lipid-alginate interaction did not generate the destruction of the liposome structure.²⁶⁾

To confirm the structural integrity of EPC liposomes, the liposomal CF content was examined by gel chromatography before and after 8 h incubation of the liposomes in a 2% alginate solution at 25°C. The liposomal peak was found at the same void volume, giving about 96% recovery of the liposomes (data not shown), although about 40% of the drug leaked in 8 h, as seen in Fig. 1a. This result indicates that alginate enhances the leakage of EPC liposomes but that the liposomal membrane integrity is maintained.

Figure 1 also shows the leakage of the drug from EPC/Cho liposomes under the same conditions as EPC liposomes. These liposomes were more resistant to the

leakage than EPC liposomes. For example, the time required for 50% leakage of the drug ($t_{1/2}$) for EPC liposomes with 4% alginate at 37°C was about 2 h, while at 25°C it was about 7 h. Cho incorporation into the membrane dramatically reduced the leakage, probably by rendering the bilayer structure more rigid²⁷⁾; the $t_{1/2}$ was about 7 h at 37°C.

A similar gel chromatographic study also showed no destruction of the liposomes. Therefore, the mixing step should be completed as quickly as possible at relatively low temperatures, although this may depend on the scale of mixing and alginate concentrations. When a droplet of the alginate solution containing liposomes contacts calcium ions, gelation occurs instantly on the surface of the droplet, subsequently proceeding into the center of the gel body along with the calcium migration. The cross-linked polymer therefore shows little interaction with the liposomes, probably due to its limited motion. This would be favorable for drug retention in the vesicles. Hence, the short exposure of about 15 min at room temperature in this study is not likely to have affected leakage of the drug in Tables 1 and 2.

Effect of Calcium Ions on Leakage from Liposomes

Calcium ions are used as a gelation inducer in the preparation of alginate gel beads; liposomes are therefore exposed to calcium and sodium ions. Calcium ions are known to fuse membranes, particularly those containing acidic phospholipids,^{28,29)} and they also affect, to some degree, the permeability of neutral phosphatidylcholine liposomes.³⁰⁾ Figure 2 shows leakage of the drug from EPC and EPC/Cho liposomes in the calcium and sodium solutions to which no alginate was added. EPC/Cho liposomes remarkably showed higher resistance against the calcium-induced leakage at each temperature. Little sodium effect was observed.

Although calcium ions are consumed for curing of the polymer, an abundance of them would be supplied from the bulk medium containing CaCl_2 and the loaded liposomes would remain exposed to the ions. To minimize the effect of calcium ions, the exposure time may be controlled depending on the desired degree of curing of the gel body. This includes keeping exposure time as short as possible but physically strong enough to hold the gel body, or displacement of the resulting gel beads in a diluted calcium solution.

Release of Drug from Curing Beads and Fully-Cured Beads Immediately after the alginate solution contacts calcium ions, the gelation begins with squeezing of the interior water out of the gel body, resulting in its

Table 1. Physical Dimensions of Droplets of Alginate Solution Containing Liposomes

Alginate concn. ^{a)} (%) (w/w)	Lipid composition (molar ratio)	$P_d^{b,c)}$ (nmol)	$F_d^{c,d)}$ ($\times 10^{10}$ mol)	Weight ^{c,e)} (mg)	Water content (%)
4	EPC	32.7	7.89	14.1	95.8
2	EPC	30.8	7.22	13.8	97.8
4	EPC/Cho (2:1)	28.5	7.51	13.8	95.8
2	EPC/Cho (2:1)	28.7	7.66	14.0	97.8

a) Initial concentration of alginate. b) Amount of phosphorus (liposomes) in a single droplet. c) Average value of 15 droplets. d) Amount of CF in a single droplet. e) Weight of a single droplet.

Table 2. Physical Dimensions of Liposome-Loaded Alginate Gel Beads

Alginate concn. ^{a)} (%) (w/w)	Lipid composition (molar ratio)	$P_i^{b,c)}$ (nmol)	$F_i^{c,d)}$ ($\times 10^{10}$ mol)	Radius ^{e)} (mm)	Volume (mm ³)	Weight ^{c,f)} (mg)	Density (g/cm ³)	Water content (%)
4	EPC	32.1	6.68	1.37	10.7	11.3	1.05	94.4
2	EPC	31.7	6.58	1.27	8.63	9.21	1.07	96.5
4	EPC/Cho (2:1)	27.8	7.27	1.36	10.4	11.1	1.06	94.5
2	EPC/Cho (2:1)	27.9	7.42	1.27	8.66	9.04	1.04	96.5

Beads were cured in 0.12 M CaCl_2 solution (pH 7.4) at 25°C for 15 min. a) Initial concentration of alginate. b) Amount of phosphorus (liposomes) in a single bead. c) Average value of 15 beads. d) Amount of CF in a single bead. e) Average value of 5 beads. f) Weight of a single bead.

contraction. This squeezing pressure may force some of the drug to leach out, especially the untrapped drug.

In the previous sections, the term "leakage" was used because the studies focused on the effects of alginate and calcium ions on the leakage from liposomes alone, which occurs prior to the incorporation of liposomes into gel bodies. Where drug migration behavior is concerned, the term "release" will hereafter be more appropriate than "leakage," because the local incorporation of drug-containing liposomes by gelation can also be applied at a local area or an injection site.

As seen in Tables 1 and 2, the liposomes were kept well within the gel body despite the squeezing outflow of water during curing. Accordingly, the effect of empty liposomes loaded in the bead on the release of CF was examined. For this purpose, the physical dimensions of the droplets containing different amounts of liposomes and their

fully-cured beads are shown in Tables 3 and 4.

Figure 3 shows the release behavior of free CF from curing beads and fully-cured beads containing different amounts of empty liposomes. These results indicate that

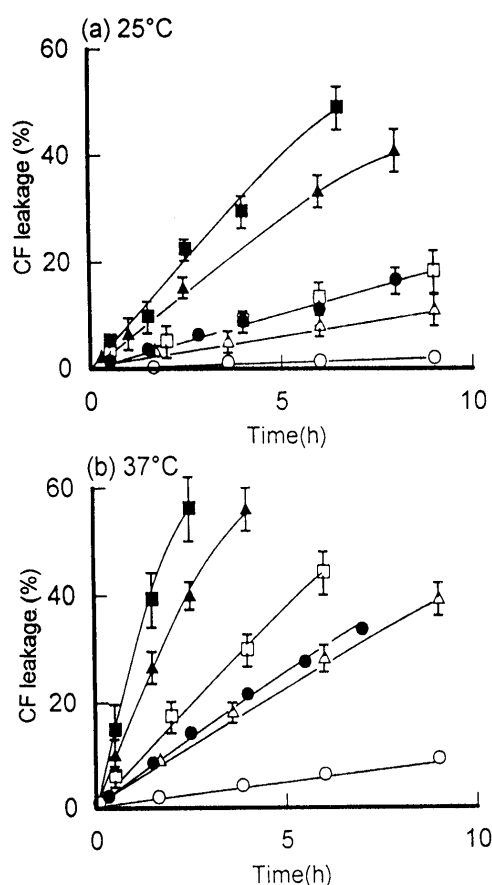


Fig. 1. Effect of Alginate on Leakage of CF from Liposomes in 10 mM Tris-HCl Buffer (pH 7.4) with 0.15 M NaCl

Each plot represents the mean \pm S.D. ($n=3$). Concentration of alginate: ■, 4%; ▲, 2%; ●, 0%. Liposomes: closed symbols, EPC; open symbols, EPC/Cho.

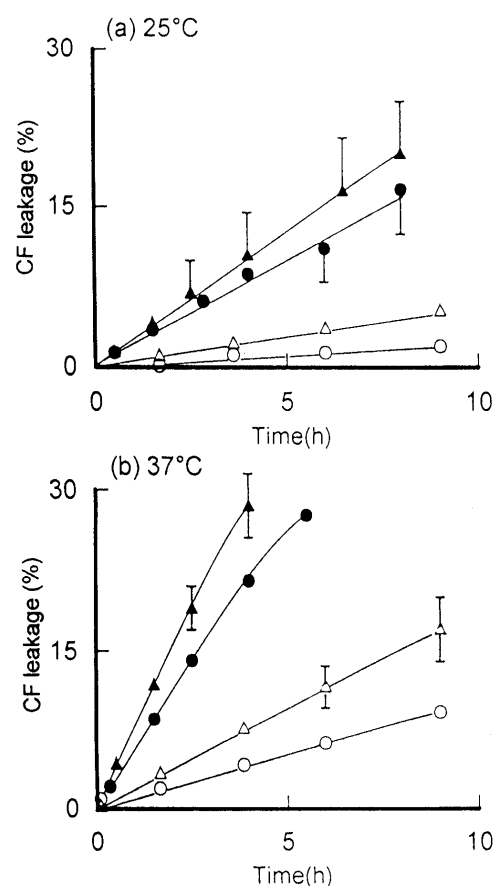


Fig. 2. Effect of Calcium on Leakage of CF from Liposomes in 10 mM Tris-HCl Buffer (pH 7.4)

Each plot represents the mean \pm S.D. ($n=3$). Concentrations of calcium and sodium: ▲, 0.12 and 0 M; ●, 0 and 0.15 M. liposomes: closed symbols, EPC; open symbols, EPC/Cho.

Table 3. Physical Parameters of Droplets of Alginate Solution Containing Free CF and Various Amounts of "Empty" EPC Liposomes

Droplet ^{a)}	$P_d^{b)}$ ($\times 10^7$ mol)	$F_d^{c,d)}$ (nmol)	Weight ^{c,e)} (mg)	Water content (%)
A	0	6.46	14.5	95.5
B	1.61	6.41	14.3	95.1
C	8.77	6.57	14.5	92.1

a) Initial alginate concentration was 4% (w/w). b) Amount of phosphorus (liposomes) in a single droplet. c) Average value of 15 droplets. d) Amount of CF in a single droplet. e) Weight of a single droplet of alginate solution.

Table 4. Physical Dimensions of Alginate Gel Beads Containing Free CF and Various Amounts of "Empty" EPC Liposomes

Bead ^{a)}	$P_i^{b,c)}$ ($\times 10^7$ mol)	$F_i^{c,d)}$ (nmol)	Radius ^{e)} (mm)	Volume (mm ³)	Weight ^{c,f)} (mg)	Water content (%)	Density (g/cm ³)
A	0	4.41	1.32	9.55	10.0	93.0	1.05
B	1.58	4.55	1.33	9.72	10.3	92.2	1.06
C	8.75	4.42	1.35	10.4	10.8	89.4	1.05

a) Beads were prepared with an initial alginate concentration of 4% (w/w) and cured in 0.12 M CaCl₂ solution (Tris-HCl, pH 7.4) containing CF for 1 d at 25°C. b) Amount of phosphorus in a single bead. c) Average value of 15 beads. d) Amount of CF in a single bead. e) Average value of 5 beads. f) Weight of a single bead.

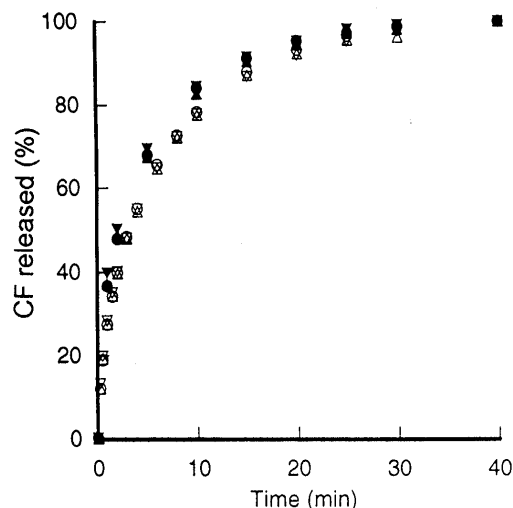


Fig. 3. Effect of Loading of "Empty" Liposomes on Release of CF from Curing Beads and Fully-Cured Beads in 0.12 M CaCl_2 Solution (pH 7.4) at 25°C

Initial concentration of alginate was 4% (w/w). Curing beads mean those being cured during the release test by dropping alginate solution in the release medium. Fully-cured beads were cured in the calcium solution for 3 d at 25°C. Each plot represents an average value of 3 runs. Keys (see Table 3 for curing beads and Table 4 for fully-cured beads): ●, A; ▲, B; ▼, C. Closed symbols, curing beads; open symbols, fully-cured beads.

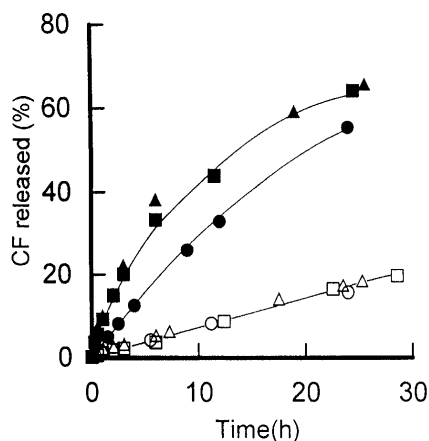


Fig. 4. Effect of Bead Gelation on Release of CF from Liposomes Loaded in Beads in 0.12 M CaCl_2 (pH 7.4) at 25°C

Each plot represents an average value of 3 runs. Initial alginate concentration: ■, 4%; ▲, 2%; ●, 0%. Liposomes: closed symbols, EPC; open symbols, EPC/Cho.

the liposomes loaded up to the amounts tested did not affect the release characteristics of small molecules which do not interact with alginate and liposomes such as CF.²³⁾ Also, the release from the curing bead was only slightly faster than that from the fully-cured bead, indicating that the squeezing outflow of water little affects the release of the drug in the continuous phase, which is considered to be mainly diffusion-controlled. Diffusion coefficients of CF from the fully-cured beads were almost the same and were $2.85 \pm 0.02 \times 10^{-6} \text{ cm}^2/\text{s}$, which was comparable to the diffusion coefficient of small molecules in water.¹⁰⁾

Release of Liposomally-Entrapped Drug from Curing Beads In the incorporation of a drug into gel beads, two different liposomal suspensions could be employed: one contains a liposomally-entrapped drug and its free form, and the other a liposomally-entrapped drug alone. When the alginate solution containing both liposomal and

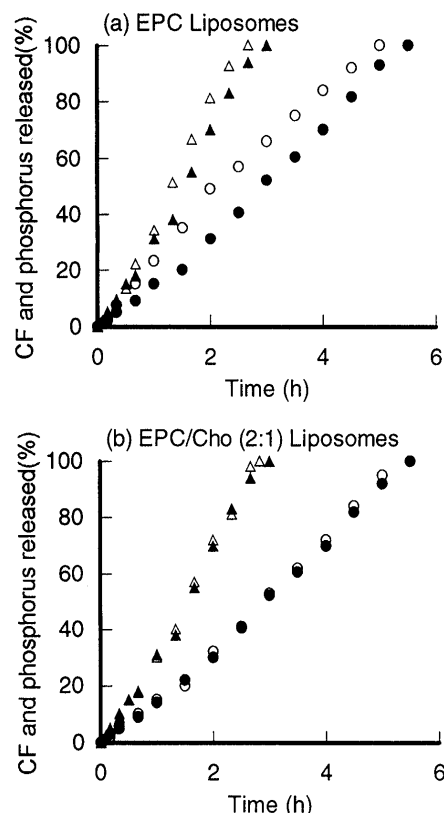


Fig. 5. Release of CF and Phosphorus (Liposomes) from Liposome-Loaded Beads in Tris-HCl Buffer (pH 7.4) at 37°C

Beads were cured in 0.12 M CaCl_2 solution (pH 7.4) for 15 min at 25°C. Each plot represents an average value of 3 runs. Open symbols, CF; closed symbols, phosphorus. Initial alginate concentration: ●, 4%; ▲, 2%.

free drug is used, the drug in the curing bead would be released in series: release of the free drug and the subsequent slow release from liposomes. After the rapid release of the free drug, as described above, a main concern is whether the release of liposomally-entrapped drug is affected by curing of the gel body.

Figure 4 shows the release characteristics of CF from liposomes dispersed in the curing bead. To minimize the effect of alginate, liposomes were mixed with the polymer solution at a low temperature, and the suspension was dropped on a calcium solution (0.12 M) at 25°C. The release of the drug from EPC liposomes was enhanced by curing of the bead compared with that from the liposomes alone (as control), and there was little difference in terms of the initial alginate concentration even on a very early stage of gel curing where the cross-linked structure should be still poor. EPC/Cho liposomes released the drug at considerably slower rate and there was no effect of the curing stage on the drug release.

Release of Liposomally-Entrapped Drug from Eroding Beads Alginate beads are gradually eroded in the neutral pH region where calcium concentration is low. The total drug release is therefore thought to be influenced by the erosion of a bead body which liberates the liposomes to the bulk phase. Also to be released is the drug from the liposomes being liberated. Each bead in Table 2 was placed in the release medium (pH 7.4, Tris-HCl) at 37°C which assumed biological pHs and temperature, and the release behaviors of the drug and liposomes were examined.

As seen in Fig. 5, the CF release generally followed a

Table 5. Ratio of Remaining CF in Liposomes after Complete Liberation of Vesicles from Beads in Tris-HCl Buffer (pH 7.4) at 37°C

Concn. ^{a)} (%) (w/w)	Lipid composition (molar ratio)	Remaining CF ^{b)} (%)
4	EPC	20.6
2	EPC	60.7
4	EPC/Cho (2:1)	85.6
2	EPC/Cho (2:1)	87.9

a) Initial alginate concentration. b) Ratio of remaining CF in liposomes after the release test (average value of 3 runs)

sigmoid pattern as well as the liposome liberation, except for the EPC liposomes loaded in the 4% bead. This result indicates that the total drug release was largely controlled by the erosion in favor of sustained release. It was expected qualitatively that EPC liposomes were more leaky than EPC/Cho liposomes during liposome liberation, as observed in earlier studies. The sigmoid pattern reflects a slight lag time needed for the bead erosion, which seems to correspond to the time required to dissolve gel-forming portions of the polymer and/or to unfold entangled polymer at the very surface of the bead body. The release was finished after about 2.5 h and about 6 h for the 2% and 4% beads, respectively, most probably due to the degree of entanglement of the polymer molecules in the bead.

Table 5 shows the percentage of remaining CF in the liposomes immediately after complete liposome liberation from beads, *i.e.*, complete bead erosion. EPC liposomes retained about 60% of the drug in the 2% bead and only about 20% in the 4% bead, whereas EPC/Cho liposomes retained more than 85% of the drug regardless of the initial alginate concentration. In connection with the drug release and liposome liberation (Fig. 5), the remaining fraction of the drug is dependent to a large extent on the erosion rate of the bead body. The 4% bead needs longer erosion time than the 2% bead, during which most drug was released from the liposomes, especially EPC liposomes. It should also be noted that the total drug released from EPC and EPC/Cho liposomes followed almost the same pattern, but most of the drug remained entrapped even after complete erosion in the EPC/Cho-4% bead while only about 20% in the EPC-4% bead.

During the bead erosion described above, free alginate and Ca^{2+} are gradually released in the local area and may affect the release of the drug from the loaded liposomes. Compared with the leakage from liposomes alone with no alginate (Fig. 1), the combined effects of the eroding gel components are appreciable. For example, the remaining fraction of the drug decreased from 90% to 20.6% in 6 h for the EPC-4% bead (see Table 5), while it decreased from 95% to 85.6% for the EPC/Cho-4% bead. These results allow estimation of the difference in membrane resistance of the EPC and EPC/Cho liposomes against the action of the gel components: EPC/Cho liposomes are about six times more resistant than EPC liposomes, while being only twice as resistant in terms of sole drug permeability.

In conclusion, we have described effects of various processes associated with the loading of drug-containing

liposomes into alginate gel beads and drug release behavior. Alginate reduced the entrapping efficiency of a water-soluble drug incorporated in the liposomes as the mixing time was prolonged. Calcium ions, a gel inducer, also stimulated the leakage of the drug from the liposomes. The periods of contact should therefore be carefully controlled. EPC and EPC/Cho liposomes (25–40 nm in diameter) were well loaded without any loss in the gel bead despite the squeezing out of water during gel curing. However, such curing stimulated the leakage of the drug from the EPC liposomes in the very early stage while no effect was observed in the EPC/Cho liposomes. In the eroding medium (pH 7.4, Tris-HCl), the total drug release was controlled by the erosion rate of the bead body which was dependent on the initial alginate concentration. However, the fractions of the free and liposomal drug released immediately after complete erosion were dependent on the lipid components of the liposomes. The results obtained in this initial study will provide useful information on applicability of a gel-loaded liposome delivery system.

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