

## Application of Alginate Gel as a Vehicle for Liposomes. II. Erosion of Alginate Gel Beads and the Release of Loaded Liposomes<sup>1)</sup>

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The possibility of producing calcium-induced alginate gel beads as a vehicle for liposomes was explored. The maximal loading of egg phosphatidylcholine liposomes (ca. 26 nm in diameter) in a fully-cured bead (1.2 mm in radius, initial alginate concn. of 4%) was  $2.9 \times 10^{-6}$  mol/bead or ca. 18%, and the size of the bead slightly increased with an increase in liposome loading. The liposomes were well maintained within both fully-cured and washed beads. The liposome release from the fully-cured bead was much slower than that from the corresponding washed bead in a pH 7.4 releasing medium. The greater the liposome loading, the faster the release of the vesicles. The liposome release was investigated in terms of liposome loading, swelling of the gel body, calcium discharge and gel erosion, using washed beads. The liposome loading did not affect the bead erosion or calcium discharge but did the initial swelling ratio and liposome release. The results suggest that the loaded liposomes are not uniformly distributed in the bead but are rather gradually concentrated to the center. Such an inhomogeneous distribution of liposomes is possibly due to the fact that the gelation occurred instantly on the surface of the droplets, and the resulting gel network or layer acts as a semipermeable membrane for liposomes and forces the vesicles to move into deeper concentric sections as gelation proceeds to the interior. As the liposome loading increases, the forced migration might be very limited because of concentrically decreasing extra room to accommodate the vesicles in the bead.

**Key words** alginate; gel matrix; liposome; erosion; calcium discharge; release

Alginic acid is a linear polysaccharide found in intercellular tissues of brown algae.<sup>2)</sup> The polysaccharide consists of (1—4)-linked residues of  $\beta$ -D-mannuronic acid (M) and  $\alpha$ -L-guluronic acid (G) arranged in a block fashion.<sup>3,4)</sup> It is well known that alginic acid can form translucent or transparent gels in association with calcium ion in which GG blocks are mainly responsible.<sup>5,6)</sup> An alginate gel matrix has been proposed as an oral drug delivery vehicle,<sup>6—8)</sup> and also as an immobilization bed of enzymes and living cells<sup>9—11)</sup> because of low toxicity, ease of gel formation and mild erosion of the gel body at neutral pHs. The gel matrix could also be applied as a delivery vehicle for dispersed systems such as emulsions and suspensions.

Liposomes are microscopic vesicles formed from phospholipids and other amphiphiles, and their biomedical and pharmaceutical applications have been extensively investigated, especially as promising drug carriers.<sup>12,13)</sup> With regard to liposomal drug delivery, the route of administration and dosage forms are not always of prime concern or are implicitly assumed to be a systemic administration. Although systemic application is now considered mainstream, thanks to possible improvements in the physicochemical and biopharmaceutical nature of dispersed systems, other dosage forms are also explored for oral administration, topical or percutaneous application and direct local delivery.<sup>12—15)</sup> For the oral route, the hydrolytic influence of enzymes and the solubilizing action of bile salts decrease their stability in the intestinal tract.<sup>16,17)</sup> As another drawback, liposomes given as an aqueous dispersed system are not convenient for routine oral administration. To overcome these disadvantages, one possible solution is considered to be an immobilized form of drug-containing liposomes in a gel matrix, e.g. a gel bead whose final form may be a dried

one.

In the previous paper, we prepared calcium-induced alginate gel beads in which liposomes were immobilized, then examined the stability of the liposomes in terms of leakage of the entrapped 5(6)-carboxyfluorescein,<sup>1)</sup> focusing on the effects of environmental factors to which the liposomes are exposed during the loading and gel preparation. In the present work, we studied the liposome release from gel beads in a neutral pH solution in terms of the amount of liposome loading, swelling of the gel body, calcium discharge and polymer erosion, to gain basic knowledge of liposome loading and its effect on the gel properties.

### Experimental

**Materials** Sodium alginate was purchased from Tokyo Kasei Kogyo, Tokyo and was used after dialysis against distilled water using Visking cellulose tubing (36/32) for 3 d (three water displacements/d), followed by lyophilization. The viscosity average molecular weight of the alginate was determined by the method previously described.<sup>1)</sup> The molecular weight was  $2.03 \times 10^5$  and the 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) was used. The molecular weight of EPC was assumed to be 780. All other chemicals were of reagent grade.

**Preparation of Liposomes** Liposomes were prepared using EPC according to the method of Bangham *et al.*<sup>18)</sup> Briefly, EPC in stock solution (hexane-ethanol, 98:2) was placed in a round bottomed flask and the solvent was completely removed on a rotary evaporator. The dried lipid film was hydrated by adding water with intermittent vortexing at room temperature for 30 min. The suspension was then subjected to ultrasonic irradiation (Tomy Seiko Co., Tokyo) under N<sub>2</sub> gas and centrifuged at  $2000 \times g$  for 20 min to remove titanium fragments. The resulting sonicated liposomes were allowed to equilibrate at room temperature for several hours and were then filtered through a 0.1  $\mu$ m polycarbonate filter (Nuclepore Co., Pleasanton, CA). The mean diameter of the liposomes was determined by dynamic light scattering (DLS-700, Otsuka Electronics, Osaka) and was  $26 \pm 4$  nm (mean  $\pm$  S.D.,  $n = 3$ ). Liposomes were freshly prepared before mixing them with alginate

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solution.

**Preparation of Fully-Cured and Washed Gel Beads** Alginate gel beads were prepared by the method previously described.<sup>1)</sup> Briefly, sodium alginate solution (2 or 4%, w/w) or its liposome-dispersed solution was allowed to fall by drops into 0.1 M CaCl<sub>2</sub> solution 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.). Alginate solution was mixed with liposome suspensions before dropping, during which various amounts of EPC were dispersed. The beads were allowed to cure in the CaCl<sub>2</sub> solution for various periods of time. Beads which cured for more than 3 d were then referred to as fully-cured beads. Since the beads prepared as described above contain excess calcium ions that are not associated with gelation, the excess calcium was washed every 10 min for 1 h with freshly distilled water. These beads were referred to as washed beads.

**Determination of Liposomes (Phosphorus) Loaded in Gel Beads** Liposomes were assayed for phosphorus content. Liposome-loaded beads were dissolved in 5 mM EDTA–50 mM Tris–HCl buffer (pH 7.4). After complete dissolution 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 Mrsny method.<sup>19)</sup>

**Size of Gel Beads** The size of beads was determined by taking a photograph of each bead ( $\times 20$ , 10 beads) and measuring its diameter at three different positions.

**Water Contents in Gel Beads** Beads were dried by heating 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 was represented as the volume of water, assuming  $d = 1.0$ . The average value of 15 beads was taken.

**Determination of the Amount of Calcium Ions in Gel Beads** Three beads were digested in nitric acid (61%, 1 ml) on a hot plate at 130 °C for 1 h. After cooling, 1 ml of perchloric acid (70%) was added, and the content was heated again to 130 °C until the solution became colorless. After cooling, the volume was appropriately adjusted by adding distilled water. The amount of calcium was then measured using an atomic absorption analyzer (AA-630-12, Shimadzu, Kyoto) with reference to a calibration curve constructed with known amounts of CaCl<sub>2</sub>.

**Determination of Alginic Acid** Uronic acids were determined by the phenol–sulfuric acid method.<sup>20)</sup> An aliquot (0.5 ml) of the sample solution was mixed well with 0.5 ml of phenol (5%) to which 2.5 ml of conc. sulfuric acid was quickly added. After 30 min of standing at 25 °C, the absorbance at 480 nm was measured. A calibration curve of purified alginate (up to 100 mg/ml) was constructed.

**Release Behavior of Phosphorus (Liposomes) from Gel Beads** Ten beads were placed in 50 ml of a buffer solution (50 mM Tris–HCl, pH 7.4, 0.15 M NaCl) in a thermostatted double-jacketed beaker, and the

solution was gently stirred by a magnetic stirrer. An aliquot of the bulk solution (1 ml) was withdrawn for a phosphorus assay at appropriate time intervals. The same amount of the solution sampled was added to the bulk solution. The temperature was 37 °C and the ionic strength of the solution was adjusted to 0.2 with NaCl.

**Dissolution Behavior of Gel Beads** The dissolution of a gel body was measured by uronic acid assay as described above.

## Results and Discussion

**Characterization of Liposome-Loaded Gel Beads** Table 1 shows the physical parameters of droplets of alginate solutions containing various amounts of EPC that were dispersed as liposomes. The liposome loading little affected the weight of the droplets, but reduced the fraction of interior water. These droplets were cured in a calcium solution, and the physical dimensions of the resulting almost spherical beads, both the fully-cured and washed, are listed in Table 2.

The gelation of alginate occurred instantly on the surface of the droplet, followed by the contraction of the gel body.<sup>6)</sup> This process is accompanied by a massive squeezing out of the interior water. However, the liposomes were well entrapped, more than 95% both in

Table 1. Physical Parameters of Droplets of Alginate Solutions Containing Various Amounts of Liposomes

Droplet	$P_d^{a)}$ ( $\times 10^7$ mol)	Weight <sup>b)</sup> (mg)	Water content <sup>c)</sup> (%)
N	0	13.1	96.0
A	0.727	13.1	96.1
B	1.74	13.3	95.7
C	3.50	13.3	94.2
D	5.14	13.1	93.4
E	9.08	13.1	91.8
F	17.0	13.3	87.6
G	23.1	13.3	83.0

Initial concentration of alginate: 4%. a) Amount of phosphorus (liposomes) in a single droplet (average value of 10 droplets). b) Weight of a single droplet (average value of 15 droplets). c) Average value of 15 droplets.

Table 2. Physical Dimensions of Fully-Cured and Washed Beads

Bead <sup>a)</sup>	$P_i^{b,c)}$ ( $\times 10^7$ mol)	Radius <sup>c)</sup> (mm)	Volume (mm <sup>3</sup> )	Weight <sup>c,d)</sup> (mg)	Water reserve <sup>e)</sup> (g/g)	Density (g/cm <sup>3</sup> )	$M_i^{e,f)}$ ( $\times 10^7$ mol)
Fully-cured							
N	0	1.19	7.19	7.79	13.7	1.08	17.1
A	0.714	1.20	7.30	7.93	14.0	1.08	17.2
B	1.68	1.20	7.31	8.00	13.8	1.10	17.6
C	3.34	1.22	7.61	8.26	14.0	1.09	17.8
D	5.13	1.22	7.62	8.32	14.1	1.09	17.4
E	8.87	1.24	7.93	8.38	13.9	1.06	17.3
F	16.4	1.27	8.48	9.24	14.1	1.09	17.1
G	22.7	1.28	8.72	9.41	13.1	1.08	17.9
Washed <sup>g)</sup>							
N	0	1.26	8.47	9.07	16.3	1.07	10.6
A	0.709	1.27	8.54	9.14	16.4	1.07	10.2
B	1.65	1.27	8.64	9.33	16.4	1.08	11.3
C	3.28	1.28	8.81	9.46	16.3	1.07	11.1
D	5.06	1.29	8.93	9.51	16.4	1.07	10.8
E	8.83	1.29	8.99	9.54	16.2	1.06	10.6
F	16.0	1.30	9.25	10.1	15.9	1.09	10.9
G	21.5	1.32	9.59	10.4	15.3	1.08	10.9

Initial alginate concentration: 4%; cured in 0.1 M CaCl<sub>2</sub> solution at 25 °C for 3 d. b) Amount of phosphorus (liposomes) in a single bead. c) Average value of 15 beads. d) Weight of a single bead. e) Weight ratio of water to alginate in 15 beads. f) Total amount of calcium in a single bead. g) Washed with distilled water for several times at 25 °C.

the fully-cured and washed beads, irrespective of the amount of liposomes loaded. While the size of the loaded liposomes was less than  $0.1 \mu\text{m}$  in diameter, the gel network formed in the beads seemed to be sufficiently dense to keep the vesicles within them. As the liposome loading increased, the volume of the bead increased. However, spherical beads with a smooth surface were no longer obtained when the initial lipid concentration was more than about  $2.9 \times 10^{-6}$  mol/droplet or 18%, and  $2.3 \times 10^{-6}$  mol/droplet or 14%, in 4% and 2% alginate, respectively. The liposomes were well reserved in the beads and the weight loss caused by gelation, which was a measure of water discharge, ranged from about 40% for the empty beads (bead N) to about 30% for the beads containing the EPC of  $2.27 \times 10^{-6}$  mol/bead (bead G).

By washing fully-cured beads to remove excess calcium ions that are not associated with gelation, the size of the beads swelled slightly compared with the parent beads, whereas the spherical integrity of the beads was well maintained, with a distinct boundary between the surface and the bulk medium after washing, even for the almost maximally liposome-loaded bead. The calcium content of the washed beads remained unchanged at about 60% of the fully-cured beads, regardless of liposome loading, indicating that calcium-associated gelation was not hindered by the loading. The liposomes, however, were lost a little: the total loss of lipid during gelation and washing processes was 2–5%. This is probably due to a slight omission of the liposomes at the very surface of the droplet and the washed bead.

**Liposome Release from Fully-Cured Beads and Washed Beads** Figures 1 and 2 show the release of liposomes from fully-cured beads and washed beads in Tris-HCl buffer (pH 7.4, 0.15 M NaCl) at  $37^\circ\text{C}$ , respectively. Some characteristics of the release are as follows: (1) The release generally followed a sigmoid manner. (2) The release from the washed beads was faster than that from the fully-cured beads. (3) The lag time of the release became shorter as the liposome loading increased.

As described in the Experimental section, the fully-cured bead contains an excess of calcium ions, while the washed bead is forcibly washed before the release experiment. Immediately after the fully-cured bead was placed in the releasing medium, a large fraction of the excess calcium ions should have been rapidly discharged before appreciable erosion of the gel body started, as if the beads were to be washed. Then, the calcium concentration in the medium would have reached approximately  $1 \times 10^{-4}$  M, and it was only about 1/1000 in the original gel reaction medium ( $0.1\text{M CaCl}_2$ ), being diluted far below the concentration to maintain the integrity of the fully-cured bead. However, the release of liposomes from the fully-cured bead was much slower than that from the washed bead. This result may mean that it takes a certain amount of time for the fully-cured gel structure, which is often represented by an “egg box junction model,”<sup>5</sup> to have a somewhat loose structure for swelling and erosion, while the washed bead is already in the state of maximal swelling in the distilled water but is strong enough to maintain the gel body.

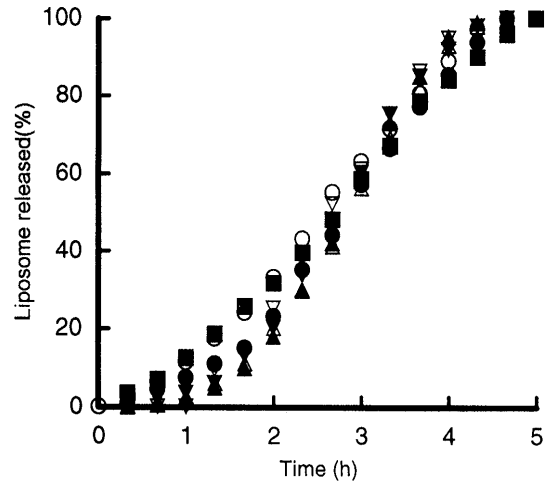


Fig. 1. Liposome Release from Fully-Cured Beads Containing Different Amounts of Liposomes in 50 mM Tris-HCl Buffer (pH 7.4, 0.15 M NaCl) at  $37^\circ\text{C}$

Beads were prepared with an initial alginate concentration of 4%. Each plot represents an average value of 3 runs. Keys (see Table 2): A,  $\blacktriangle$ ; B,  $\triangle$ ; C,  $\blacktriangledown$ ; D,  $\triangledown$ ; E,  $\bullet$ ; F,  $\circ$ ; G,  $\blacksquare$ .

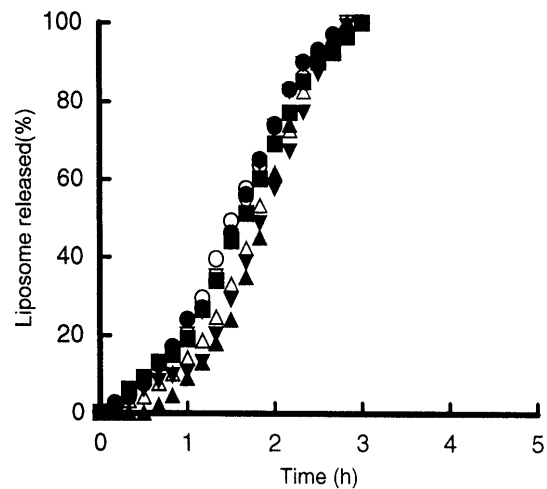


Fig. 2. Liposome Release from Washed Beads Containing Different Amounts of Liposomes in 50 mM Tris-HCl Buffer (pH 7.4, 0.15 M NaCl) at  $37^\circ\text{C}$

Beads were prepared with an initial alginate concentration of 4%. Each plot represents an average value of 3 runs. Keys (see Table 2): A,  $\blacktriangle$ ; B,  $\triangle$ ; C,  $\blacktriangledown$ ; D,  $\triangledown$ ; E,  $\bullet$ ; F,  $\circ$ ; G,  $\blacksquare$ .

As described earlier, the initial release rate of liposomes increased as the liposome loading increased. Assuming that the liposomes were uniformly distributed in the beads, irrespective of the amount of liposomes loaded, the release rate should be the same for all the beads. Although the size of the bead only slightly increased with increasing the loading, the gel structure seemed to be little affected by such a slight swelling because the associated calcium for gelation remained unchanged. The results leave us wondering why smaller liposome loading brings about a slower release of liposomes from the bead.

It is too complicated at this time to understand the whole mechanism of the release behavior of liposomes in which a variety of parameters are involved. To simplify the gel erosion and liposome release processes, the release behavior from washed beads containing various amounts

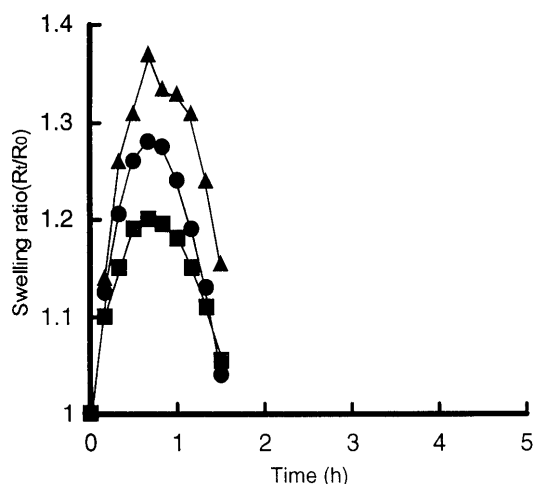


Fig. 3. Magnitude of Swelling of Washed Beads Containing Different Amounts of Liposomes in 50 mM Tris-HCl Buffer (pH 7.4, 0.15 M NaCl) at 37°C

Beads were prepared with an initial alginate concentration of 4%. Each plot represents an average value of 3 runs.  $R_0$ , radius of the bead ( $t=0$ );  $R_t$ , radius of the bead at time  $t$ . Keys (see Table 2): A,  $\blacktriangle$ ; E,  $\bullet$ ; G,  $\blacksquare$ .

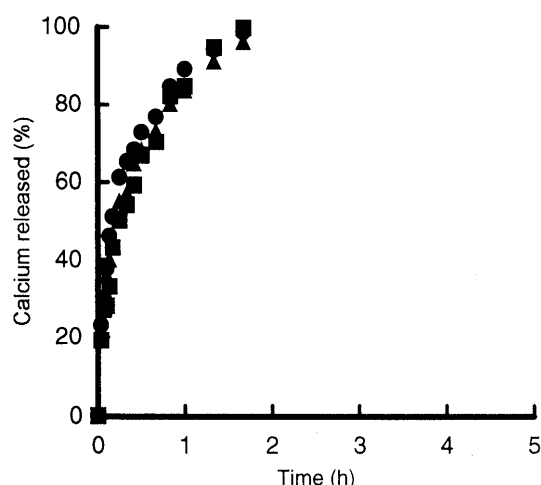


Fig. 4. Calcium Discharge from Washed Beads Containing Different Amounts of Liposomes in 50 mM Tris-HCl Buffer (pH 7.4, 0.15 M NaCl) at 37°C

Beads were prepared with an initial alginate concentration of 4% (w/w). Each plot represents an average value of 3 runs. Keys (see Table 2): A,  $\blacktriangle$ ; E,  $\bullet$ ; G,  $\blacksquare$ .

of liposomes was examined in terms of the magnitude of swelling, discharge of calcium associated with gelation and polymer erosion (estimated as uronic acid). The initial liposome release governed by the synchronized processes may be characterized more easily as long as the spherical integrity of the beads is maintained.

Figure 3 shows the swelling of the liposome-loaded beads with time, reaching a maximum size at about 40 min, with subsequent reduction in size. The spherical integrity was maintained for about 90 min. After this time the surface of the gel body started losing its clear boundary and collapsing to a gum-like lump. Increasing the liposome loading decreased the swelling ratio but did not affect the time required to reach a maximal swelling size of each bead, indicating that the liposomes hindered the polymer molecules from unfastening and spatial swelling.

The associated calcium that contributes to forming the tight gel structure was discharged in a bursting manner,

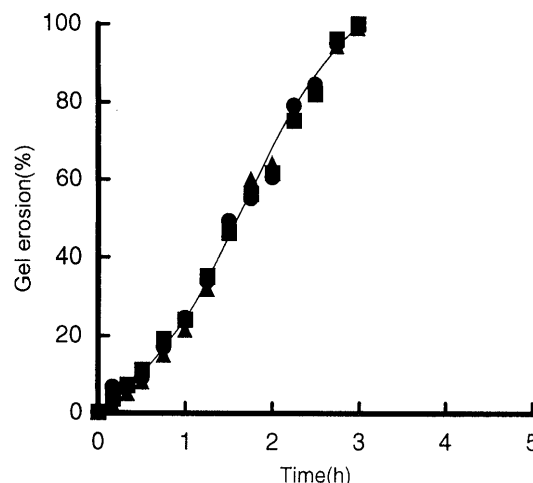


Fig. 5. Erosion Rate of Washed Beads Containing Different Amounts of Liposomes in 50 mM Tris-HCl Buffer (pH 7.4, 0.15 M NaCl) at 37°C

Beads were prepared with an initial alginate concentration of 4%. Polymer erosion was measured by a uronic acid assay. Each plot represents an average value of 3 runs. Keys (see Table 2): A,  $\blacktriangle$ ; E,  $\bullet$ ; G,  $\blacksquare$ .

which was completed in 2 h (Fig. 4). The calcium discharge is caused by ion exchange with coexisting ionic substances.<sup>21)</sup> In this study, these were mainly sodium ions (0.15 M) in the buffer. This discharge of the calcium implies that the junctions are unfastened and the polymer molecules are ready to dissolve and diffuse.

Changes in the uronic acid amount in the bulk represent the erosion of the polymer molecules from the surface of the gel body. The time course was in an almost linear fashion, and little difference was seen among the different liposome loadings (Fig. 5). Erosion of the gel body is primarily due to changes in the unionized and ionized fractions of the COOH groups when placed in the pH 7.4 releasing medium from distilled water. The ionized groups repel each other and at the same time attract more water, resulting in an expansion of the gel body and subsequent erosion.

No lag time of the swelling and calcium discharge indicates that pH changes rapidly occurred from the surface to the interior immediately after coming into contact with the releasing medium. Also, more than 95% of the calcium was already discharged before the spherical integrity of the bead was lost (Figs. 3 and 4). However, the calcium discharge and the gel erosion did not match each other (Figs. 4 and 5), indicating that polymer erosion encounters, to a large extent, resistance of chain entanglement after the gel structure is canceled by a rapid calcium discharge. Accordingly, the liposome release is basically controlled by gel erosion, but the effect of liposome loading on the initial release remains unclear. For example, during an early period of having reached the maximal swelling, in about 40 min, the liposomes were little released from bead A and were released about 15% from bead G. In about 90 min, at which the spherical integrity was lost, the liposome release was 25% from bead A and 50% from bead G.

Figure 6 shows the relationship between gel erosion and liposome release, taken from Figs. 2 and 5. It should be noted here that the spherical integrity of the beads was maintained up to about 50% gel erosion. If the loaded

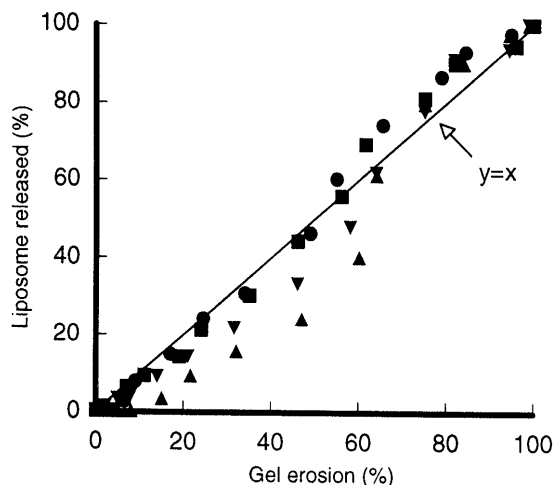


Fig. 6. Relationship between Erosion of Washed Beads and Liposome Release

Replot of the data from Figs. 2 and 5. Medium: 50 mM Tris-HCl Buffer (pH 7.4, 0.15 M NaCl); Temperature, 37 °C. Keys (see Table 2): A, ▲; C, ▼; E, ●; G, ■.

liposomes were uniformly distributed in any beads and the release were solely controlled by gel erosion, the relationship should be represented with a single line, irrespective of the liposome loading. However, when the liposomes were loaded to a greater extent (beads E and G), the release was proportional to the gel erosion, which was represented by  $y=x$  up to about 50% gel erosion. As the liposome loading was smaller (beads A and C), the release deviated from linearity in the early period. The results suggest that the liposomes are not uniformly distributed in the bead, but are rather gradually concentrated to the center, since there was little difference in the gel erosion among the beads examined.

Inhomogeneous distribution of liposomes in the bead is possibly due to the fact that the gelation occurred instantly on the surface of the droplets, so that the resulting gel layer acts as a semipermeable membrane for liposomes and forces the vesicles to move to deeper concentric sections as gelation proceeds to the center. When greater amounts of liposomes were loaded, such a forced migration might be very limited because there is little extra room to accommodate the vesicles, resulting in a relatively uniform distribution.

Skjåk-Bræk *et al.* reported that there was a concentration gradient of polymer in calcium-induced gels<sup>22</sup>; higher at the surface than in the center. They suggest that the relative rate of diffusion of polymer molecules and gelling cations and strong and irreversible binding of cross-linking ions are responsible. Although the gel they used was formed as a large right circular cylinder (14 mm in diameter and 19 mm in length), a similar concentration gradient to the center may be possible even in the small bead we used. Such phenomena would cooperatively drive loaded liposomes deep, from the surface to the center of the bead.

In conclusion, calcium-induced alginate gels can hold liposomes maximally up to about 18% and 14% in the 4% bead and 2% bead, respectively. The liposomes were well maintained in the bead, even after being washed in distilled water. The magnitude of liposome loading affected the release behavior in the early stage: the more the liposome loading, the faster the liposome release, while little difference of the bead erosion rates was realized. The results suggest that a gelling layer forced loaded liposomes to move into the center, resulting in inhomogeneous distribution of the vesicles in the bead. This phenomena will be an important factor to be noted when not only liposomes but other dispersed systems are attempted to be accommodated in alginate gel matrices.

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