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Remote loading of diclofenac, insulin and fluorescein isothiocyanate labeled insulin into liposomes by pH and acetate gradient methods

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Abstract

Remote loading of the model drugs diclofenac, insulin and fluorescein isothiocyanate labeled insulin (FITC-insulin) into liposomes by formation of transmembrane gradients were examined. A trapping efficiency of almost 100% was obtained for liposomal diclofenac, by the calcium acetate gradient method, whereas liposomes prepared by the conventional reverse-phase evaporation vesicle method had 1–8% trapping efficiencies. Soybean-derived sterol was a better stabilizer of the dipalmitoylphosphatidylcholine bilayer membrane than cholesterol, as shown from trapping efficiencies and drug release. The pH gradient method resulted in a 5–50% of FITC-insulin liposomal trapping efficiency, while insulin could not be loaded by this method. Liposomes released calcein in response to insulin, showing insulin interacts with the liposomal membrane in the presence of a transmembrane gradient. The present work has demonstrated a remote loading method for weak acids such as diclofenac into liposomes by the acetate gradient method. From the result of remote loading of FITC-insulin into liposomes by the pH gradient method, this method may be available for the preparation of liposomal peptides.

Keywords: Liposome; Remote loading; Acetate gradient; Diclofenac; pH Gradient; Insulin

1. Introduction

Liposomes, which are lipid bilayer vesicles, have gained increasing attention as drug carriers which reduce the toxicity and increase the pharmacological activity of various drugs. Various

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methods are available for the preparation of liposomes, but disadvantages remain. The conventional reverse-phase evaporation vesicle (REV) method, and ether or ethanol injection methods are based on passive entrapment of the drug during formation of the lipid bilayer vesicles. However, these processes require vigorous conditions, namely organic solvents, sonication and high temperature, which result in loss of drug efficiency and inactivation, particularly of peptides (Weiner, 1994).

Several methods exist for improved loading of the drugs, including remote (active) loading methods which load drug molecules into preformed liposomes using pH gradients and potential differences across liposomal membranes (Čeh and Lasic, 1995).

Weak bases like doxorubicin (Mayer et al., 1986, 1993; Harrigan et al., 1993) and vincristine which coexist in aqueous solutions in neutral and charged forms (Mayer et al., 1990, 1993), have been successfully loaded into preformed liposomes via the pH gradient method. Chakrabarti et al. (1992, 1994) reported that short modified peptides can be efficiently accumulated in large unilamellar vesicle (LUV) via pH gradients (inside acidic). However, there are only a few reports of using remote loading for weak acids, and no reports for insulin. Proteins with a high percentage of α-helix, such as apoproteins (Lund-katz et al., 1990) or serum albumin (Law et al., 1988), have a high affinity for liposomes. The α-helix and amphoteric properties of the insulin molecule may facilitate incorporation into liposomes.

In this study, remote loading of weak acids and peptides into liposomes was investigated. The lipid composition of liposomes, the optimal conditions for drug loading, and the characteristics of liposome loaded drugs were also examined. Diclofenac and insulin or fluorescein isothiocyanate labeled insulin (FITC-insulin) were used as weak acid and peptide drug models, respectively.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC) was purchased from NOF (Tokyo, Japan). Soybean-derived sterols (SS) used in this study was a mixture of β-sitosterol (49.9%), campesterol (29.1%), stigmasterol (13.8%), and brassicasterol (7.2%). SS was kindly supplied by Ryukakusan Co. Ltd. Diclofenac sodium (DNa), crystalline bovine pancreas insulin (crystalline; zinc content; approx. 0.5%) and FITC-insulin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cholesterol (Ch), 4-hydroxybenzoic acid *n*-hexyl ester, trifluoroacetic acid, HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethansulfonic acid) and calcein were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Sephadex G-100 was purchased from Pharmacia Biotech (Sweden). All other chemicals were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and were analytical grade.

2.2. Preparation of liposomal diclofenac

The preformed liposomes (empty liposomes) were prepared according to the REV method (Szoka and Papahadjopoulos, 1978) as described previously (Qi et al., 1995). Diclofenac was loaded in empty liposomes by pH gradient and acetate gradient methods. Briefly, DPPC and SS or Ch (7:2 molar ratio, SS liposomes, Ch liposomes, respectively) were dissolved in chloroform and dried in an evaporator. The dried lipid film was redissolved in chloroform and mixed with a 10-fold dilution of phosphate-buffered saline (1/10 PBS, pH 7.4), 150 mmol/l sodium acetate solution (pH 7.9), or 120 mmol/l calcium acetate solution (pH 7.3) for empty liposomes. Each mixture was mixed by vortexing, followed by sonication in a bath-type sonicater (Honda Electronics, Tokyo, Japan) to give a homogeneous w/o emulsion. Each emulsion was then placed on a rotary evaporator and the organic solvent was removed to obtain liposome suspension. The suspension was extruded through polycarbonate membranes (Nuclepore, Costar Scientific Corporation, USA) with pore sizes of 0.4 and 0.2 μm at about 60°C. Liposomes preformed in 1/10 PBS (inside pH 7.4) were incubated with DNa in 1/10 PBS at a pH adjusted to 4.0 (outside) by 0.1 mol/l hydrochloric

acid at 60°C for 60 min (pH gradient method). Liposomes preformed in sodium acetate (inside pH 7.9) or calcium acetate solution (inside pH 7.3) were washed twice with 120 mmol/l sodium sulfate solution (outside pH 5.9) by ultracentrifugation (100 000 × *g*, 4°C, 60 min) to create an acetate concentration gradient across the liposomal membrane, and then were incubated with DNa in 120 mmol/l sodium sulfate solution (sodium acetate and calcium acetate gradient methods). The osmotic pressure of each aqueous medium was adjusted.

Conventional liposomes as reference were prepared by REV method as described above except using 1/10 PBS containing DNa instead of only buffer solution as aqueous phase.

Liposomes loaded with diclofenac by the conventional and remote loading methods were separated from free drug by ultracentrifugation (100 000 × *g*, for 60 min) at 4°C. The process was repeated three times by making a suspension of the precipitate in sodium sulfate solution.

2.3. Preparation of liposomal insulin

Liposome entrapped insulin and FITC-insulin were prepared by conventional REV method and remote loading method. The former one uses PBS, and HEPES (HBS, 20 mmol/l HEPES and 150 mmol/l sodium chloride, pH 7.5), or citric acid buffer solution (CBS, 300 mmol/l citric acid, pH 4.0).

The latter cases consist of acetate and pH gradient methods. The liposomes were preformed in 120 mmol/l calcium acetate solution (inside pH 7.3) or 120 mmol/l zinc acetate solution (inside pH 6.0). Then, the liposomes were incubated with insulin or FITC-insulin in sodium sulfate solution or CBS (outside pH 2.1) (calcium or zinc acetate gradient methods). The liposomes preformed in CBS were gel filtered with HBS. The resulting filtrate containing liposomes were incubated in HBS containing insulin or FITC-insulin (inside pH 4.0 and outside pH 7.5, pH gradient method). After incubation, the liposome were separated from free insulin by ultracentrifugation or by gel filtration for the pH gradient method. In the case of pH gradient method, the liposome suspension was passed through Sephadex G-100 column (1.5 × 26 cm, Pharmacia Biotech, Sweden) with the HBS in all fractions.

2.4. Determination of solubility of diclofenac in various aqueous solutions

Five mililiters of each aqueous solution was added to enough DNa to ensure saturation at 50°C. After the temperature was reduced to 25°C, excess diclofenac salts were crystallized. The system was left to equilibrate for 72 h at 25°C. The samples were centrifuged, filtered (Ekicrodisc, 0.45 μm, Gelman Sciences Japan Ltd., Japan), and assayed by high performance liquid chromatography (HPLC).

2.5. Determination of DPPC, diclofenac, insulin, FITC-insulin and calcein concentrations

The concentration of DPPC in liposome suspension was determined by enzymatic assays (Phospholipid B Test Wako, Wako Pure Chemical Industries Ltd., Osaka, Japan) (Qi et al., 1995). Diclofenac (Maitani et al., 1994) and insulin (Nakazawa and Nagase, 1986) concentrations were determined by HPLC, as previously reported. The HPLC system consisted of a Shimadzu LC-9A liquid chromatography, SPD-6A UV detector, and C-R6A chromatopac. The concentration of calcein and FITC-insulin were determined using a fluorophotometer (excitation at 490 nm and emission at 520 nm for calcein, excitation at 480 nm and emission at 516 nm for FITC-insulin, respectively; Hitachi F-4010, Tokyo, Japan).

2.6. Determination of size distribution of liposomes

The size distribution of liposomes was determined using a light scattering instrument (Model ELS-800, Otsuka Electronics, Japan) and dynamic laser light scattering methods.

2.7. Calculation of trapping efficiency

From the amount of DPPC and drug determined before and after separation of liposomal drug from free drug, the trapping efficiencies were calculated according to the following equation:

$$\text{Trapping efficiency (\%)} = \frac{M^{a}_{drug} M^{b}_{DPPC}}{M^{a}_{DPPC} M^{b}_{drug}} \times 100 \quad (1)$$

where M^{b}_{drug} and M^{a}_{drug} are the amount of drug in liposome suspension, and M^{b}_{DPPC} and M^{a}_{DPPC} are the amount of DPPC in liposome suspension determined before and after separation, respectively.

2.8. Theoretical trapping efficiency by the REV method

The theoretical trapping efficiency can be expressed as $100 \times n \times V_L/V_T$, where V_L and V_T represent the internal volume of a liposome and the total volume of the prepared liposome suspension (3×10^{-3} l), respectively, and n is the number of liposomes. The V_L value was calculated using liposomal diameter (193 nm) assuming that the liposomes are unilamellar and form a uniform population of spherical vesicles. The n value was calculated from the prepared lipid amount of DPPC (70×10^{-6} mol), the size of liposomes, the Avogadro's number (A), and the outer (0.60 nm^2) and the inner (0.49 nm^2) surface area per DPPC head group in DPPC/SS (7: 2) liposome and the bilayer thickness (4.4 nm), from the results of DPPC/Ch liposomes (Huang and Mason, 1978; Lis et al., 1982), under the assumption of that the effect of SS and Ch on the packing geometry of lipids within the liposomal bilayer are similar. The theoretical capture volume of liposomes per mol of DPPC is $V = V_L \times A/n_{DPPC} = 4.6$ l/mol, where n_{DPPC} is the number of DPPC molecule per unilamellar liposome (4.07×10^5). The molar ratio of DNa entrapped in liposomes to DPPC (y) can be expressed as $y = V \times x = 0.0046 \times x$, where x is the initial concentration (mmol/l) of DNa in solution (Fig. 1B). The theoretical trapping efficiency is $100 \times n \times V_L/(3 \times 10^{-3}) = 10.9\%$.

2.9. Measurement of drug release from liposomal diclofenac

Three mililiters of liposome suspension was placed in a cellulose membrane bag (Viskase Co.,

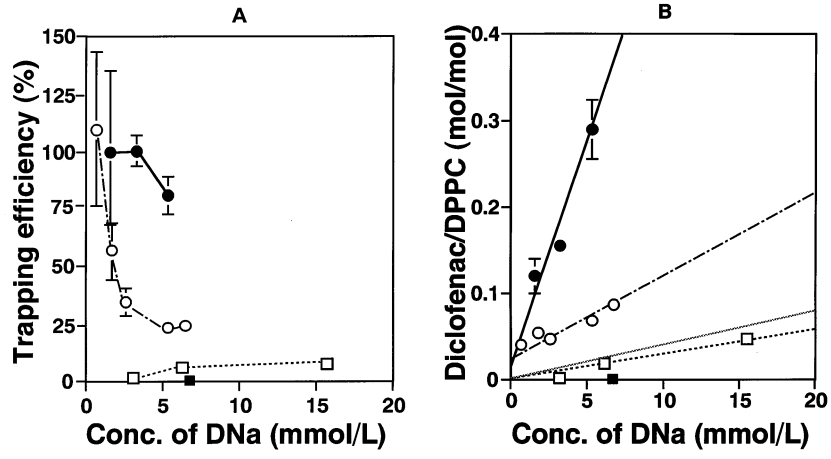

Fig. 1. Effects of various loading methods on trapping efficiency (A) and the molar ratio of entrapped drug to DPPC (B) vs. initial concentration of DNa in SS liposomes. Method: □, REV; ■, pH gradient; ○, sodium acetate gradient; ●, calcium acetate gradient. Each value represents the mean ± S.D. ($n = 3$). The concentration of DPPC in the incubation media was 20–30 mmol/l, except for the pH gradient method (10–12 mmol/l). The theoretical values for REV are represented by the shaded line at 23.3 mmol/l of DPPC ($y = 0.0046 \times x$). Remote loading incubation by sodium or calcium acetate gradient methods were performed at 37°C for 1 h and for the pH gradient method at 60°C for 1 h.

Chicago, USA), which was pretreated by soaking in water at 60°C overnight, and washed with deionized water. The dialysis bag was soaked in PBS at 37°C and drug release from the liposome at appropriate time intervals was determined by HPLC. The release rate constant k was obtained according to the following equation:

$$\ln(C_{\text{retained}}/C_{\text{initial}}) = -kt \quad (2)$$

where C_{retained} and C_{initial} indicate the concentrations of drug retained in liposomes at time t and 0, respectively. C_{retained} was calculated from the amount of drug released at time t and the initial amount in liposomes at time 0, assuming that the volume of liposome suspension is constant.

2.10. Measurement of calcein leakage from the liposomes entrapped calcein induced by insulin

Liposomes entrapped calcein were prepared by the REV method using CBS (pH 4.0) or HBS (pH 7.5) containing calcein of 0.0505 mmol/l, and passed through gel filtration eluted with HBS in all fractions. The resulting liposomes with entrapped calcein were incubated in HBS containing insulin and a pH gradient at 25°C. Leakage of calcein from the liposomes entrapped calcein was determined from the measurement of the release of calcein from the liposomes. The final phospholipid concentration was 0.816 mmol/l. The time course of fluorescence intensity change corresponding to calcein efflux was recorded immediately after the addition of insulin solution with rapid stirring. The percentage of calcein released at time t was obtained according to the following equation:

$$\text{Calcein release } (\%) = 100(F_t - F_0)/(F_T - F_0) \quad (3)$$

where F_0 is the fluorescence intensity of calcein in the liposome suspension in the absence of insulin, F_t is the fluorescence intensity at time t after addition of insulin, F_T is the fluorescence intensity when the liposomes with entrapped calcein were disrupted by Triton X-100. Leakage kinetics were monitored at different molar ratios of insulin to DPPC after adding aliquots of insulin solution.

3. Results

3.1. Trapping efficiency and loading conditions of diclofenac in liposomes

The effects of various loading methods on trapping efficiency and the ratio of entrapped drug to DPPC versus initial concentration of DNa in SS liposomes are shown in Fig. 1. The trapping efficiency of diclofenac by the REV method was approximately 1–8%, equivalent to a molar ratio of diclofenac to DPPC of 0.002–0.059. Diclofenac precipitated in the liposomes exterior because of the low solubility of diclofenac in acidic aqueous solutions in the pH gradient method. The resulting precipitate was washed three times with 1/10 PBS or 1/10 PBS containing ethanol (10 or 20%) and ultracentrifuged to remove unloaded diclofenac. The trapping efficiency in SS liposomes by the pH gradient method was about 0.03%.

The effects of temperature on trapping efficiencies of SS liposomes were examined preliminarily using calcium acetate gradient methods. Samples were incubated for 60 min at 4, 37, or 60°C, and exhibited trapping efficiencies of about 95.6 ± 1.1, 101.6 ± 6.3 and 56.2 ± 12.3%, equivalent to molar ratios of diclofenac to DPPC of 0.122 ± 0.040, 0.156 ± 0.009 and 0.072 ± 0.007, respectively. The initial concentrations of DNa and DPPC in the incubation media for these experiments were about 3.4 and 30 mmol/l, respectively. When SS liposomes were incubated with DNa solution for 60 min at 60°C, the trapping efficiencies were lower than for 20 min at 60°C (95.0 ± 9.8%, equivalent to the molar ratio of diclofenac to DPPC of 0.127 ± 0.047). These results suggest that the optimal incubation conditions for this loading process are 37°C for 1 h or 60°C for less than 20 min, and therefore 37°C for 1 h is used for loading.

The trapping efficiency was over 50% of the initial drug amount for the sodium acetate gradient method, equivalent to a 0.04 molar ratio of diclofenac to DPPC, at mmol/l concentrations of DNa under 1.7, over this concentration it decreased to approximately 25% (Fig. 1). On using the calcium acetate gradient method, trapping efficiencies approaching 100% were obtained at

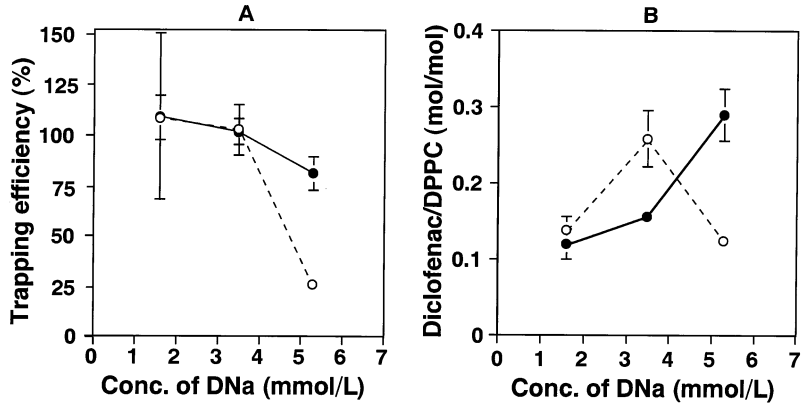

Fig. 2. Effect of SS liposomes and Ch liposomes on trapping efficiency (A) and the molar ratio of entrapped drug to DPPC (B) vs. initial concentration of DNa for the calcium acetate gradient method at 37°C for 1 h. ○, Ch liposome; ●, SS liposome. Each value represents the mean ± S.D. ($n = 3$). The initial concentration of DPPC in the incubation media was approximately 20 mmol/l.

low concentrations of DNa. Even if the concentration of DNa increased to 5.3 mmol/l, about a 70–80% trapping efficiency was maintained (Fig. 1A), equivalent to a molar ratio of diclofenac to DPPC of 0.2–0.3 (Fig. 1B). Sodium and calcium acetate gradient methods had higher entrapment efficiencies for diclofenac than the REV method.

Effect of SS liposomes and Ch liposomes on trapping efficiency and the ratio of entrapped drug to DPPC versus initial concentration of DNa by calcium acetate method at 37°C for 1 h are shown in Fig. 2A and B. The initial concentration of DPPC in the incubation media was approximately 20 mmol/l. At high concentrations of DNa in the incubation medium, trapping efficiencies of diclofenac were about 80% in SS liposomes, and about 25% in Ch liposomes (Fig. 2A).

The drug release profiles from diclofenac loaded Ch liposomes and SS liposomes by the calcium acetate gradient method in PBS at 37°C are shown in Fig. 3. The initial concentrations of DPPC and DNa in each liposome suspension were approximately 30 and 5 mmol/l, respectively. Faster drug release was observed from Ch liposomes than from SS liposomes.

Change in size distribution of diclofenac loaded SS liposomes and Ch liposomes by the calcium acetate gradient method versus the initial concentration of DNa in the incubation media is shown in Fig. 4.

3.2. Insulin or FITC-insulin in SS liposomes

Fig. 5 shows trapping efficiency of FITC-insulin into SS liposomes by the pH gradient method. FITC-insulin was loaded at a 5–50% efficiency into SS liposomes, whereas insulin could not be loaded.

The time course of leakage of calcein from the SS liposomes with entrapped calcein triggered by

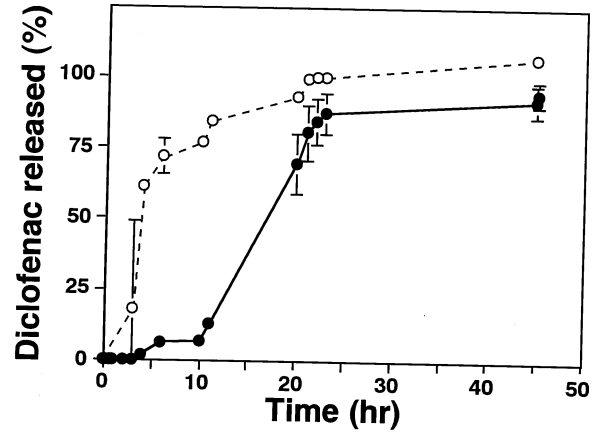

Fig. 3. Release profiles of diclofenac from diclofenac loaded SS liposomes and Ch liposomes by the calcium acetate gradient method in PBS at 37°C. ○, Ch liposome; ●, SS liposome. Each value represents the mean ± S.D. ($n = 3$). The concentrations of DPPC and DNa in each liposome suspension were approximately 30 and 5 mmol/l, respectively.

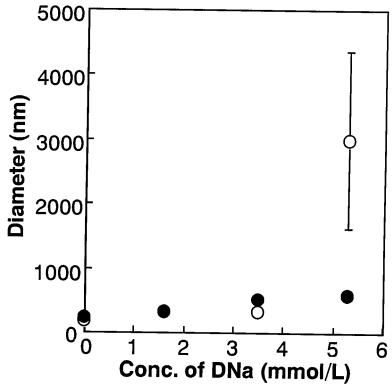

Fig. 4. Change in size distribution of diclofenac loaded SS liposomes and Ch liposomes by the calcium acetate gradient method vs. the initial concentration of DNa. ○, Ch liposome; ●, SS liposome. Each value represents the mean ± S.D. ($n = 3$). The initial concentration of DPPC in the incubation media was approximately 20 mmol/l.

insulin in a pH gradient at 25°C was examined. Fig. 6A and B represent the calcein leakage (%) and the rate constant k of calcein leakage in the early period, respectively.

4. Discussion

A pH gradient across the membrane of liposomes can drive the loading of drug molecules. Usually this pH imbalance is generated by a two-step process: first the vesicles are prepared in a certain pH solution, then the external medium is exchanged by gel-exclusion chromatography with a different pH solution. This approach has been developed further by using transmembrane differences in salt concentrations, such as sodium acetate or calcium acetate, i.e. acetate gradient method.

Acetate gradient methods take advantage of the large difference in permeability coefficients across lipid bilayers of the cation and acetic acid molecule, generated by the dissociation of the acetate anion, to produce an increase of the liposome internal pH (Clerc and Barenholz, 1995). This pH imbalance (inside base, outside acid) serves as an efficient driving force to load and accumulate weak acids inside lipid vesicles, since the dissociated part of the acid can not permeate through the liposomal membrane. A pH gradient method can be applied to load acidic, basic and amphoteric drugs changing the internal and external pH. Acetate gradient method can be applied to load acidic drugs coexisting in neutral and charged form in aqueous solutions since the internal pH increases and the external pH of liposomes is scarcely changed.

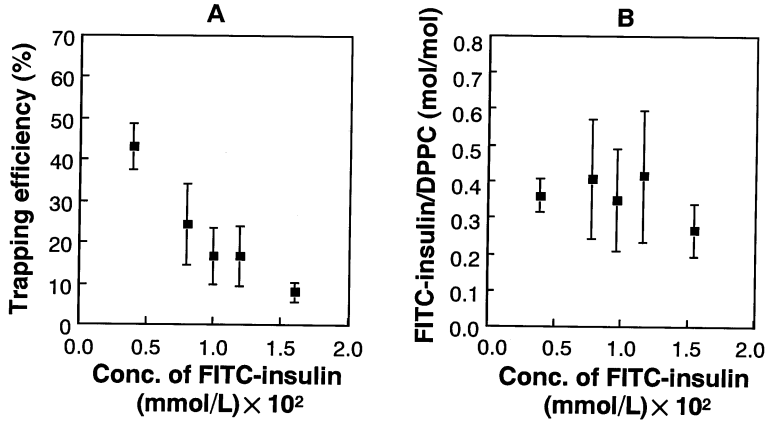

Fig. 5. Trapping efficiency of FITC-insulin (A) and the molar ratio of FITC-insulin to DPPC (B) into SS liposomes by the pH gradient method. The concentration of DPPC in the incubation media was 4.486 mmol/l. Each value represents the mean ± S.D. ($n = 3$).

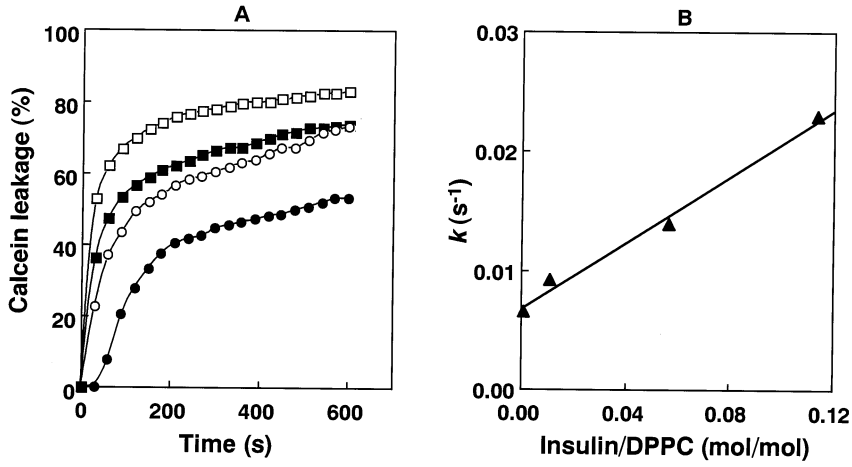

Fig. 6. The time course of leakage of calcein from SS liposomes entrapped calcein triggered by insulin with a pH gradient in HBS at 25°C. The calcein leakage (%)-time profile (A) and rate constant k of calcein leakage (B) according to the concentration of insulin. The concentration of DPPC in the incubation media was 0.816 mmol/l. Insulin/DPPC (mol/mol): □, 0.114; ■, 0.0570; ○, 0.0114; ●, 0.00114.

4.1. Trapping efficiency of diclofenac by various methods

In conventional REV method, since the solubility of DNa in low pH is very low, 1/10 PBS (pH 7.4) was used. The shaded line in Fig. 1B represents the theoretical molar ratios of entrapped diclofenac to DPPC at 23.3 mmol/l of DPPC in the REV method, i.e. the theoretical trapping efficiencies (10.9%). The trapping efficiencies (1–8%) by the REV method were lower than predicted, particularly at low concentrations of DNa.

As loading of DNa by pH gradient and acetate gradient methods, each pH condition was selected since undissociated form of diclofenac can penetrate the liposomal membrane. Concerning pH gradient method, the low trapping efficiency was observed, compared to the REV method or to acetate gradient methods (Fig. 1A). This may be due to the low solubility of DNa in acidic aqueous solution (pH 4.0), which was liposome exterior by the pH gradient method. Under these conditions, sufficient concentration gradient of DNa can not be formed, resulting in low drug loading.

In the case of acetate gradient methods, the solubility of DNa in external aqueous phase (pH 5.9) was high enough because the transmembrane pH gradient can be obtained just by the dilution with acetate-free solution. However, despite the formation of similar pH gradients by the sodium acetate and calcium acetate gradient methods, the latter was more effective than the former in terms of trapping efficiency. Two reasons are suggested for this difference: (1) differences in intraliposomal solubility of loaded drugs; and (2) ion-pairing of diclofenac with sodium ions in liposomes in the sodium acetate gradient method.

The solubility of diclofenac in various solutions was 1/10 PBS (34.3 ± 0.5 mmol/l) > sodium acetate solution (14.9 ± 1.0 mmol/l) > sodium sulfate solution (11.2 ± 1.3 mmol/l) > calcium acetate solution (0.7 ± 0.1 mmol/l). The lower the solubility of diclofenac in the internal aqueous phase, the higher the diclofenac trapping efficiency (Fig. 1). These results may indicate that diclofenac in calcium acetate solution may precipitate in liposomes and maintain the concentration gradient of diclofenac throughout the liposomal membrane. On the other hand, diclofenac loaded into liposomes may be released from liposomes via increased permeability due to formation of the ion pair $D^- Na^+$, especially when high concentrations of DNa are used. Maitani et al. (1994) reported that diclofenac salts permeate through membranes in an ion pair state. In addition,

Quintanar-Guerrero et al. (1997) reported that ion-pairing effectively increases lipophilicity of charged drug molecules.

4.2. SS liposomes versus Ch liposomes loaded diclofenac

We have reported that SS stabilized DPPC liposomes more effectively than Ch (Muramatsu et al., 1994; Qi et al., 1995, 1996). The effects of SS liposomes or Ch liposomes for the calcium acetate gradient method on trapping efficiency, drug release and the change of particle size were examined.

Both SS liposomes and Ch liposomes had about 100% trapping efficiency, equivalent to a molar ratio of diclofenac to DPPC of 0.1–0.2, at low concentrations (< 3.4 mmol/l) of drug (Fig. 2). However, at 5.3 mmol/l, Ch liposomes had the low trapping efficiencies (about 25%) while SS liposomes still had approximately 100% efficiencies. The change of particle size of Ch liposomes (Fig. 4) as well as the low trapping efficiency at 5.3 mmol/l supported that Ch liposomes are less stable than SS liposomes.

The release of loaded drug from SS liposomes and Ch liposomes was also examined to verify if differences in loading of drug is due to the liposomal membrane stability and the specific interaction of liposomal membrane. SS liposomes had remarkably slower release of diclofenac in PBS at 37°C than Ch liposomes (Fig. 3). The k values using Eq. (2) for up to 10 h were 0.195 and 0.012 h^{-1} for Ch liposomes and SS liposomes, respectively. These results indicate that the stabilizing effect of SS on DPPC liposomes is greater than Ch, and the loaded drug inside liposomes is released completely without interaction of drug with liposomal membrane.

4.3. Loading of insulin or FITC-insulin to SS liposomes

Conventional REV method is applied using drug dissolved in various pH buffered solutions except isoelectric point (pH 5.5). The trapping efficiencies of the REV method were about 5–15% (PBS, pH 7.4 or CBS, pH 4.0) or about 10–20% (HBS, pH 7.5) for insulin and FITC-insulin, respectively. However, it is expected that remote loading of peptides has various advantages, such as improved drug loading and prevention of drug inactivation. We selected the acetate gradient methods and pH gradient method for remote loading of FITC-insulin and insulin, because insulin is an amphoteric molecule and its charge is changed according to pH of solution. In the case of the inner phase of DNa loading, calcium acetate was better than sodium acetate. In neutral solutions of the inner phase of liposomes, insulin is mostly polymerized as a zinc-containing hexamer, eventually leading to the precipitation of higher order aggregates. High retention of insulin in liposomes by intraliposomal polymerization is expected. On the base of these reasons, we selected calcium acetate and zinc acetate as internal aqueous phase of acetate gradient methods. Acidic solution (pH 2.1) was selected for external phase of liposomes, because of the high solubility of insulin in it. However, loading of insulin and FITC-insulin into liposomes was not possible via the calcium or zinc acetate gradient methods. For the acetate gradient method, both insulin (isoelectric point, $pI = 5.5$) and DPPC may be positively charged and repulsed in the external phase.

On the other hand, the pH gradient method was examined (inside pH 4.0, outside pH 7.5), which was effective for remote loading of basic molecule into liposomes. For this method the interaction of insulin to liposomal membrane was examined by measuring the release of the calcein from liposomes into insulin solution. Calcein leakage induced by insulin from calcein entrapped liposomes was dependent on the molar ratio of insulin to DPPC in the media in the pH gradient method (Fig. 6A and B). There was no leakage of calcein induced by insulin when the internal and external aqueous phase was HBS, i.e. without a pH gradient. As a result, we expected that the liposomes can associate with insulin by this method.

The pH gradient method had a trapping efficiency for FITC-insulin of about 5–50% (Fig. 5A). Insulin loading was not observed. Differences in trapping efficiency between insulin and FITC-insulin using the pH gradient method may

be due to differences in aggregation or hydrophobicity between insulin and FITC-insulin. In neutral pH solutions, insulin is mostly polymerized. FITC-insulin contains from 1.2 to 1.8 bound fluorescein groups per mole of insulin (Tietze et al., 1962). The primary site of binding of FITC appears to involve the terminal amino group of phenylalanyl residue in B chain of insulin. Substitution of the terminal amino group of phenylalanyl residue altered the structure, perhaps mediated by disruption of intermolecular forces (Bromer et al., 1967). Their sedimentation studies showed that zinc insulin was highly aggregated, zinc-free insulin was less aggregated, and mono-FITC-insulin was probably in monomer form. In general, neutral forms of transported compounds are considerably more permeable or associable with liposomes than charged forms. FITC-insulin may enter or bind on the surface of preformed liposomes by the pH gradient method because of the hydrophobic character of the largely planar fluorescein. Insulin has 42% α-helix (at pH 7.0) (Pocker and Biswas, 1980) that shows a high affinity for liposomes, but it could not be sufficient for insulin to penetrate liposomes. It is suggested that the introduction of FITC moiety to the protein prevents aggregation of insulin or increase of hydrophobicity, resulting in loading of FITC-insulin into liposomes.

5. Conclusions

The present work has demonstrated remote loading of weak acid molecules into preformed liposomes, and described optimal conditions for the calcium acetate gradient method. Our results suggest that the pH gradient method may be useful for preparation of liposomal peptide using the basic group and hydrophobic group of peptide, although further study is required.

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