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Encapsulation efficiency of water-soluble and insoluble drugs in liposomes prepared by the microencapsulation vesicle method

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Abstract

The microencapsulation vesicle (MCV) method is a liposome preparation technique that reproducibly produces liposomes with homogeneous particle sizes with a high encapsulation efficiency. Liposomes encapsulating water-soluble drugs, lipophilic drugs and an amphiphilic drug were prepared by the MCV method and the encapsulation efficiency of the drugs was examined. Three kinds of egg yolk lecithin with different iodine values, i.e., purified egg yolk lecithin (PEL), partially hydrogenated purified egg yolk lecithin (R-20) and completely hydrogenated purified egg yolk lecithin (R-5), were used for membrane materials in order to explore the possible effects of membrane rigidity or surface area on the encapsulation efficiency of the drug. Water-soluble 5-fluorouracil showed 12–15% encapsulation efficiency, which was higher than those reported in the literature (less than 10%). With the MCV method, theoretically the initial drug-containing water phase was always separated from the dispersion medium by the lecithin-containing oil phase, which was advantageous to maintaining a higher encapsulation efficiency of the water-soluble drug. The encapsulation efficiency of lipophilic ibuprofen and flurbiprofen was around 90%. As for ketoprofen and liposomes were not formed when using hydrogenated egg yolk lecithin R-5, while the encapsulation efficiency using PEL or R-20 was around 80%. Amphiphilic amitriptyline hydrochloride resulted in a slightly higher encapsulation efficiency when dissolved in the water than the chloroform. Among the three kinds of lecithin, the most unsaturated PEL tended to show a higher encapsulation efficiency, probably due to differences in the packing geometry of the hydrophobic carbon chains in the membrane bilayer. The encapsulation efficiency of these drugs strongly correlated to the $\log P_{\text{octanol/water}}$ and also tended to correlate to the $\log P_{\text{chloroform/water}}$ for the order of the $\log P_{\text{chloroform/water}}$ was almost the same as the order of the $\log P_{\text{octanol/water}}$ in the drugs examined. As far as the results of this study, the $\log P_{\text{octanol/water}}$ was considered to be a better indicator of the encapsulation efficiency of a drug in the MCV method.

Keywords: Liposome; Encapsulation efficiency; Partition coefficient; Hydrogenated egg yolk lecithin

1. Introduction

Liposomes are used for effective medication by enclosing an aqueous solution with a membrane of

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phospholipids. Encapsulating a sufficient amount of the therapeutic agent is one of the most desirable properties for their usage (Mainardes and Silva, 2004; Heurtault et al., 2003; Sharma and Sharma, 1997). The liposomes encapsulate a hydrophilic drug within an aqueous component, while liposomes also entrap the lipophillic drug within the lipid bilayer (Barenholz, 2003). Factors affecting the encapsulation efficiency of the drug in the liposomes are various and come from the properties of both the liposomes and encapsulated drugs. Concerning the encapsulated drugs, the encapsulation efficiency is affected by hydrophilic or lipophilic properties and tended to interact with the membrane bilayer (Barenholz, 2003; Kulkarni et al., 1995). As for the liposome properties, aqueous volume, membrane rigidity, surface area (Kulkarni et al., 1995) and preparation methods (Kirby and Gregoriadis, 1984) are reported to have influenced the encapsulation efficiency.

The microencapsulation vesicle (MCV) method is a preparation technique in which liposomes are formed through a two-step emulsification and dispersion with mechanical agitation (Ishii et al., 1988). We have previously reported that the technique reproducibly produced liposomes with homogeneous particle sizes, a high encapsulation efficiency and good stability in preparations with purified egg yolk lecithin and its hydrogenated derivatives (Ishii et al., 1988; Ishii, 1993; Nii et al., 2003). The MCV method is expected to have advantages in preparing drug-loaded liposomes, for a drug can theoretically be encapsulated easily if it dissolves either in a water phase or an oil phase. A drug solution, regardless of the water phase or the oil phase, forms a w/o emulsion first, which then forms a w/o/w emulsion and finally the drug-loaded liposomes are generated. The encapsulation efficiency of drugs with different solubility profiles in the liposomes when applying the MCV method is of interest.

In this study, water-soluble drugs, one being a hydrophilic compound and the other a salt form of an ionizable compound, drugs-soluble in the oil phase and an amphiphilic drug were studied to examine the encapsulation efficiency of the liposomes prepared by the MCV method. Three kinds of purified egg yolk lecithin with different degrees of saturation in their acyl hydrocarbon chains were used for membrane materials in order to explore the possible effects of membrane rigidity or surface area on the encapsulation efficiency of the drug.

2. Materials and methods

2.1. Materials

Purified egg yolk lecithin (PEL, iodine value, IV = 75), hydrogenated purified egg yolk lecithin with two different iodine values, i.e., R-20 (IV = 20) and R-5 (IV = 2) were kindly provided by Asahi Chemical Industries Co. Ltd. (Tokyo, Japan). The compositions of the acyl hydrocarbon chains for these lecithins were provided by Asahi Chemicals Industries Co. Ltd. (unpublished in-house analysis data) and are shown in Table 1. Chloroform, ethanol, methanol, potassium dihydrogen phosphate (Nacalai Tesque Inc., Kyoto, Japan) and acetonitrile (Kanto Chemical Co. Inc., Tokyo, Japan) were of the highest grade. Diclofenac sodium, flurbiprofen, ketoprofen, ibuprofen, amitriptyline hydrochloride (Wako Pure Chemical Industries Ltd. Osaka, Japan) and 5-fluorouracil (Sigma–Aldrich, St. Louis, MO, USA) were used as the model drugs to be loaded into liposomes.

Table 1
Composition of acyl hydrocarbon chains in egg yolk lecithin[a]

	PEL	R-20	R-5
C-16			
16:0	30.4	29.4	29.3
16:1	1.5	0.0	0.0
C-18			
18:0	15.2	35.8	55.8
18:1	27.7	20.0	0.0
18:2	15.5	0.0	0.0
18:3	0.0	0.0	0.0
C-20			
20:0	0.0	3.2	7.7
20:1	0.0	3.6	0.0
20:2	0.0	0.6	0.0
20:4	5.5	0.0	0.0
C-22			
22:0	0.0	2.9	7.0
22:1	0.0	3.0	0.0
22:2	0.0	1.2	0.0
22:5	0.0	0.0	0.0
22:6	4.1	0.0	0.0
Others	0.1	0.2	0.2
Saturated	45.6	71.3	99.8
Unsaturated	54.4	28.6	0.2

Data represent relative amounts (%).
[a] In-house analysis data provided by Asahi Chemicals Industries Co. Ltd.

2.2. Preparation of drug-loaded liposomes

The liposome suspensions in which the model drug was loaded were prepared according to the MCV method (Ishii et al., 1988). Briefly, the phospholipids were dissolved into an organic solvent and mixed with a solution to form a w/o emulsion. The mixture was then added to another solution to form a w/o/w emulsion. The organic solvent was evaporated during the second agitation and a liposomal suspension was generated.

In this experiment, chloroform was used for the organic solvent phase and distilled water was used for the water phase. Each test drug was dissolved either in chloroform or water. Concentrations of the drug solutions used in this study were as follows: 5-fluorouracil; 1.0 mg/mL (7.7 mM) in water, diclofenac sodium; 1.2 mg/mL (3.8 mM) in water, flurbiprofen; 12.2 mg/mL (50 mM) in chloroform, ibuprofen; 10.3 mg/mL (50 mM) in chloroform, ketoprofen; 12.7 mg/mL (50 mM), amitriptyline hydrochloride; 31.4 mg/mL (100 mM) in water or 15.7 mg/mL in chloroform (50 mM).

PEL, R-20 or R-5 (1.05 mM) was dissolved in 10 mL of chloroform and then, 5 mL of water was added to the lecithin solution. In the experiments using 5-fluorouracil and diclofenac sodium, 5 mL of water was substituted with 5 mL of a water solution of each drug. In the experiments using flurbiprofen, ibuprofen and ketoprofen, 10 mL of chloroform was substituted with 10 mL of a chloroform solution of each drug. As for amitriptyline hydrochloride, either 10 mL of a chloroform solution or 5 mL of a water solution of the drug was substituted to compare the encapsulation efficiency of both preparation methods. The mixture was emulsified with a sealed-type homogenizer (type DX-T, Nihonseiki Co. Ltd., Tokyo, Japan) at 7000 rpm for 10 min to form 15 mL of w/o-type emulsion (first emulsification). The product was then immediately added to 10 times the volume of water (150 mL) in a spherical reaction flask kept at 45 °C under agitation with a chemistirrer (type B-100, Tokyo Rikakikai Co. Ltd., Tokyo, Japan) at 520 rpm to form a w/o/w type complex emulsion (second emulsification). Stirring was continued for 120 min to remove the chloroform from the suspension by evaporation and finally to generate a drug-loaded liposome suspension. A flow chart of the liposome preparation is shown in Fig. 1.

2.3. Measurement of drugs encapsulated in liposomes

The encapsulation efficiency was calculated according to a method as we reported previously (Ishii and Nagasaka, 2001).

$$\text{Encapsulation efficiency (\%)} = \frac{C_{\text{total}} - C_{\text{out}}}{C_{\text{total}}} \times 100$$

where C_{out} is the liposome suspension diluted with water and ultrafiltered through a Millipore filter (type UFP1 THK BK; Nihon Millipore Kogyo Co. Ltd.) to remove the liposomes and C_{total} is the liposome suspension diluted with heated ethanol (70 °C) in order to disrupt the liposomes completely and release the encapsulated drugs to the solvent. The ethanol solution was cooled and ultrafiltered through a Millipore filter.

Concentrations of the drug in the filtrate of C_{out} and C_{total} were quantitatively analyzed using HPLC with UV detection (Shimadzu LC-6A liquid chromatography, SPD-6A UV detector and C-R6A chromatopac). The mobile phases used in this study were as follows: 5-fluorouracil; water/methanol (97:3, v/v), diclofenac sodium, flurbiprofen, ibuprofen and ketoprofen; methanol/water/acetic anhydride (70:30:0.1, v/v/v), amitriptyline hydrochloride; 0.025 M potassium dihydrogen phosphate/acetonitrile/water (50:45:5, v/v/v).

2.4. Partition coefficient of test drugs between chloroform and water

The partition between chloroform and water was estimated with the shake-flask method. Diclofenac sodium (4 mg/1 mL), 5-fluorouracil (10 mg/2 mL) and amitriptyline hydrochloride (30 mg/1 mL) were dissolved in water and were put into a 30 mL test tube with a stopper. Chloroform was then added to each test tube to a total of 10 mL, i.e., 9 mL for diclofenac sodium and amitriptyline hydrochloride and 8 mL for 5-fluorouracil. Flurbiprofen (62.5 mg/0.5 mL) and ketoprofen (250 mg/0.5 mL) were dissolved in chloroform and put into a 30 mL test tube with a stopper and then, 9.5 mL of water was added to a total of 10 mL. Test tubes were sealed and gently shaken by hand for 1 min. Solutions were then placed in a reciprocating shaker and mechanically shaken for 2 h at 180 rpm. The solutions were left for 4 h for separation of the two phases. For each drug, concentrations in chloroform and water

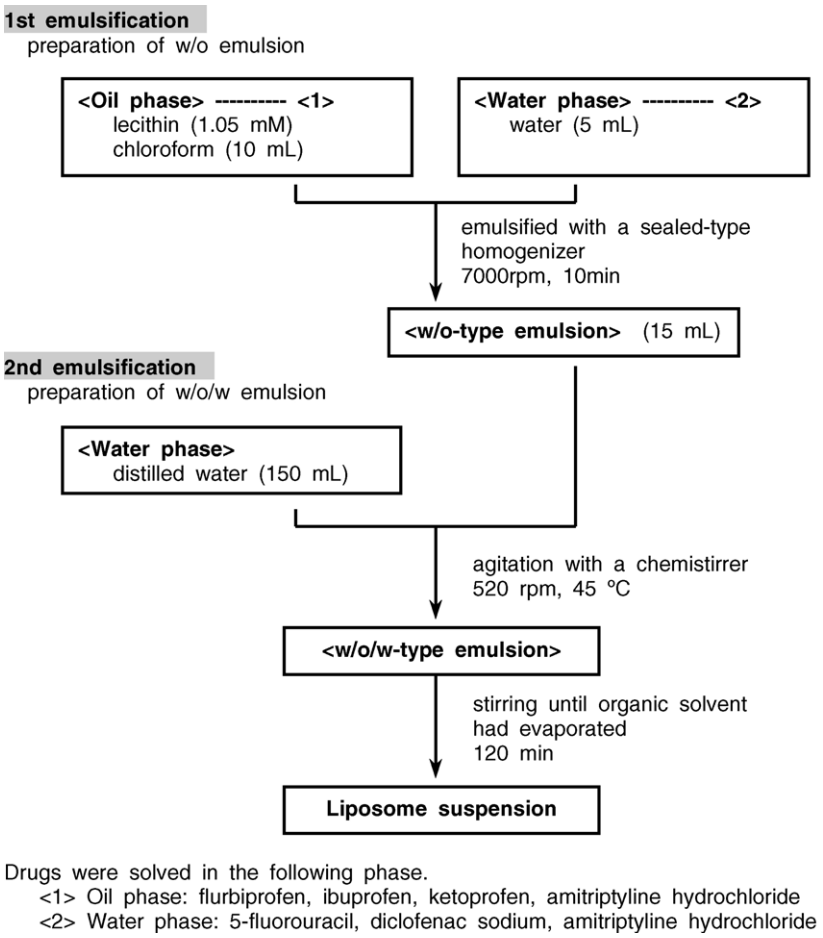

Fig. 1. Flow chart showing the preparation of drug-loaded liposomes using the MCV method.

were spectrophotometrically determined. Partition coefficient of each drug between chloroform and water was calculated according to the equation to calculate $\log P$, i.e., $\log \text{Partition}_{\text{chloroform/water}} = \log$ (drug concentration in chloroform/drug concentration in water).

2.5. Statistics

The encapsulation efficiencies of each test drug among PEL, R-20 and R-5 were analyzed by one-factorial ANOVA followed by the Scheffe's *F*-test. The encapsulation efficiency of amitriptyline hydrochloride prepared by the two different methods was analyzed with a *t*-test following the *F*-test. The correlation between the mean diameters and the estimated partition coefficient of the compounds was analyzed with Spearman's correlation coefficient using the rank test.

3. Results and discussion

3.1. Drugs dissolved in the water phase

The encapsulation efficiency of 5-fluorouracil and diclofenac sodium in liposomes using each lecithin is presented in Fig. 2. With 5-fluorouracil, the encapsulation efficiencies of those prepared with PEL, R-20 and R-5 were 11.6, 14.8 and 18.4%, respectively. Although there was no significant difference, the encapsulation efficiency tended to increase as the degree of

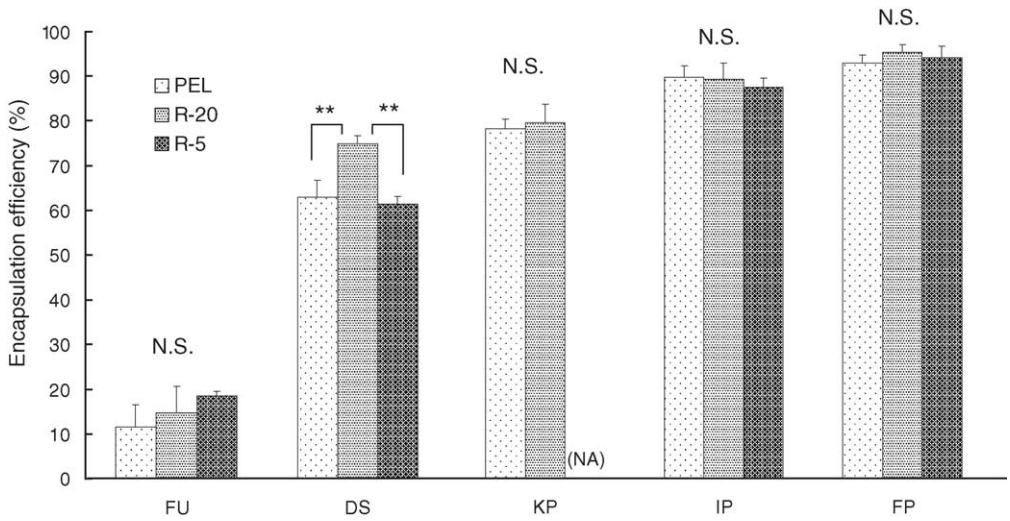

Fig. 2. Encapsulation efficiency of various drugs in liposomes prepared with egg yolk lecithin (mean ± S.D., $n = 3$), FU: 5-fluorouracil, DS: diclofenac sodium, KP: ketoprofen, IP: ibuprofen and FP: flurbiprofen. $^{**}p < 0.01$, N.S.: not significant and Scheffe's F-test.

saturation of the lecithin used for the liposome membrane increased. 5-Fluorouracil has frequently been used as a model of a water-soluble drug for liposomal loading. Its encapsulation efficiency in phospholipid liposomes was reported to be less than 10%, regardless of the types of liposomes, e.g., MLVs, SUVs or LUVs (Fresta et al., 1993; Elorza et al., 1993; Sasaki et al., 1987) or membrane rigidity (El Maghraby et al., 2001). 5-Fluorouracil was trapped in encapsulated water. With the MCV method, the drug solution firstly formed the w/o emulsion and then the emulsion was dispersed in water to form the w/o/w emulsion. Therefore, theoretically, the initial drug-containing water phase was always separated from the outer water phase (i.e., dispersion medium) by the lecithin-containing oil phase, which was advantageous to the maintenance of a higher encapsulation efficiency of the water-soluble drug compared with the other solvent dispersion preparation methods such as the reverse-phase evaporation vesicle (REV) method (Szoka and Papahadjopoulos, 1978). Later, however, when the organic solvent in the oil phase was evaporated and the lecithin formed liposome membranes, the encapsulated drug leaked out by diffusion through the lecithin bilayer. Membrane fluidity was one of the major factors affecting the encapsulation efficiency of the drug (Kulkarni et al., 1995). Unsaturated phospholipids increased the fluidity of the membrane, which were expected to facilitate the leakage of trapped drugs (Oku, 1994). The encapsulation efficiency of 5-fluorouracil tended to decrease as the degree of saturation of the lecithin used for liposome membrane decreased.

In diclofenac sodium, the encapsulation efficiencies of those prepared with PEL, R-20 and R-5 were 62.9, 74.8 and 61.3%, respectively. It was reported that the encapsulation efficiency of diclofenac sodium was influenced by the partition of the drug between the membrane lipids and the water (Balon et al., 1999). It might have been one of possible causes of the different encapsulation efficiencies among the three kinds of lecithin observed in this study.

3.2. Drugs dissolved in the oil phase

The encapsulation efficiencies of ketoprofen, ibuprofen and flurbiprofen in liposomes using each lecithin are also presented in Fig. 2. Ibuprofen and flurbiprofen were highly encapsulated with the three kinds of lecithin. The encapsulation efficiency prepared with PEL, R-20 and R-5 were 89.6, 89.3 and 87.5% in ibuprofen and 92.8, 95.2 and 94.1% in flurbiprofen, respectively. There was no significant difference among these three kinds of lecithin in both drugs. As for ketoprofen, liposomes were not formed when using R-5. The exact reason remains to be elucidated; however, it might have been that a thick bubbling covered

the mixture during the second emulsification. It was thought that the bubbling slowed the evaporation of the organic solvent and consequently obstructed the formation of the liposomes from the w/o/w-type emulsion during the second emulsification. With PEL and R-20, some bubbling was also observed but the liposome formation was not affected. The encapsulation efficiencies of those prepared with PEL and R-20 were 78.0 and 79.4%, respectively, and there was no significant difference between them.

The encapsulation efficiency was very high in all the above water-insoluble drugs, for the water-insoluble drugs were trapped in a hydrophobic region between the double layers of the phospholipids (Kulkarni et al., 1995). Unlike the water-soluble drugs, these drugs did not leak due to diffusion and thus, a high encapsulation efficiency was maintained with all the three kinds of lecithin, regardless of the differences in the degrees of saturation.

3.3. An amphiphilic drug dissolved in the water phase or oil phase

The encapsulation efficiency of amitriptyline hydrochloride, as a model of an amphiphilic drug, dissolved in either water or chloroform is shown in Fig. 3. The encapsulation efficiencies of those prepared with PEL, R-20 and R-5 were 26.9, 20.0 and 19.5%, respectively, when dissolved in water, while they were 24.1, 18.3 and 19.3%, respectively, when dissolved in chloroform. With the three kinds of lecithin, the encapsulation efficiency was comparable and the difference was not significant between the different preparatory methods. The results suggested that the encapsulation efficiency of amitriptyline hydrochloride was mainly determined by the affinity of the drug for the used for the liposome membrane. In all cases using the three kinds of lecithin, the encapsulation efficiency was slightly higher when dissolved in water. This tendency was relatively clear in PEL while there was no substantial difference in R-5. The observation suggested that the partition coefficient of the drug between the lecithin and the water was also a factor that affected the encapsulation efficiency. If the drug was dissolved in water, this initial water phase was theoretically separated from the outer water phase as described above. If the drug was dissolved in the oil phase, in contrast, the drug could diffuse through both the inner and outer water phase from the liposome membrane. The fluidity of the membrane bilayer was thought to be another factor that controlled the diffusion of the drug through the membrane. In this context, dissolving amphiphilic drugs in the water phase was more advantageous to achieve a higher encapsulation efficiency when the liposome was prepared with the MCV method; in particular, using an unsaturated lecithin such as PEL. Among the three kinds of lecithin, the most unsaturated PEL tended to show a higher encapsulation efficiency compared with

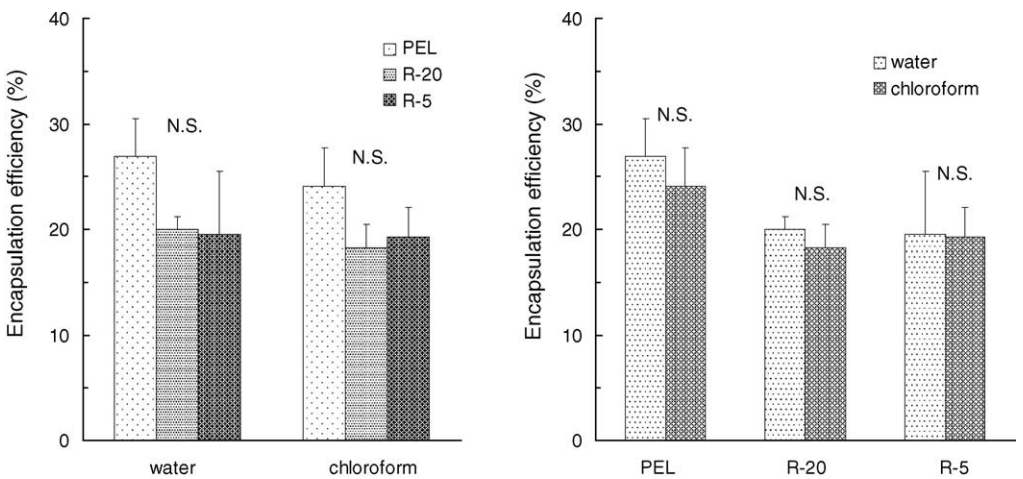

Fig. 3. Encapsulation efficiency of amitriptyline hydrochloride in liposomes when dissolved in water or chloroform (mean ± S.D., $n = 3$), N.S.: not significant and Scheffe's F-test.

hydrogenated R-20 or R-5, though the difference was not significant. The result was probably due to differences in the packing geometry of the hydrophobic carbon chains among these three kinds of lecithin. It was reported that amphiphilic drugs perturbed the acyl hydrocarbon chains of the phospholipid bilayer. A rigid gel state was more resistant to the effect of amphiphilic drugs, while a fluid liquid crystal state allowed for the amphiphilic drug molecules to be embedded into bilayers and thereby disrupted their structure (Elorza et al., 1997). As shown in Table 1, PEL contained 54.4% unsaturated acyl hydrocarbon chains that were bent at unsaturated bonds and became flexible with a high fluidity in a bilayer. In contrast, the cylinder-shaped saturated acyl hydrocarbon chains were dominant in R-20 and particularly R-5, which formed a dense and rigid bilayer. Consequently, the packing of the PEL bilayer was considered sparse and fluid compared with the bilayer of R-20 or R-5, which could embed a larger amount of amitriptyline hydrochloride with in the bilayer structure.

3.4. Encapsulation efficiency and partition coefficient of the drugs

As the liposome was used as a biomembrane model, it was reported that the encapsulation efficiency of drugs correlated to the partition coefficient between 1-octanol and water (Smith et al., 1975). Fig. 4 plots the encapsulation efficiency to $\log P_{\text{octanol/water}}$. The $\log P_{\text{octanol/water}}$ of 5-fluorouracil (−0.78), flurbiprofen (4.11), ibuprofen (3.72) and ketoprofen (2.81) were cited from SciFinder Scholar in which the value was calculated using Advanced Chemistry Development (ACD) Software Solaris V 4.67. The $\log P_{\text{octanol/water}}$ of diclofenac sodium (0.70) was cited from the literature (Balon et al., 1999). Amitriptyline hydrochloride was not plotted, as we were not able to find published $\log P$ data. The encapsulation efficiency of the five drugs strongly correlated to the $\log P_{\text{octanol/water}}$ (rs = 0.97). It was reported that the liposomes favorably encapsulated drugs with either a low or high $\log P_{\text{octanol/water}}$ but could not hold drugs having an intermediate $\log P_{\text{octanol/water}}$ that was around 2–3 (Defrise-Quertain et al., 1984). In the current study, ketoprofen fell under this category, but the encapsulation efficiency was rather high and similar to ibuprofen and flurbiprofen. Other model drugs in this range of $\log P_{\text{octanol/water}}$ would be tested to explore the further relationship between the $\log P_{\text{octanol/water}}$ and the drug encapsulation efficiency.

With the MCV method, the liposomes were formed from a w/o/w emulsion. Accordingly, it was anticipated that the encapsulation efficiency of a drug might have some correlation to the water and the chloroform, the solvent for the oil phase. Therefore, a possible relationship between the encapsulation efficiency and the partition coefficient of the drugs between the water and the chloroform was also examined. The partition coefficient of the drugs was determined by experiment. As we

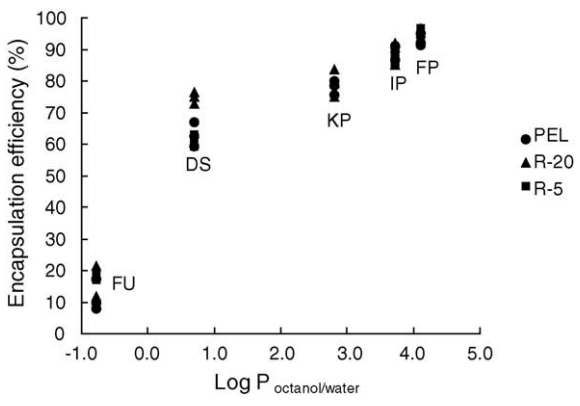

Fig. 4. Encapsulation efficiency and partition coefficient of drug between 1-octanol and water, FU: 5-fluorouracil, DS: diclofenac sodium, KP: ketoprofen, IP: ibuprofen and FP: flurbiprofen.

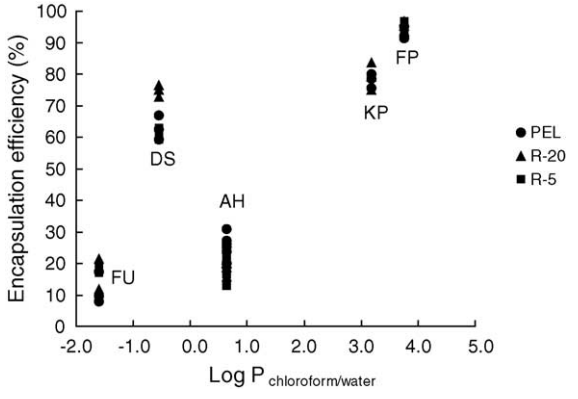

Fig. 5. Encapsulation efficiency and partition coefficient of drug between chloroform and water, FU: 5-fluorouracil, DS: diclofenac sodium, AH: amitriptyline hydrochloride, KP: ketoprofen and FP: flurbiprofen.

were not able to determine the concentration of ibuprofen in the water phase, the remaining five drugs were used for plotting. The results are shown in Fig. 5. There was still a tendency to increase the encapsulation efficiency as the $\log P_{\text{chloroform/water}}$ increased (rs = 0.70). The result was probably because that the order of the $\log P_{\text{chloroform/water}}$ was almost the same as the order of the $\log P_{\text{octanol/water}}$ in these drugs. As far as the results of this study, the $\log P_{\text{octanol/water}}$ was considered to be a better indicator of the encapsulation efficiency of a drug in the MCV method.

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