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Physicochemical specification of drug carrying liposomes for the quality control in the industrial production

Sadao Hirota *

Visiting Professor, School of Pharmacy, University of California, Part-time Lecturer, Department of Material Science and Engineering, Tokyo Denki University, Japan

Abstract

Animal data of liposomal drugs in a laboratory environment are often not reproduced on industrial scale products. One of the most important reasons is in the fact that the colloid-chemical properties of the liposomes manufactured on a small scale are not reproduced in a large scale production. In this review, colloid chemical characterization of liposomes is introduced, fixed aqueous layer thickness (FALT) as an example, and its importance in a quality control of a liposomal product in an industrial scale production is shown. Methoxy-polyethylenglycol-diacylgricerol (PEG-DAG) with varying PEG chain length and acyl chains were synthesized, FALT of liposomes coated with PEG-DAG determined and tissue distribution in tumor bearing mice studied. The higher incorporation ratio of PEG-DAG into liposomal membrane was observed with PEG-DAG with short acyl chains (myristoyl) and a small PEG molecular weight (1000). The easier to be incorporated, the easier to be stripped in the serum. The disposition data in the mice well reflected the colliod chemical and in vitro data of the PEG liposomes. Galactosyl-carbonyl-propionyl-polyethyleneglycol-diacylglycerol (Gal-PEG-DAG) with oxyethylene number, $n = 10$, 20 and 40 were synthesized. The exposure of galactosy residue beyond the fixed aqueous layer of liposomes coated with Gal-PEG-DAG was monitored by RCA lectin induced agglutination, half life in the blood and organ distribution after i.v. injection into rats determined and intrahepatic distribution studied. Only the liposomes containing Gal-PEG_{10}DAG aggregated with the lectin, indicating that only with this derivative the galactose group was adequately exposed. The Gal-PEG_{10}-DAG liposomes were cleared from plasma with a half life of 0.3 h. The plasma elimination could be attributed entirely to increased uptake by the liver. The increased liver uptake was almost entirely attributed to increased uptake by the non-parenchymal cell. Incorporation of PEG-DSPE in the Gal-PEG_{10}DAG liposomes caused (1) a 3-fold increase in blood circulation time, (2) a small but significant decrease in hepatic uptake after 20 h and (3) a significant shift in intrahepatic distribution in favor of the hepatocytes, comparable to that of the control liposomes. In conclusion, therapeutic efficacy and safety of liposomes can be controlled by their colloid chemical, more exactly, surface chemical properties. By setting up reasonable quality control specifications of the properties in the laboratory and examining the specifications satisfied in upscaling, the efficacy and safety are reproduced in a large scale product.

Keywords: Physicochemical; Liposomes; Industrial production

* Present address: 6-6-18 Higashikaigan Minami, Chigasaki 253, Japan.

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1. Introduction

Efficacy and safety data of liposomal drugs in a laboratory environment are often not reproduced on an industrial production scale. This is largely due to the fact that the physico-chemical properties of the liposomes manufactured on a small scale are not reproduced in large scale production. The size and the electric charge of liposomes are measured and are adequately specified in relation to the bio-distributions in most developments of liposomes (Hirota et al., 1996). Uniformity of lipid components, exposure of bio-chemically important functional groups on the outer surface of liposomes (Yoshioka et al., 1993), fixed aqueous layer thickness (FALT), number of the lipid bilayers, etc. are also dependent upon the scale of production. Nevertheless, these properties are not always exactly specified. Uniformity, especially of the functional groups on the membrane surface, can be assessed chemically or bio-chemically with fractionated samples and FALT can be easily determined through electro-chemical means (Shimada et al., 1995). According to Gouy-Chapman's theory, the logarithm of ζ potential, ζ, is related to Debye-Hückel parameter, κ, by the following equation

$$\ln \zeta = A - L\kappa \quad (1)$$

where A is a constant related to the surface charge density and L is the FALT. The thickness of the diffuse electrical double layer is $1/\kappa$. When the electrolyte in the aqueous phase is univalent such as NaCl, κ can be expressed in 1/nm unit as

$$\kappa = 3.3\sqrt{C} \quad (2)$$

where C is the molarity of the electrolyte in the outer aqueous phase. When nonionic PEG lipid is employed, L is not affected by the environmental electrolyte concentration and can be regarded as constant. Changing the electrolyte concentration C, so that κ, the ζ is determined, then $\ln \zeta$ is plotted against κ and a straight line is obtained. The slope gives L, FALT in nm unit.

PEG derivatives of phosphatidylethanolamine (PEG-PE) have been widely used as a lipid component to prepare sterically stabilized liposomes and have been extensively studied (Klivanov et al., 1990; Lasic et al., 1991). However, pharmaceutical formulation is restricted because PEG-PE is an anionic lipid as a result of which the net surface negative charge density is dependent upon the PEG amount. Even if the hidden negativity of the particle is proved to be related to dysopsonization, the optimal negativity will not be formulated independently from FALT. When cationic liposome is used as a gene-carrier, PEG-PE will be preventing the incorporation of the gene in or around the liposome. Nonionic PEG lipids are appropriate in these cases for coating liposomes.

Methoxy polyethyleneglycol diacylglycerol (PEG-DAG) were synthesized using different PEG molecular weights of 1000, 2000 and 5000 and using different anchor acyl groups, namely dimyristoyl ($C = 14$), distearoyl ($C = 18$) and dibehenoyl ($C = 22$) (Figs. 1 and 2).

These lipids are bio-degradable and easily produced on an industrial scale. The FALT around liposomes with and without adriamycin (ADR) coated with PEG2000-DMG was determined to be 2–3 nm (Table 1, Fig. 2a,b).

An attempt was made to compare more in detail the effects of acyl- and PEG-chain length on the incorporation of the PEG-lipids into the lipid bilayers of liposomes during the liposome preparations and the stripping of the PEG-lipids from the lipid bilayer in the fetal bovine serum. For this purpose, PEG on the surface of liposomes and in the outer aqueous phase (as micellar solution) were determined colorimetrically by picrates methods. Briefly, 10 ml of sodium nitrate-

Fig. 1. Chemical structure of methoxy-polyethyleneglycol diacylglyceride (PEG-DAG).

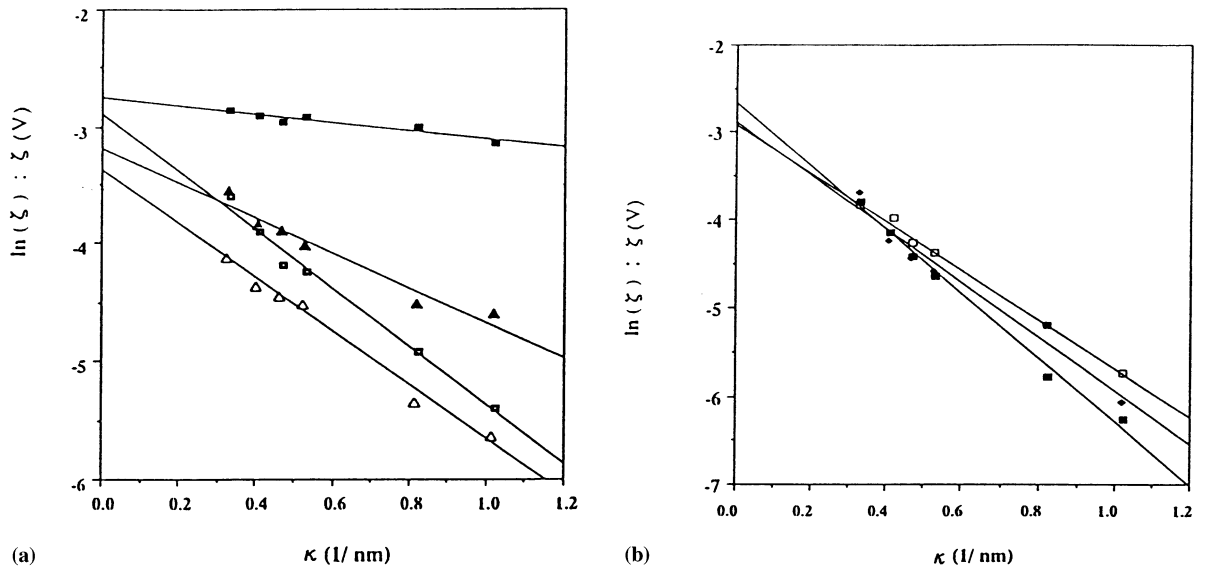


Fig. 2. (a) Plots of natural logarithm of ζ potential versus Debye Hückel parameter for PEG-DAG-coated liposomes containing ADR.

Changing the electrolyte concentration C, so that κ, the ζ is determined, then $\ln \zeta$ is plotted against κ and a straight line is obtained. The slope gives L, FALT in nm unit. (b) Plots of natural logarithm of ζ potential versus Debye Hückel parameter for PEG-DAG-coated liposomes without ADR. Changing the electrolyte concentration C, so that κ, the ζ is determined, then $\ln \zeta$ is plotted against κ and a straight line is obtained. The slope gives L, FALT in nm unit. ■, plain; ▲, PEG 2000-3.7; □, PEG 2000-5.4; △, PEG 2000-7.0.

picrate reagent (3.3 mM sodium nitrate and 20 mM picric acid in 0.1 M NaCl solution) was added to the sample solution containing PEG-lipids (5 ml) and allowed to stand for 1 h at room temperature. 1,2-Dichloroethane (5 ml) was added to the mixture and extracted by shaking it vigorously. After centrifugation at $15\,000 \times g$ for 10 min, the organic layer was collected and spectrophotometrically measured at 378 nm (Table 2).

Table 1
FALT around liposome coated with polyethyleneglycol dimyristoylglycerol PEG2000-DMG

Mol% of PEG2000-DMG	FALT (nm)	
	With ADR	Without ADR
0	0.31	NT
3.7	1.2	3.2
5.4	2.1	3.2
7.0	1.9	3.1

Liposomes composed of DSPC/CH/DSPG (10/10/6 molar ratio) and additional 5.4 mol% PEG2000-DAG were extruded through two stacked polycarbonate membrane filters of 0.2 and 0.1 nm pore size and applied to a Sepharose CL-4B column (ϕ 20–200 nm) with 10 mM Tris–HCl buffer (pH 7.4) containing 150 mM NaCl (method A). Both liposomally incorporated PEG2000-DAG and unincorporated PEG2000-DAG micelles were fractionated with Sepharose CL-4B and both fractions were evaporated to dryness. After dilution of each with distilled water, the amount of PEG2000-DAG both in liposomes and micelles was determined colorimetrically with picrate. Briefly, 10 ml of sodium nitrate-picrate reagent (3.3 mM sodium nitrate and 20 mM picric acid in 0.1 M NaCl solution) was added to the sample solution containing PEG-lipids (5 ml) and allowed to stand for 1 h at room temperature. 1,2-Dichloroethane (5 ml) was added to the mixture and extracted by vigorous shaking. After centrifugation at

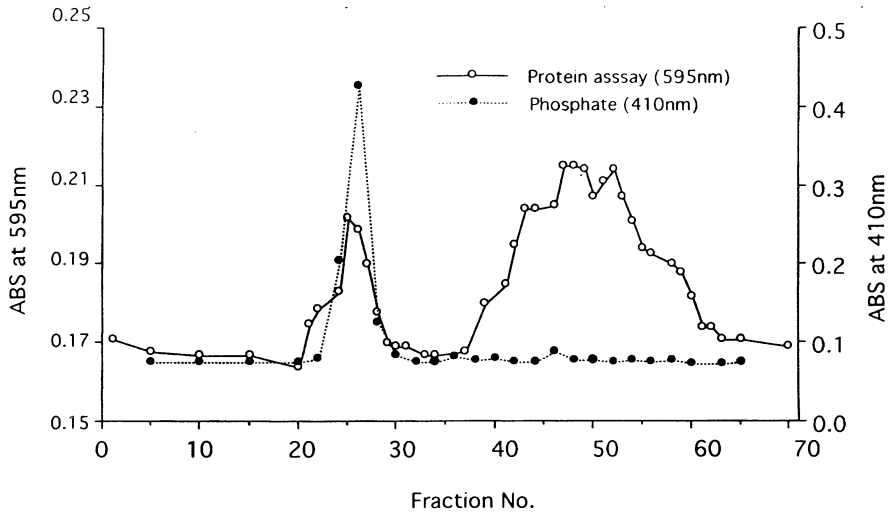


Fig. 3. Elution profile of PEG-liposome containing methoxy PEG2000-DSG. An aliquote of PEG-liposome solution was chromatographed on Sepharose CL-4B column and fractionated. PEG-lipids in each fraction were detected spectrophotometrically by Bio-Rad protein assay (for PEG2000-DSG) and phosphate assay (for dimtyristoylphosphatidylcholine (DMPC), respectively.

15 000 × *g* for 10 min, the organic layer was collected and spectrophotometrically measured at 378 nm. Based on these data, the incorporation of PEG2000-DAG in the liposomes was determined and is expressed here in terms of percentage incorporation. Data represents the mean ± S.D. ($n \geqq 3$). Initial concentration of PEG-lipid was 5.8 mol.% for liposomal lipids (Tables 2 and 3).

Incorporation ratio of PEG-lipid into liposomal membrane was determined by picrate method. Initial concentration of PEG-lipid was 5.8 mol% for liposomal lipids.

Liposomes composed of DSPC/CH/DSPG (19/10/6 molar ratio) were extruded through two stacked polycarbonate membrane filters of 0.2 and 0.1 nm pore size and co-incubated with the concentrated PEG2000-DAG micelle solution (5.4 mol% PEG2000-DAG to total lipid) followed by sonication for 1 h at 65°C. The mixture was applied to a Sepharose CL-4B column (ϕ 20–200 nm) with 10 mM Tris–HCl buffer (pH 7.4) containing 150 mM NaCl (method B). Both liposomally incorporated PEG2000-DAG and unincorporated PEG2000-DAG micelles were separately collected and evaporated to dryness. After dilution with distilled water, the amount of PEG2000-DAG both in liposomal and micellar fractions was determined colorimetrically with picrate. Based on these data, incorporation ratio of PEG-lipid into liposomal membrane relative to total PEG2000-DAG incorporation

Table 2
Effect of acyl chain length on PEG2000-DAG incorporation into liposomal membrane during liposome formation

	Incorporation (mol%)	±S.D.
PEG2000-DMG	81.8	0.96
PEG2000-DTG	53.3	1.33
PEG2000-DBG	44.5	3.23
PEG2000-DSPE	60.9	3.95

Mixing method A with PEG2000-DSPE as a positive standard.

Table 3
Effect of PEG chain length on PEG-DBG incorporation into liposomal membrane during liposome formation

	Incorporation (mol%)	±S.D.
PEG1000-DBG	79.5	4.89
PEG2000-DBG	44.5	0.48
PEG5000-DBG	22.25	1.90
PEG2000-DSPE	60.93	3.95

Mixing method A with PEG2000-DSPE as positive standard.

Table 4
Effect of acyl chain length on PEG2000-DAG incorporation into preformed liposomal membrane during liposome formation

	Incorporation (mol%)	±S.D.
PEG2000-DMG	86.5	0.11
PEG2000-DSG	18.5	1.75
PEG2000-DBG	12.86	0.76

Mixing method B with PEG2000-DSPE as a positive standard.

was determined. Initial concentration of PEG-lipid was 5.8 mol% for liposomal lipids (Table 4).

Liposome suspensions (20 mM total lipid) composed of DSPC/CH/DSPG/PEG2000-DAG were prepared either by mixing method A or B, and centrifuged a $30\,000 \times g$ for 2 h to obtain the liposomal pellet. The pellet was resuspended with an identical volume of 10 mM Tris–HCl buffer (pH 7.4) containing 150 mM NaCl. The liposome sample (3 ml) suspension and 3 ml of FBS (or 10 mM Tris–HCl buffer (pH 7.4) containing 150 mM NaCl) were co-incubated at 37°C. At the indicated times, 0.5 ml samples were taken from the mixture and the PEG2000-DAG stripped from the liposomal membrane were isolated by centrifugation at $30\,000 \times g$ for 2 h. The amount of the PEG-lipids was determined by colorimetry of the picrates. The PEG2000-DAG remaining in the liposome was expressed as the percentage of the total PEG2000-DAG added. The total PEG2000-DAG were determined just after the addition of 10 mM Tris–HCl buffer (pH 7.4) (3 ml) to the liposome suspension (3 ml). Data represents the mean ± S.D. ($n \geqq 3$).

With PEG coated liposomes, the coverage of PEG on the membrane surface, i.e. the ratio of PEG on the lipid membrane to PEG in the outer aqueous phase, was dependent upon hydrophilic lipophilic balance, i.e. the ratio of PEG chain length to anchor alkyl chain length (Tables 5 and 6).

From the PEG incorporation experiments in Tables 2–4, it was found that:

(1) Contrary to expectation, the longer was the acyl chain, the smaller was the PEG2000-DAG incorporation into the liposomal membrane during liposome formation.

(2) As it was expected, the longer was the PEG chain length, the smaller was the PEG-DBG incorporation into the liposomal bilayer.

From the PEG stripping experiments with FBS in Tables 5 and 6, it was found that:

(3) As it was expected, the longer the acyl chain length, the smaller the stripping and the longer the PEG chain length, the larger the stripping,

(4) PEG2000-DSG and PEG2000-DSPE showed similar incorporation and stripping behaviors.

It was also found that FALT was dependent upon the hydrophilic lipophilic balance of the PEG lipid (data not shown). For the stable retaining of FALT of 2–3 nm which is necessary to avoid the reticuloendotherial system (RES), the most suitable PEG molecular weight was found to be 2000 and the most appropriate anchor acyl group to be distearoyl or dibehenoyl.

It is well known that a detergent with single alkyl chain and PEG chain forms spherical micelles of uniform size above critical micelle concentration at a temperature above Kraft point and the lipid with double acyl chain forms edgeless liquid crystal membrane, i.e. vesicle, above the phase transition temperature. However, the state of PEG-lipids in aqueous media is not yet clear. The wide peak of PEG-lipid, without phospho-lipid from fraction 37–60 in Fig. 3 suggests the existence of PEG-lipid disk micelles or other kinds of lipid molecular assemblies which are not uniform in size.

A correlation was observed between the bio-distributions of these PEG coated liposomes in the tumor bearing mice and the above obtained physicochemical properties of the liposomes (Tables 7–9).

Plain liposomal ADR, PEG-coated liposomal ADR and free ADR in 10 mM Tris–HCl buffer (pH 7.4) containing 150 mM NaCl were administered via the tail vein of Ehrlich ascites carcinoma bearing male CDF1 mice (5-weeks-old, 10 days after subcutaneous inoculation of 5×10^5 cells/animal in the back of the mice) at a dose of 2.5 mg ADR/kg body weight. After the injection (4–24 h), the mice were sacrificed by cervical dislocation and blood, tumor liver, or spleen samples were

Table 5
Effect of acyl chain length on stripping of PEG2000-DAG from liposomal membrane in FBS

Time (h)	PEG-DMG	PEG-DSG	PEG-DBG	PEG-DSPE	PEG-DBG mixing method B	PEG-DMG in buffer
0	100	100	100	100	100	100
1	64.5 ± 2.0	75.9 ± 1.7	77.1 ± 5.6	76.9 ± 1.3	5.7 ± 1.4	
3	62.4 ± 1.2	66.8 ± 7.1	73.2 ± 4.8	59.0 ± 1.5	9.5 ± 1.8	
8	46.2 ± 1.5	57.5 ± 2.8	63.5 ± 2.8	61.3 ± 1.3	6.6 ± 0.3	95.9 ± 3.5
24	30.8 ± 8.5	39.5 ± 1.3	45.0 ± 4.7	33.5 ± 4.8	9.1 ± 1.9	98.7 ± 1.9

taken from the animal. The amount of ADR was determined by fluorescence assay. Briefly, each blood sample was centrifuged at $1200 \times g$ for 10 min to obtain serum which was extracted with chloroform:isopropanol (1:1 v/v). Following vigorous shaking of the mixture and a brief centrifugation, the organic layer was collected. The fluorescence of the organic layer was determined (excitation wave length: 500 nm and emission wave length: 550 nm) Data represent the mean ± S.D. ($n \geqq 3$) (Tables 8, 9 and Table 10).

These findings demonstrate the necessity for accurate physico-chemical specification of liposomes for up-scaling and quality control in liposome production.

It is easy to deliver drug carrying liposomes to the Kupffer cell in a laboratory environment. However, the Kupffer cell targeting has not yet been established in an industrial environment. Industrial liposomal products are filtered through membrane to eliminate micro-organisms, but liposomes smaller than 200 nm diameter after the membrane filtration are not recognized nor caught by the Kupffer cells. Although the Kupffer cell targeting efficiency is enhanced by coating the surface with phosphatidylserine, this coating also results in the distribution of the liposomes to the macrophages of the whole body particularly to the macrophages of the spleen and the bone marrow. It was found that liposomes covered by galactose residues on their outer surface selectively accumulate to the Kupffer cells even when their size is below 200 nm.

Kupffer cell targeting without affecting the whole body immune system is now possible on an industrial scale. This technique will be useful for the therapy of hepatitis or other diseases needing liver specific immuno-suppression by incorporating an immuno-suppressor into galacto-liposomes. Liver specific immuno-activation may also be possible with an immuno-activator.

The study on galacto-liposomes was first aimed at the targeting of liposomes to the hepatocytes. Avoiding Kupffer cells by PEG coating of liposomes and exposing galactosyl residues across the PEG aqueous layer, enables the liposomes to be taken up selectively by the hepatocytes through the asialo-receptors on the surface of the hepatocytes. For the preparation of galacto-liposomes, spacer of at least $n = 7$ oxyethylene groups (the largest oxygen to oxygen distance is 0.29 nm for one oxyethylene unit) between galactosyl group and the anchor alkyl group was considered to be necessary for the exposure of galactosyl group to the membrane surface across the 2 nm FALT with PEG. Galactosyl-(oxyethylene)$_{10}$-dipalmitoylglycerol (Gal-OE$_{10}$-DPG), galactosyl (oxyethylene)$_{20}$-distearoylglycerol and galactosyl-(oxyethylene)$_{40}$-distearoylglycerol were synthesized.

Chemical structures of galactosyl-(oxyethylene)$_n$-diacylglycerol (Gal-OE$_n$-DAG).

Exposures of Gal residues on the fixed aqueous layer were examined by agglutinations of the Gal-OE$_n$-DAG coated liposomes with lectin, ricinus communis agglutinin (RCA-120) (Fig. 4a,b,c).

The most efficient n was found to be 10. Slow but steady agglutination took place with a co-existence of PEG2000-DMG.

The intravenous injections of these liposomes to the tumor bearing mice indicated that almost all the liposomes were accumulated in the liver and distribution to the spleen was minor (Tables 11 and 12).

Contrary to expectations, the increased liver uptake was induced by the nonparenchymal cells

Table 6
Effect of PEG chain length on stripping of PEG2000-DAG from liposomal membrane in FBS

Time (h)	PEG1000-DBG	PEG2000-DBG	PEG5000-DBG	PEG-DSPE
0	100	100	100	100
1	64.6 ± 4.2	77.0 ± 5.6	40.4 ± 1.9	76.9 ± 1.3
3		73.1 ± 2.8	21.5 ± 1.8	59.0 ± 1.5
8	61.3 ± 2.1	63.5 ± 2.8	18.3 ± 1.6	61.3 ± 1.3
24	33.5 ± 8.5	45.0 ± 4.7	21.0 ± 2.5	33.5 ± 4.8

of the liver, specifically by the Kupffer cells (data not shown), while the hepatocytes were not significantly involved. Incorporation of PEG2000-DSPE in the Gal-OE_{10}-DPG liposmes only partially reversed the effect of the galactolipid with respect to liver and spleen uptake as well as

Table 7
Plasma levels of ADR encapsulated in liposomes coated with PEG-DAG in tumor-bearing mice

	ADR conc. (μg/ml serum)	
	4 h	24 h
Free ADR solution in saline	ND	ND
ADR in plain liposomes DSPC/CH/DSPG = 10/10/2[a]	20.8 ± 8.9	ND
DSPC/CH/DSPG/PEG2000-DMG = 10/10/2/1.5[a]	17.2 ± 4.9	ND
DSPC/CH/DSPG/PEG2000-DSG = 10/10/2/1.5[a]	36.7 ± 8.2	4.6 ± 1.6
DSPC/CH/DSPG/PEG2000-DBG = 10/10/2/1.5[a]	39.8 ± 7.6	17.2 ± 3.7
DSPC/CH/DSPG/PEG1000-DBG = 10/10/2/1.5[a]	36.4 ± 9.0	6.1 ± 0.5
DSPC/CH/DSPG/PEG5000-DBG = 10/10/2/1.5[a]	20.2 ± 8.0	ND
DSPC/CH/DSPG/PEG2000-DSPE = 10/10/2/1.5[a]	46.8 ± 4.4	5.4 ± 1.4
DSPC/CH/DSPG/PEG2000-DBG = 10/10/2/1.5[b]	6.8 ± 2.7	ND

[a] Prepared by mixing method A.
[b] Prepared by mixing method B.

Table 8
Tumor levels of ADR encapsulated in liposomes coated with PEG-DAG in tumor-bearing mice

	ADR conc. (μg/ml serum)	
	4 h	24 h
Free ADR solution in saline	0.96 ± 0.39	1.5 ± 0.17
ADR in plain liposomes DSPC/CH/DSPG = 10/10/2[a]	2.4 ± 1.0	2.9 ± 1.0
DSPC/CH/DSPG/PEG2000-DMG = 10/10/2/1.5[a]	2.9 ± 0.3	2.8 ± 1.1
DSPC/CH/DSPG/PEG2000-DSG = 10/10/2/1.5[a]	3.1 ± 0.14	5.5 ± 1.6
DSPC/CH/DSPG/PEG2000-DBG = 10/10/2/1.5[a]	3.2 ± 0.4	4.7 ± 1.4
DSPC/CH/DSPG/PEG1000-DBG = 10/10/2/1.5[a]	3.1 ± 0.6	5.5 ± 1.7
DSPC/CH/DSPG/PEG5000-DBG = 10/10/2/1.5[a]	2.4 ± 0.8	4.2 ± 0.7
DSPC/CH/DSPG/PEG2000-DSPE = 10/10/2/1.5[a]	3.9 ± 0.15	5.9 ± 1.6
DSPC/CH/DSPG/PEG2000-DBG = 10/10/2/1.5[b]	1.6 ± 0.18	2.7 ± 0.17

[a] Prepared by mixing method A.
[b] Prepared by mixing method B.

Table 9
Liver levels of ADR encapsulated in liposomes coated with PEG-DAG in tumor-bearing mice

	ADR conc. (μg/ml serum)	
	4 h	24 h
Free ADR solution in saline	7.2 ± 0.07	4.0 ± 1.1
ADR in plain liposomes DSPC/CH/DSPG = 10/10/2[a]	11.2 ± 2.3	14.0 ± 1.0
DSPC/CH/DSPG/ PEG2000-DMG = 10/10/2/1.5[a]	12.6 ± 0.9	12.4 ± 0.4
DSPC/CH/DSPG/ PEG2000-DSG = 10/10/2/1.5[a]	5.6 ± 2.7	4.8 ± 2.0
DSPC/CH/DSPG/ PEG2000-DBG = 10/10/2/1.5[a]	3.7 ± 0.6	6.5 ± 1.5
DSPC/CH/DSPG/ PEG1000-DBG = 10/10/2/1.5[a]	6.1 ± 0.9	6.8 ± 0.9
DSPC/CH/DSPG/ PEG5000-DBG = 10/10/2/1.5[a]	11.2 ± 1.7	11.3 ± 4.1
DSPC/CH/DSPG/ PEG2000-DSPE = 10/10/2/1.5[a]	6.0 ± 1.3	6.6 ± 1.1
DSPC/CH/DSPG/ PEG2000-DBG = 10/10/2/1.5[b]	13.8 ± 2.1	12.1 ± 0.2

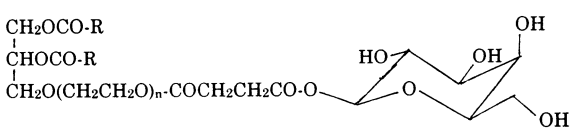
[a] Prepared by mixing method A.
[b] Prepared by mixing method B.

Table 10
Spleen levels of ADR encapsulated in liposomes coated with PEG-DAG in tumor-bearing mice

	ADR conc. (μg/ml serum)	
	4 h	24 h
Free ADR solution in saline	1.0 ± 0.3	1.2 ± 0.1
ADR in plain liposomes DSPC/CH/DSPG = 10/10/2[a]	6.5 ± 2.3	10.7 ± 4
DSPC/CH/DSPG/ PEG2000-DMG = 10/10/2/1.5[a]	6.5 ± 0.6	11.0 ± 0.6
DSPC/CH/DSPG/ PEG2000-DSG = 10/10/2/1.5[a]	2.2 ± 0.4	3.8 ± 3.6
DSPC/CH/DSPG/ PEG2000-DBG = 10/10/2/1.5[a]	1.7 ± 0.3	3.8 ± 1.0
DSPC/CH/DSPG/PEG 1000-DBG = 10/10/2/1.5[a]	2.5 ± 0.3	9.1 ± 1.2
DSPC/CH/DSPG/ PEG5000-DBG = 10/10/2/1.5[a]	6.6 ± 1.0	9.9 ± 1.5
DSPC/CH/DSPG/ PEG2000-DSPE = 10/10/2/1.5[a]	2.6 ± 0.3	6.2 ± 0.5
DSPC/CH/DSPG/ PEG2000-DBG = 10/10/2/1.5)[b]	9.0 ± 0.3	12.9 ± 0.7

[a] Prepared by mixing method A.
[b] Prepared by mixing method B.

intrahepatic distribution (Shimada et al., 1997). Although these results did not satisfy the initial intention, the results indicated clearly the existence of galactose receptors on the surface of the Kupffer cells that are stronger than the asialoreceptors of the hepatocytes. In spite of attempts to avoid Kupffer cells, i.e. size reduction and PEG coating, liposomes were accumulated in the Kupffer cells. The targeting efficiency was much larger than the plain liposomes without galactose coating. The industrial importance of this finding is enormous as stated above and this is obviously another example of the importance of physicochemical specification of drug carrying liposomes for the quality control in industrial production.



Scheme 1. Chemical structure of galactosyl(oxyethylene)$_n$-diacylglycerol (Gal-OEn-DAG).

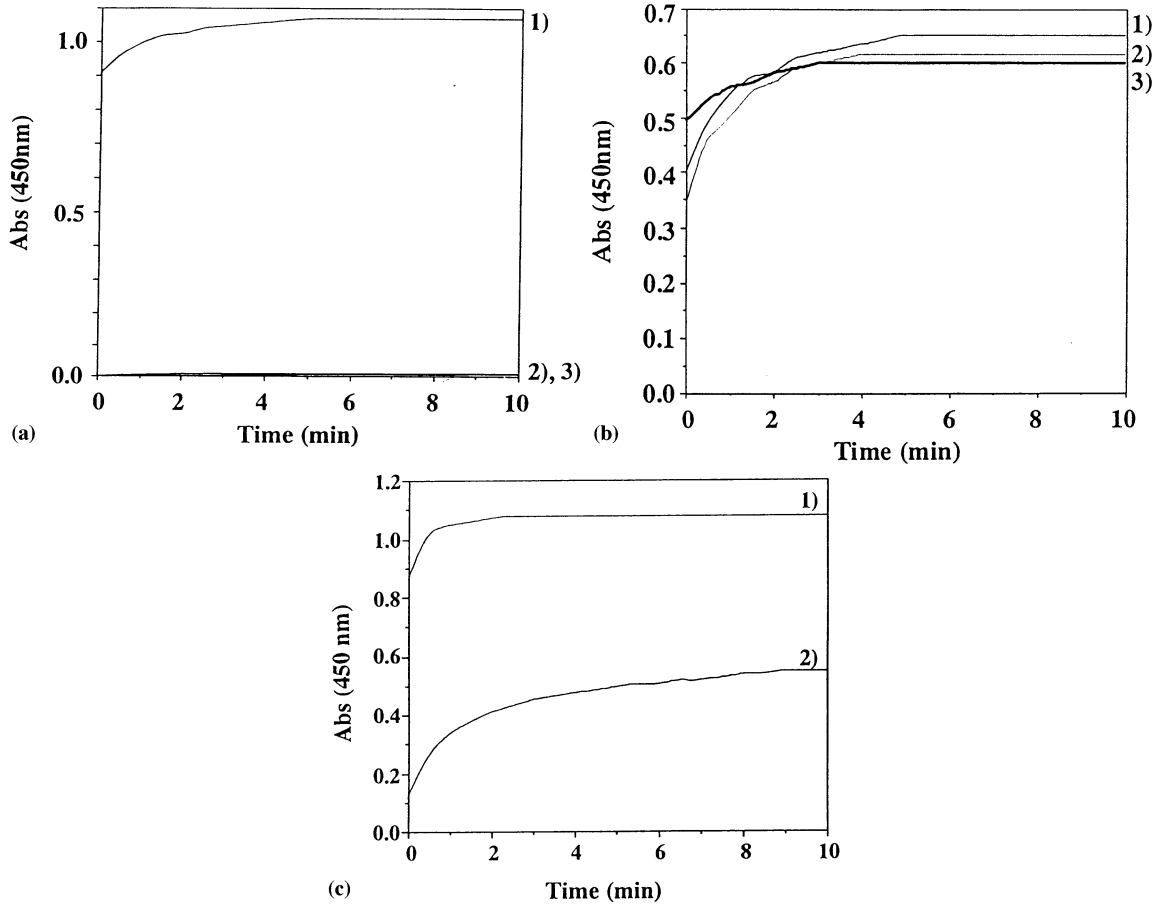


Fig. 4. (a) RCA-120-induced agglutination of Gal-OE_n-DAG coated liposomes. Liposomes (1.0 mM lipid, 2 ml) composed of egg PC and Gal-OE_n-DAG (8:2 molar ratio) were mixed at 25°C with 10 mM Tris–HCl/150 mM NaCl (pH 7.4), containing 0.5 mg/ml RCA-120 and agglutination was monitored spectrophotometrically at a wavelength of 450 nm. Trace (1) Gal-$(oxyethylene)_{10}$-dipalmitoylglycerol, trace (2) Gal-$(oxyethylene)_{20}$-distearoylglycerol and trace (3) Gal-$(oxyethylene)_{40}$-distearoylglycerol. (b) RCA-120-induced agglutination of Gal-OE_{10}-DPG coated liposomes. Liposomes (0.5 mM lipid, 2 ml) composed of egg PC and Gal-OE_{10}-DPG (8:2 molar ratio) were mixed at 25°C with 10 mM Tris–HCl/150 mM NaCl (pH 7.4), containing 0.25 mg/ml RCA-120 and agglutination was monitored spectro-photometrically at a wavelength of 450 nm. Trace (1) egg PC: Gal-$(oxyethylene)_{10}$-dipalmitoylglycerol is 9:1, trace (2) egg PC: Gal-$(oxyethylene)_{10}$-dipalmitoylglycerol is 9.5:0.5 and trace (3) egg PC: Gal-$(oxyethylene)_{10}$-dipalmitoylglycerol is 0:10. (c) RCA-120-induced agglutination of liposomes doubly coated with Gal-OE_{10}-PMG and PEG2000-DMG. Liposomes (0.8 mM lipid, 2 ml) composed of egg PC and Gal-OE_{10}-DMG (8:2 molar ratio) with or without 5 mol% PEG2000-DMG were mixed at 25°C with 10 mM Tris–HCl/150 mM NaCl (pH 7.4), containing 0.5 mg/ml RCA-120 and agglutination was monitored spectrophotometrically at a wavelength of 450 nm. Trace (1) without PEG2000-DMG and trace (2) with PEG2000-DMG.

Conclusion

Therapeutic efficacy and safety of liposomes can be controlled by their physico-chemical or surface chemical properties. By setting up reasonable physico-chemical specifications of the properties in the laboratory and ensuring that the specifications are satisfied in the up-scaling process, the efficacy and safety can be reproduced in quality control of a large scale production.

Table 11
Organ distribution of liposomes coated with Gal-OE_{10}-DPG (performed at GUIDE)

	Distribution (% of dose)				Liver/spleen
	Blood	Spleen	Liver	Recovery	
Control	35.0	5.7	19.1	60.1	3.4
10% Gal-OE_{10}-DPG	0.4	0.8	84.9	86.1	106.0
10% Gal-OE_{10}-DPG+2% PEG2000-DSPE	7.5	1.4	70.5	79.4	50.3
20% Gal-OE_{10}-DPG	0.4	1.0	90.5	91.5	90.5

Table 12
Intrahepatic distribution

	% Of liver uptake (normalized)		
	NPC	PC	NPC/C
Control	76.9 (72.2)	29.5 (27.8)	2.6
10% Gal-OE_{10}-DPG	63.2 (91.1)	6.2 (8.9)	10.2
10% Gal-OE_{10}-DPG+2% PEG2000-DSPE	80.6 (81.7)	18.0 (18.3)	4.5
20% Gal-OE_{10}-DPG	69.7 (93.8)	4.6 (6.2)	15.2

NPC, Non-parenchymal cell; and PC, parenchymal cell; of liposomes coated with Gal-OE_{10}-DPG (performed at GUIDE).

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