ELSEVIER

Biochimica et Biophysica Acta 1329 (1997) 370–382

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Enhancement of the in vivo circulation lifetime of L-α-distearoylphosphatidylcholine liposomes: importance of liposomal aggregation versus complement opsonization

Patrick L. Ahl, Suresh K. Bhatia [1], Paul Meers, Patricia Roberts, Rachel Stevens, Richard Dause, Walter R. Perkins, Andrew S. Janoff *

The Liposome Company, Inc., Princeton Forrestal Center, One Research Way, Princeton, NJ, 08540-6619, USA

Received 21 May 1997; accepted 22 May 1997

Abstract

Incorporation of *N*-(ω-carboxy)acylamido-phosphatidylethanolamines (-PEs) into large unilamellar vesicles (LUVs) of L-α-distearoylphosphatidylcholine (DSPC) was found to dramatically increase the in vivo liposomal circulation lifetime in rats, reaching a maximal effect at 10 mol.% of the total phospholipid. Neither pure DSPC liposomes nor those with the longest circulating derivative, *N*-glutaryl-dipalmitoylphosphatidylethanolamine (-DPPE), were found to significantly bind complement from serum. Therefore, the relatively short circulation time of pure DSPC liposomes did not appear to be related to greater complement opsonization leading to uptake by the reticuloendothelial system. However, *N*-(ω-carboxy)acylamido-PEs were particularly efficient inhibitors of a limited aggregation detected for pure DSPC liposomes. The aggregation tendency of DSPC liposomes incorporating various structural analogs of *N*-glutaryl-DPPE correlated inversely with the circulation lifetimes. Therefore, it is concluded that such PE derivatives enhance the circulation time by preventing liposomal aggregation and avoiding a poorly understood mechanism of clearance that is dependent on size but is independent of complement opsonization. At high concentrations of *N*-glutaryl-DPPE (above 10 mol.%), the liposomes exhibited strong complement opsonization and were cleared from circulation rapidly, as were other highly negatively charged liposomes. These data demonstrate that both the lack of opsonization and the lack of a tendency to aggregate are required for long circulation. Liposomal disaggregation via *N*-(ω-carboxy)acylamido-PEs yields a new class of large

Abbreviations: AUC, area under the curve; Chol, cholesterol; DOPE, L-α-dioleoylphosphatidylethanolamine; DPPE, L-α-dipalmitoylphosphatidylethanolamine; DSPC, L-α-distearoylphosphatidylcholine; DSPG, L-α-distearoylphosphatidylglycerol; EDTA, ethylenediaminetetraacetic acid; EPC, egg phosphatidylcholine; GM_1, monosialoganglioside; IFV, interdigitation-fusion vesicle; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; PE, phosphatidylethanolamine; PEG, poly(ethylene glycol) 2000; PEG-DSPE, L-α-distearoylphosphatidylethanolamine–*N*-[poly(ethylene glycol) 2000]; QELS, quasi-elastic light scattering; RES, reticuloendothelial system; SUV, small unilamellar vesicle; T_m, gel to liquid-crystalline phase transition temperature; CH50, serum dilution at which 50% hemolysis is observed (see Section 2) ; GVB^{2+}, gelatin veronal buffer (see Section 2)

* Corresponding author. Fax: +1 609 520 8250; E-mail: ajanoff@mail.lipo.com

[1] Present address: Ranbaxy Laboratories Limited, Gurgaon, India.

PII S0005-2736(97)00129-6

unilamellar DSPC liposomes with circulation lifetimes that are comparable to those of sterically stabilized liposomes.

Keywords: Phospholipid; Long-circulating liposome; Opsonization; Complement; Drug delivery

1. Introduction

The propensity of liposomal drug carriers to be taken up by the phagocytic cells of the reticuloendothelial system (RES) can affect many important pharmaceutical properties, especially the in vivo circulation lifetime. Designing long-circulating liposomes has been a major goal of liposome research [1–5].

Complex interactions with plasma opsonins and phagocytic cells can determine the ultimate in vivo consequences for liposomes. In particular, C3 is a crucial component in the serum complement cascade that opsonizes particles and liposomes and plays a major role in their clearance by the RES [6–9]. Liposomal size, surface charge, surface hydrophilicity and specific lipid composition can influence the rate of RES uptake. For instance, liposomes that contain cholesterol and a high surface charge density, imparted by phospholipids such as phosphatidylglycerol, are strong activators of C3 [10], leading to rapid clearance by the RES.

A number of liposomal surface-modifying materials, such as glycolipids [11–16], or phospholipids linked to hydrophilic polymers [17–28], have been introduced to inhibit RES uptake. Prior to the invention of these additives, small unilamellar vesicles (SUVs) containing rigid gel-phase phospholipids, such as distearoylphosphatidylcholine (DSPC), were considered the best long-circulating liposomes [29–36]. However, the small size requirement was not always optimal for drug delivery.

The circulation lifetimes of some liposomal formulations are also significantly enhanced by derivatives of phosphatidylethanolamine (PE), prepared by the reaction of dicarboxylic acids with the free amine group of PE to give *N*-(ω-carboxy)acylamido-PEs (see Fig. 1) [37]. Therefore, it was of interest to examine this relatively new mediator of enhanced circulation time in terms of mechanism and efficacy. This was tested in intermediate-to-large vesicles (~100 nm diameter) composed of DSPC, which do not exhibit the relatively long circulation time characteristics of small sonicated vesicles. We report here on the enhancement of circulation lifetimes of such liposomes and an investigation of the role of complement and effective particle size. Portions of this work were presented previously in abstract form [38].

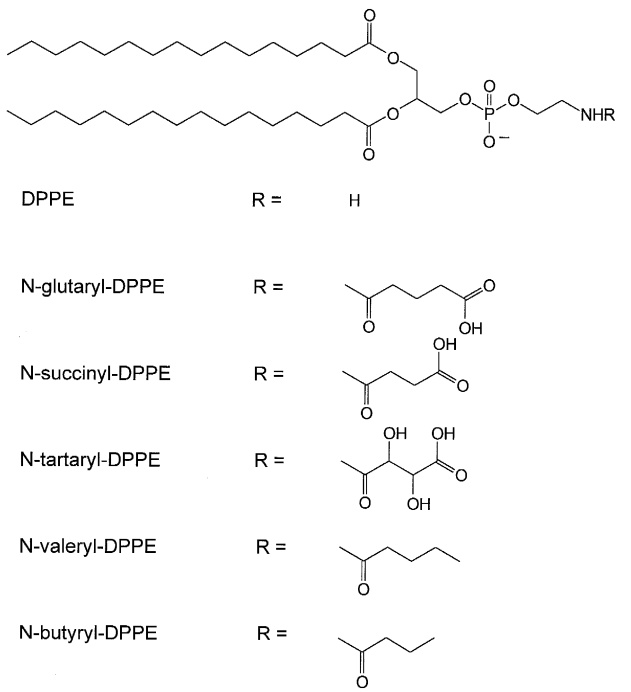

Fig. 1. Chemical structures of phosphatidylethanolamine derivatives.

2. Materials and methods

2.1. Materials

Male Sprague Dawley rats were purchased from Charles River (Wilmington, MA, USA). DSPC was purchased from Princeton Lipids (Princeton, NJ, USA). PEG-DSPE (2000 MW) and DSPG were obtained from Avanti Polar Lipids (Alabaster, AL, USA), while the cholesterol was from Sigma (St. Louis, MO, USA). The [^{3}H]cholesteryl hexadecyl ether was purchased from Dupont NEN Research Products (Boston, MA, USA). Ethanol (dehydrated 200 proof) was obtained from Pharmco Products

(Bayonne, NJ, USA). A 0.9% (w/v) saline solution, for injection, was from Abbott Laboratories (North Chicago, IL, USA). Sodium barbital, dextrose, gelatin veronal buffer (GVB^{+2}: 141 mM NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.8 mM sodium barbital, 3.1 mM barbituric acid, 0.1% gelatin, pH 7.3–7.4), antibody sensitized sheep erythrocytes (EA7S) and lyophilized rat serum were from Sigma. EDTA was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, USA).

2.2. Chemical synthesis of DPPE derivatives

N-Glutaryl-, *N*-butyryl-, *N*-valeryl- and *N*-succinyl-DPPE were prepared by heating a suspension of dipalmitoylphosphatidylethanolamine (DPPE) in anhydrous chloroform at 50–55°C until a clear solution was obtained. After cooling this solution to room temperature, triethylamine and the appropriate anhydride were added and the resulting solution was stirred at room temperature. After 3 h, chloroform was removed and the residue was chromatographed on a silica gel column that was eluted with chloroform–methanol–water (65:25:4, v/v/v). The products were dried in a vacuum oven at 60°C for 6 h to give a white crystalline material, with an approximate yield of 80%. *N*-Tartaryl-DPPE was prepared using disuccinimidyl tartrate instead of the anhydride and it was purified by passing the crude material through a Sephadex LH-20 column in chloroform–methanol (1:1, v/v) followed by silica gel column chromatography in chloroform–methanol–water (65:25:4, v/v/v). After lyophilization, the final product was obtained as a semi-solid material at a yield of 47%. All of the compounds described above gave a single spot on silica thin layer chromatography (TLC) [65:25:6 (v/v) chloroform–methanol–water on Whatman K6 silica gel 60 A plates) and were characterized as being pure by infrared (IR) and ^{1}H NMR spectroscopy. In particular, the R_f values for *N*-glutaryl, succinyl, tartaryl, butyryl and valeryl derivatives were 0.26, 0.25, 0.49, 0.44 and 0.46, respectively. ^{1}H NMR peaks from the methylenes α and β to the original diacid carboxyl groups appeared between 2 and 3 ppm from tetramethylsilane, with the correct ratio to the rest of the phospholipid protons. For the monoacid derivatives, peaks α to the amide linkage also appeared in the same range, with the appropriate amplitude. For example, for *N*-glutaryl-DPPE: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.3–5.1 (1H, m), 4.5–4.3 (1H, 2d), 4.2–4.0 (1H, m), 4.0–3.89 (4H, m), 3.6–3.4 (2H, t), 2.5–2.3 (2H, m), 2.3–2.1 (6H, m), 2.1–1.8 (2H, m), 1.8–1.5 (4H, m), 1.5–1.1 (48H, m) and 1.0–0.7 (6H, m). Infrared spectra of the *N*-glutaryl-DPPE product displayed the expected carboxylic carbonyl stretch at approximately 1700 cm^{-1}.

2.3. Liposome preparation

Large unilamellar vesicles (LUVs) were prepared from dry lipid films and extruded as described previously [39]. Radiolabeled samples contained 5 μCi/ml of [^{3}H]cholesteryl hexadecyl ether, a relatively non-exchangeable probe [40,41], in the final sample volume, giving a ratio of 1 to 4000 lipid molecules. The lipid film was hydrated and extruded above the main phase transition temperature of DSPC to a final lipid concentration of 30 mM. Pyrogen-free saline for injection (0.9%, w/v, NaCl) was used as a buffer in all animal studies. Samples for microscopy and turbidity measurements also contained 10 mM HEPES. The liposomal samples used in the in vitro complement assays were suspended in buffer A [GVB^{+2} plus 0.1% (w/v) dextrose]. The pH of samples containing *N*-(ω-carboxy)acylamido-PEs required adjustment with NaOH after hydration to bring the pH to near seven (except for the *N*-tartaryl derivative), due to dissociation of the carboxyl and/or phosphate protons.

Small unilamellar vesicles (SUVs) were prepared by probe sonication at a temperature above the T_m of the lipid, using a Branson 450 sonicator (Danbury, CT).

DSPC/*N*-glutaryl-DPPE (9:1) interdigitation-fusion vesicles (IFVs) were prepared in saline for injection from sonicated SUVs using 4 M ethanol, as previously described [42].

The phosphate content of the final LUV preparations was determined by the method of Chen et al. [43]. All liposome samples were stored at 4°C overnight before use.

2.4. Liposome sizing and turbidity measurements

The average diameter of each of the different LUV formulations was determined by quasi-elastic light

scattering (QELS) using a NICOMP 270 particle sizer (Particle Sizing Systems, Santa Barbara, CA, USA) at 60°C (to ensure that the bilayer was in the liquid-crystalline phase) and fit by a simple number-weighted Gaussian size distribution. Surface area-weighted average diameters were calculated from number-weighted histograms, with the distribution half-width defined as 2.354 times the standard deviation. All liposomes, except those in the size dependence studies, were extruded to a final surface area-weighted diameter of 120 to 144 nm, with distribution widths at half height of approximately 35 nm. One exception was the DSPC/chol/DSPG/PEG-DSPE (1:4:4:1) formulation, which had an average diameter of 69 nm.

The sizes of liposomes used in the size-dependence experiments are shown in the caption to Fig. 5. IFVs and the 200, 400, 600 and 800 nm pore size LUVs were evaluated by freeze-fracture electron microscopy, as described in Boni et al. [44] rather than by QELS. Over 1000 liposomes were measured for each size distribution histogram. The surface area-weighted average diameter and the corresponding standard deviation were calculated directly from the size distribution histograms following standard equations for weighted averages [45]. The sizes obtained by this method were very close to the number-weighted sizes from QELS, except at the larger liposome sizes, where the QELS method is very inaccurate.

The aggregation levels of the LUV samples were examined at concentrations and temperatures at which the sample was injected into the rats, using phase contrast microscopy and turbidity measurements with short path-length cuvettes. Quantitative dynamic light scattering methods are not applicable to heterogeneous aggregated samples. Therefore, phase contrast and Nomarski photomicrographs of liposome samples were taken at room temperature using either a 20× objective or a 40× oil immersion objective with an Olympus BH-2 microscope and an Olympus PM-10ADS exposure control unit (New York/New Jersey Scientific, Middlebush, NJ, USA). The turbidity measurements were made at approximately the same lipid concentration as was used in the in vivo experiments and at several dilutions of the sample concentration. The optical density (OD) at 450 nm for each LUV formulation was measured using either a 1 or 0.1 mm path-length quartz cuvette (Hellma, Jamaica, NY, USA) at room temperature in a UV-2101PC spectrophotometer (Shimadzu Scientific Instruments, Princeton, NJ, USA). Quantitative turbidity comparisons of different formulations were made by normalization of the OD at 450 nm for sample lipid concentration and cuvette path-length.

2.5. In vitro liposome-induced complement depletion assay

These assays are based on the depletion of complement from serum by liposomes and its detection by the relative ability of the treated serum to mediate complement-dependent lysis of activated red blood cells [46]. Liposome-induced complement activation was measured in 1.5 ml eppendorf centrifuge tubes in a two-step assay. First, 200 μl of liposomes were incubated for 30 min at 37°C with 100 μl of rat serum that was diluted 1:1 (v/v) with buffer A (see above). The final lipid concentrations at this step in the assay ranged from 57 to 77 mM. Samples were incubated in an Eppendorf 5436 Thermomixer (Brinkmann Instruments, Westbury, NY, USA) with gentle shaking. Then, 300 μl of buffer A were added, the sample was vortex-mixed and centrifuged at 8000 $\times g$ for 4 min. A 100-μl volume of the supernatant was removed and was two-fold serially diluted eight times in buffer A. A 100-μl aliquot of antibody-activated sheep red blood cells (EA7S) at $1 \cdot 10^8$ cells/ml was added to each tube. All the tubes were then incubated for 30 min at 37°C. The hemolysis reaction was stopped by the addition of 300 μl of ice cold buffer B (150 mM NaCl, 75 mM barbital, 0.1% gelatin and 20 mM EDTA, pH 7.5). Unlysed red blood cells were sedimented for 4 min at 8000 $\times g$. Hemolysis was determined from the OD at 415 nm of 250 μl aliquots, using a microplate reader. The fidelity of the hemolysis assay was ascertained by the smooth monotonic nature of the data and its reproducibility. Furthermore, positive and negative controls were used in each series of experiments to correct for any differences in the hemoglobin response from a given red cell preparation. In addition, measurement of both the OD at 415 nm and the use of Drabkin's reagent gave a linear response to hemoglobin in the range tested in the hemolysis experiments. Liposome light-scattering and residual

red cell hemolysis were corrected for using liposome-only and red cell-only blanks.

Hemolysis data were plotted on a log scale in terms of the serum dilution factor, which is fractional, i.e. a 1:100 dilution of serum is equal to 0.01. A shift of the curve to the right indicates that liposomes have bound complement. An alternative method of expressing the data is in terms of the CH50 [46], the serum dilution at which 50% hemolysis occurs. Hemolysis (50%) at a 1:100 dilution level would give a CH50 of 100 [46]. The CH50 values used for Table 2 were calculated by a linear fit to a log–log version of the von Krogh equation [46]. Percentage CH50 decreases were calculated relative to the negative buffer control of the sample set. A two sample *t*-test analysis, assuming equal variances (Microsoft Excel version 5.0), was used to test for statistical significance. Based on C3 concentrations in human serum and extrapolating to rats, it can be estimated that as few as three C3 molecules associated per liposome may cause total depletion of C3 under the conditions of these experiments.

2.6. Animal experiments

Male Sprague Dawley rats (between 330 and 400 g) were weighed just prior to the experiment. Liposome preparations were given by a single bolus injection in the lateral tail vein at 50 mg/kg. The injection volumes contained 4 to 5 μCi of [^{3}H]cholesteryl hexadecyl ether per rat and were typically 0.8 to 1.0 ml. The pharmacokinetics of the DSPC and DSPC/*N*-glutaryl-DPPE LUVs were essentially independent of lipid dose from 10 to 300 mg/kg (unpublished results). Whole-blood samples of 0.3 to 0.5 ml were withdrawn under CO_2 anesthesia by retro-orbital bleeding from each rat. Blood was collected in EDTA-containing microtainer tubes (Becton Dickinson, Rutherford, NJ, USA) using heparinized microhematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA, USA). The blood plasma fractions were obtained by sedimentation in a Hermle model Z230 benchtop centrifuge (Brinkmann Instruments).

The percentage of the total dose in the plasma at each time point (% dose) was calculated as $\{P_{DPM}/ml\} \times \{[plasma\ volume]/T_{DPM}\} \times 100$ where P_{DPM}/ml is the DPM per ml of plasma and T_{DPM} is the total DPM injected into the rat. Plasma volume was taken to be 4.04 ml/100 g body weight [47].

The circulation lifetimes of the different liposome formulations were compared by integration of the % dose in the plasma to give the ''area under the curve'' (AUC) for each rat, determined by summing the areas under trapezoidal segments between 0 and 24 h. The % dose at t = 0 was set at 100%. In order to make comparisons between different liposome formulations easier, each of the average AUCs were normalized to the AUC of the 200 nm pore size DSPC/*N*-glutaryl-DPPE (9:1) LUVs. The average and standard deviation of the normalized AUC was calculated for each formulation. A two sample *t*-test analysis, assuming equal variances (Microsoft Excel version 5.0), was used to test for statistical significance. When the fraction of liposomal material recovered in plasma (taking whole blood as 100%) was used to correct plasma AUC values for each liposome composition, the differences between corrected and original values were within the error of the measurements.

3. Results

3.1. N-glutaryl-DPPE enhancement of circulation lifetime

It was of interest initially to determine whether *N*-(ω-carboxy)acylamido-PEs could indeed enhance the circulation lifetimes of approximately 100 nm diameter DSPC liposomes (LUVs). Liposomes with various percentages of *N*-glutaryl-DPPE were compared to pure DSPC liposomes (Fig. 2). The plasma clearance of 100 nm DSPC LUVs was apparently biphasic, consisting of an initial phase with $t_{1/2}$ of less than 5 min followed by a much slower phase. It is clear that a dramatic increase in lifetime is afforded by incorporation of only 10 mol.% of the *N*-glutaryl derivative, primarily decreasing the very rapid clearance. The enhanced circulation lifetime was observed up to 10 mol.%, but at higher concentrations, the circulation time decreased, a phenomenon discussed below.

Large hydrophilic polyethylene glycol groups covalently linked to phospholipid headgroups are known to sterically stabilize certain liposomes, preventing complement fixation and RES uptake and leading to

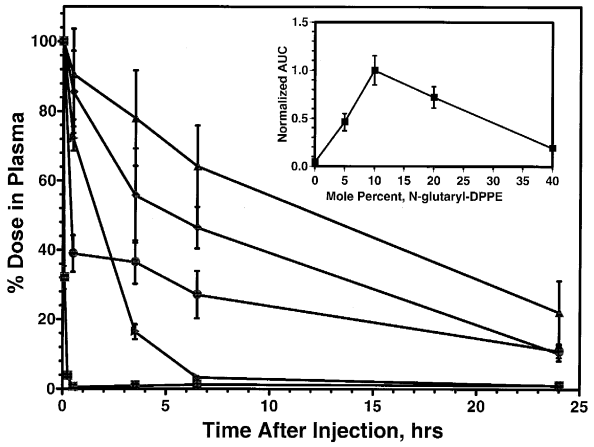

Fig. 2. Dependence of the in vivo circulation lifetime of DSPC/*N*-glutaryl-DPPE LUVs on the membrane concentration of *N*-glutaryl-DPPE. The liposomes were radiolabeled with [^{3}H]cholesteryl hexadecyl ether. Male Sprague Dawley rats were dosed at 50 mg/kg with a bolus tail vein injection. Typical time courses of percentage of original dose remaining in plasma were measured as described in Section 2. The percentage of total lipid dose still present in the plasma as a function of time after injection is shown for 0 (-■-), 5 (-●-), 10 (-▲-), 20 (-♦-) and 40 (-►-) mol.% *N*-glutaryl-DPPE in DSPC. The areas under the curves (AUCs) were calculated from such circulation lifetime profiles as described in Section 2. The AUC (±standard deviation) as a function of composition is shown in the inset. Between four and twelve experiments were averaged for each data point.

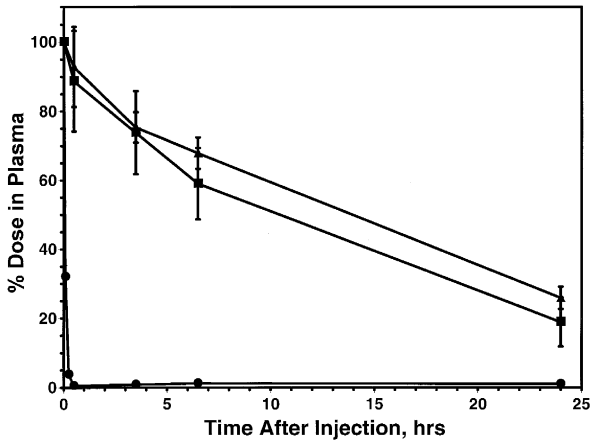

Fig. 3. In vivo circulation lifetime of different DSPC LUV formulations. The percentage of total lipid dose still present in the plasma as a function of time after injection is shown for the following LUV formulations: DSPC/*N*-glutaryl-DPPE (9:1) -■-, DSPC -●- and DSPC/PEG-DSPE (9:1) -▲-. Plasma clearance profiles for DSPC LUVs represent the combined results of two sets of experiments.

long circulation lifetimes (see Section 1). The circulation lifetime profile of DSPC/*N*-glutaryl-DPPE (9:1) LUVs are compared to the profiles of identically prepared DSPC/polyethylene glycol-distearoylphosphatidylethanolamine (PEG-DSPE) and pure DSPC liposomes in Fig. 3. The circulation lifetimes of the *N*-glutaryl-DPPE-containing liposomes were statistically comparable with the sterically stabilized liposomes ($P > 0.05$).

Comparison with other PE derivatives in Table 1 demonstrates that this effect of the dicarboxylic acid derivative was clearly dependent on its structure. For instance, the *N*-succinyl-DPPE derivative was nearly as effective in circulation time enhancement as the *N*-glutaryl derivative, but the *N*-butyryl- and *N*-valeryl derivatives were significantly less effective ($P < 0.01$). DSPC/*N*-tartaryl-DPPE does not fit this hypothesis, but also differs in other characteristics,

Table 1
Comparisons of in vivo circulation lifetimes for different DSPC LUV formulations

Classification	Formulation	Normalized AUC [b]
Controls	DSPC	0.04 ± 0.02 [a] (4)
	DSPC/chol, 2:1	0.34 ± 0.04 [a] (5)
	DSPC/PEG-DSPE, 9:1	1.16 ± 0.05 (4)
N-(ω-carboxy)acylamido-PEs	DSPC/*N*-glutaryl-DPPE, 9:1	1.00 ± 0.15 (15)
	DSPC/*N*-succinyl-DPPE, 9:1	0.81 ± 0.13 (4)
	DSPC/*N*-tartaryl-DPPE, 9:1	0.35 ± 0.09 [a] (6)
N-acylamido-PEs	DSPC/*N*-butyryl-DPPE, 9:1	0.49 ± 0.03 [a] (3)
	DSPC/*N*-valeryl-DPPE, 9:1	0.52 ± 0.05 [a] (3)

[a] Statistically significant difference in AUC from DSPC/*N*-glutaryl-DPPE (9:1) LUV formulation, $P < 0.01$.
[b] Area under the curve ± standard deviation, calculated as described in Section 2. The number of measurements is indicated in parentheses.

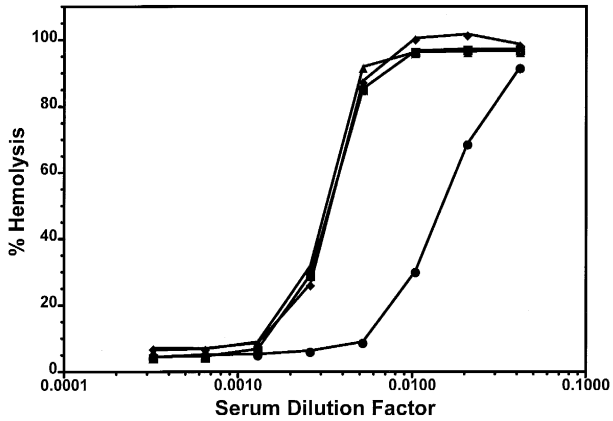

Fig. 4. Liposome-induced in vitro complement binding. Serum dilution factor is defined as a fraction, i.e. a 1:100 dilution of serum is equal to 0.01. Complement depletion (determined as described in Section 2) is indicated by a shift of the hemolysis curve to the right (as described in Section 2). Complement-induced hemolysis curves are shown for serum that was incubated with buffer (-■-), DSPC/chol/DSPG (2:4:4) LUVs (-●-), DSPC/*N*-glutaryl-DPPE (9:1) LUVs (-◆-) or DSPC LUVs (-▲-).

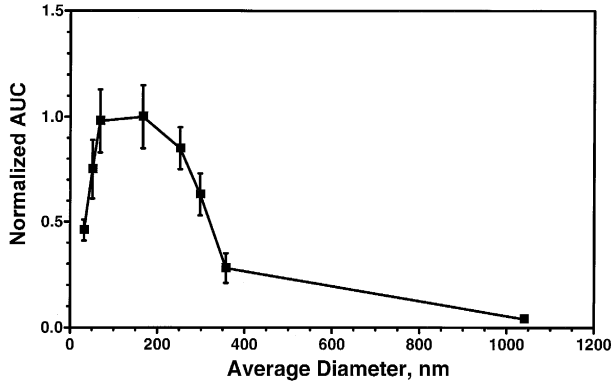

Fig. 5. Liposome size dependence of circulation lifetime. The AUCs of DSPC/*N*-glutaryl-DPPE (9:1) liposome formulations with various size distributions are plotted versus the surface area-weighted average diameter of the formulations. The AUCs were calculated as described in Section 2. Each AUC was normalized to the AUC of the DSPC/*N*-glutaryl-DPPE (9:1) LUVs extruded through 200 nm pores. The surface area-weighted diameter and distribution half-width, along with the number of experiments for each liposome formulation were the following: SUVs: 32 ± 11 nm (3); 50 nm pore: 52 ± 16 nm (5); 80 nm pore: 69 ± 17 nm (6); 200 nm pore: 167 ± 65 nm (15); 400 nm pore: 252 ± 138 nm (4); 600 nm pore: 298 ± 150 nm (3); 800 nm pore: 357 ± 254 nm (5) and IFVs: 1040 ± 580 nm (2). The weighted averages typically varied by no more than $\pm 10\%$ over multiple measurements.

such as its capability for hydrogen bonding and apparent pK (see Section 2). Modification of the DSPC chain packing by the addition of cholesterol in DSPC/chol (2:1) liposomes also does not enhance the circulation lifetime as effectively as *N*-glutaryl-DPPE, the most efficacious derivative.

3.2. Effect of N-glutaryl-DPPE on in vitro complement depletion by liposomes

The possibility that *N*-glutaryl-DPPE enhances the circulation time by reducing complement opsonization of DSPC liposomes was next investigated in vitro using an antibody-activated sheep red blood cell hemolysis assay (see Section 2). In these complement binding assays, the liposomal test samples were always compared to buffer alone, as a negative control, and the liposomal composition of DSPC/chol/DSPG (2:4:4), as a positive control. The latter is similar to compositions previously shown to strongly activate complement [10]. Fig. 4 shows typical hemolysis curves for rat serum that was pre-incubated with several DSPC-containing liposome preparations.

Table 2
Effects of *N*-glutaryl-DPPE and PEG-DSPE on circulation lifetime of a liposome composition that fixes complement

Liposome formulation	AUC [a] (relative)	% CH50 decrease [b]
DSPC/chol/DSPG, 2:4:4	0.05 ± 0.01 [c] (4)	68.3 ± 5.0 (7)
DSPC/chol/DSPG/*N*-glutaryl-DPPE, 1:4:4:1	0.02 ± 0.0007 [c] (4)	88.9 ± 1.9 (3)
DSPC/chol/DSPG/PEG-DSPE, 1:4:4:1	0.90 ± 0.13 (4)	19.1 ± 4.8 (3)

[a] Area under the curve, calculated as described in Section 2 relative to *N*-glutaryl-DPPE/DSPC (9:1).
[b] CH50 defined in Section 2 and the caption to Fig. 4. The percentage CH50 decrease is relative to the CH50 of buffer alone, which was taken as 100% (i.e. 0% decrease). A decrease of CH50 represents more complement binding by the liposomes. The number of experiments is given in parentheses.
[c] Statistically significant difference in AUC from DSPC/*N*-glutaryl-DPPE (9:1) LUV formulation, $P < 0.01$.

While the positive control, DSPC/chol/DSPG (2:4:4), produced a significant shift to the right (CH50 decrease) relative to the buffer control, indicating depletion of complement from the serum, neither the DSPC nor the DSPC/*N*-glutaryl-DPPE (9:1) formulations appeared to bind significant amounts of complement from the serum. The same result was observed in rat plasma (data not shown). Therefore, *N*-glutaryl-DPPE did not appear to enhance the circulation time of DSPC by inhibiting complement opsonization.

A related question is whether *N*-glutaryl-DPPE could reduce opsonization and/or increase the circulation time of formulations that do bind complement. Therefore, we utilized our positive complement binding control, DSPC/chol/DSPG (2:4:4), and replaced some of the DSPC with *N*-glutaryl-DPPE to give DSPC/chol/DSPG/*N*-glutaryl-DPPE (1:4:4:1). This

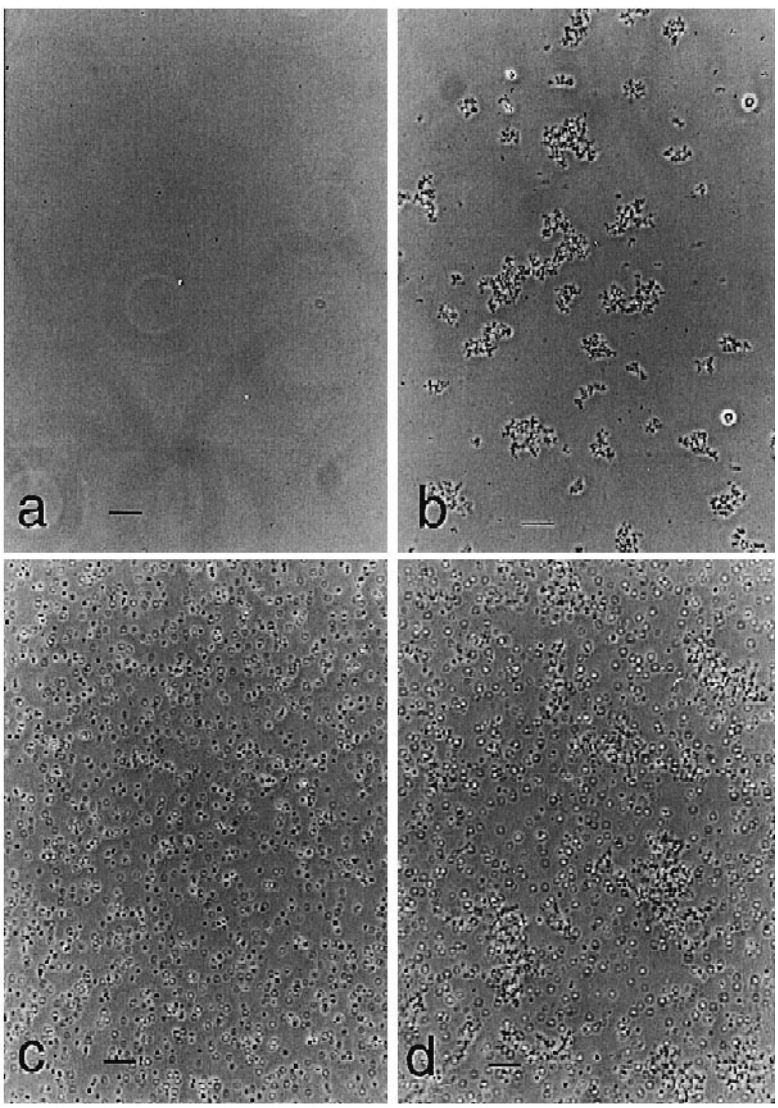

Fig. 6. Aggregation level of DSPC LUV formulations. The aggregation level of DSPC (b and d) and DSPC/*N*-glutaryl-DPPE (9:1) (a and c) in rat serum (a and b) or plasma (c and d) are shown. The lipid concentration in all samples was adjusted to approximately 2 mM, the concentration expected for complete dilution of the injected dose into the blood volume of a rat. The plasma sample contained a background of particles, largely consisting of platelets. The photomicrographs were taken using 20 × objective phase contrast imaging optics. The bar in each photomicrograph corresponds to 20 μm.

composition bound even more complement than the positive control and the clearance from plasma was also even faster (Table 2). If the sterically stabilizing phospholipid, PEG-DSPE, was used to replace DSPC instead of *N*-glutaryl-DPPE, the circulation time was greatly increased, while opsonization was greatly decreased (Table 2). These data confirm the fact that, unlike PEG-phospholipids, *N*-glutaryl-DPPE does not inhibit complement opsonization and, in fact, it can even enhance it under some circumstances.

3.3. Size dependence of DSPC / N-glutaryl-DPPE LUV circulation lifetime

Having shown the absence of an opsonization effect, it was of interest to determine if *N*-(ω-carboxy)acylamido-PEs could influence other factors that affect the circulation lifetime. Since the circulation lifetimes of other liposomes are dependent on size (see Section 4), it was of interest to first confirm this dependency for DSPC/*N*-glutaryl-DPPE (9:1) LUVs in our system. In Fig. 5, data are shown that clearly demonstrate this size-dependence under conditions where dynamic light scattering, microscopy and turbidity revealed that there was no significant level of liposomal aggregation. The size was optimal for circulation in the 100 to 200 nm diameter range.

3.4. N-glutaryl-DPPE reduction of liposome aggregation

The pure DSPC liposomes used in these experiments were shown initially by dynamic light scattering to have the same optimal 100 nm size as *N*-glutaryl-DPPE-containing liposomes (data not shown). However, after incubation at room temperature, several observations indicated that limited aggregation had occurred. Measurements by dynamic light scattering indicated that these aggregates were beyond the size range where the method is applicable (>1 μm, data not shown). Although aggregates visible to the eye did not form, phase contrast photomicrographs at room temperature demonstrated substantial aggregation in either serum or plasma, as shown in Fig. 6. DSPC liposomes (Fig. 6b and d) were highly aggregated while DSPC/*N*-glutaryl-DPPE liposomes were not aggregated (Fig. 6a and c). The majority of the observed aggregates were at least

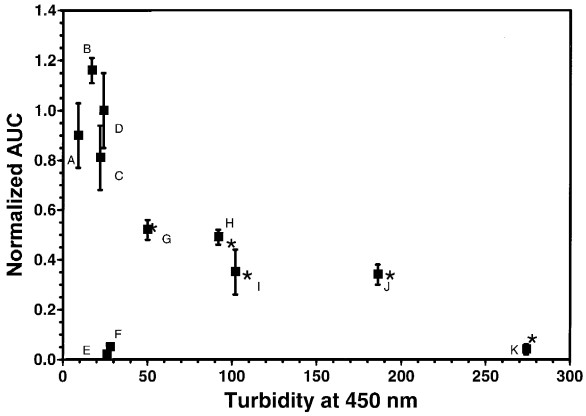

Fig. 7. Correlation between LUV aggregation level and in vivo circulation lifetime. All turbidity measurements were made at room temperature (see Section 2). The turbidity value is defined for a 1-mm path-length cuvette as the OD at 450 nm per mM lipid × 1000. The individual formulations shown in the figure are labeled as follows: (A) DSPC/chol/DSPG/PEG-DSPE (1:4:4:1); (B) DSPC/PEG-DSPE (9:1); (C) DSPC/*N*-succinyl-DPPE (9:1); (D) DSPC/*N*-glutaryl-DPPE (9:1); (E) DSPC/chol/DSPG/*N*-glutaryl-DPPE (1:4:4:1); (F) DSPC/chol/DSPG (2:4:4); (G) DSPC/*N*-valeryl-DPPE (9:1); (H) DSPC/*N*-butyryl-DPPE (9:1); (I) DSPC/*N*-tartaryl-DPPE (9:1); (J) DSPC/chol (2:1) and (K) DSPC. An asterisk (*) indicates that liposome aggregates were observed for the LUV formulation at room temperature using 40× oil immersion Nomarski microscopy.

10 μm in diameter. DSPC liposomes containing *N*-valeryl-DPPE, *N*-butyryl-DPPE or *N*-tartaryl-DPPE also showed aggregates of smaller average size than for pure DSPC (not shown). In contrast, similarly prepared DSPC/*N*-succinyl-DPPE or DSPC/PEG-DSPE LUVs at the same concentration had no tendency to form any microscopically visible aggregates at room temperature (data not shown). When DSPC liposomes and *N*-glutaryl-DPPE/DSPC liposomes were compared in buffer at the expected dilution after injection into rats, it was also clear that only the DSPC liposomes were aggregated (data not shown).

The relative turbidity of each LUV formulation was consistent with microscopic observations of aggregation. These measurements give an indication of the relative proportion of large liposomal aggregates in each of the formulations, although direct calculation of the size distributions are not possible from turbidity measurements [48]. When turbidity was plotted versus circulation lifetime in Fig. 7, it was clear that there was a strong correlation between the

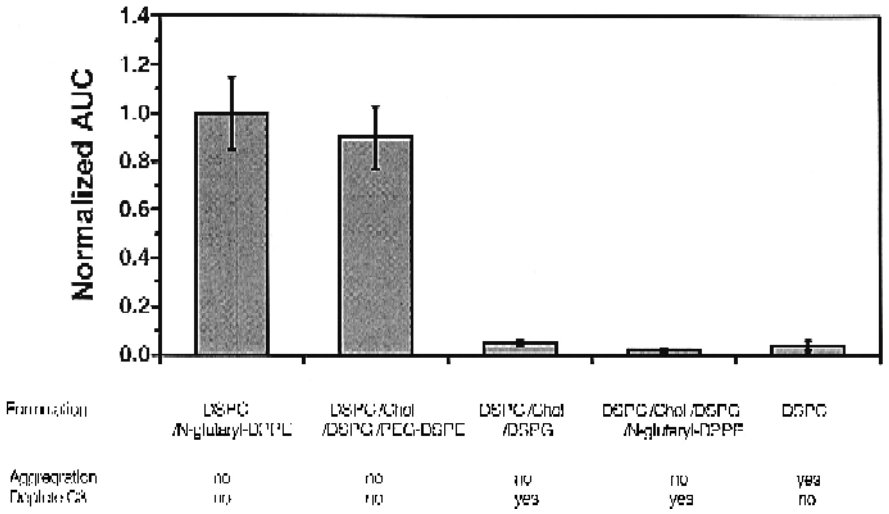

Fig. 8. In vivo circulation lifetime enhancement requires both low levels of liposomal aggregation and complement activation. Normalized AUC, aggregation and complement activation were determined as described in previous figure captions and in Section 2.

two parameters for those formulations that do not opsonize well. For opsonized formulations (the two points in the lower left-hand corner of the figure), circulation lifetimes were short, despite the lack of any tendency to aggregate. Therefore, liposomal tendency for aggregation and complement opsonization appear to be two separate and important determinants of the circulation lifetime of optimally sized DSPC LUVs. Fig. 8 demonstrates that only liposomes that were both poorly opsonized and disaggregated had long circulation lifetimes.

4. Discussion

The biphasic behavior of liposome plasma clearance profiles and the effect of liposome size on in vivo circulation lifetime have been noted previously [20,49,50], but the mechanism of size-dependent clearance remains unknown. Furthermore, the relatively large effect that a tendency to aggregate can have on in vivo liposome circulation lifetime has not been well recognized. Non-aggregated DSPC/*N*-glutaryl-DPPE LUVs clearly have an optimal particle diameter for long circulation (in the range of 100 to 200 nm) that rapidly drops off over approximately 300 nm, similar to DSPC liposomes containing PEG-DSPE [51]. Therefore, the circulation lifetime of these liposomes is highly dependent on relatively small changes in average particle diameter, which could result from even relatively low levels of liposomal aggregation into small clusters of even a few vesicles. It is clear that the observed tendency to aggregate in buffer, serum and plasma is sufficient to strongly influence the circulation lifetime. Therefore, rapid clearance of these liposomes appears to be mediated by a size-dependent mechanism that is not necessarily related to previously observed complement-mediated clearance [52–56], although a potential role for other opsonins besides those in the complement cascade has not yet been investigated. Small effects of liposomal size on complement opsonization of fluid-phase liposomes have been reported [10,59], but opposite size dependence for in vitro uptake by cultured macrophages versus in vivo clearance have also been noted, suggesting a separate macrophage-independent mechanism of size-dependent clearance in vivo [50].

This size-dependent mechanism of rapid clearance may be associated with a shift in biodistribution. Studies by Liu et al. [57] and Litzinger et al. [58] have shown that small-diameter long-circulating LUVs containing either GM_1 or PEG-linked phospholipids accumulate primarily in the liver. However, liposome accumulation shifted to spleen, when the average diameter of the liposome formulation was increased, possibly indicating a filtering mechanism. It has also been shown that very large particles can

become trapped in the capillary beds of the lungs, e.g. [66]. Similar size-dependent biodistribution shifts might be occurring for liposomal aggregates and might be the basis for the apparently biphasic DSPC LUV clearance kinetics.

Many liposomal formulations only weakly bind complement components because opsonization occurs only when certain physical criteria are met [10,60,61]. Devine et al. [10] clearly demonstrated that the density of surface charge is a crucial factor in promoting complement fixation. A relatively highly charged surface was necessary to fix complement, and this was greatly enhanced by the presence of cholesterol [10]. Pure DSPC liposomes do not meet these complement-binding criteria, but the absence of any net charge and relatively low surface hydration may allow attractive van der Waals forces to dominate liposome–liposome interactions such that aggregation becomes a factor for large DSPC vesicles [62,63]. The negative surface charge conferred by the divalent *N*-(ω-carboxy)acylamido-PEs would be expected to create an electrostatic repulsive force that disaggregates these liposomes. Some negatively charged derivatives disaggregated the liposomes better than others, indicating the importance of the divalency, effective pK values and structural features, such as the possible extension of the *N*-glutaryl carboxyl group a relatively large distance away from the membrane surface.

These results correspond in many respects to those of Park et al. [37] who showed that some *N*-(ω-carboxy)acylamido-PEs improved the circulation lifetime of fluid phase egg phosphatidylcholine–chol (EPC–chol) LUVs. Structural dependence was also observed, although with some differences, from those found in the DSPC liposomes. The mechanism of clearance of the EPC–chol liposomes was not determined, but it was suggested that lipid structure-specific dysopsonin binding may inhibit clearance of the derivatized liposomes. The possibility of limited liposomal aggregation was not considered in this study, but we have observed partial aggregation of identical EPC–chol liposomes by turbidity and microscopic observation under conditions similar to those reported (data not shown). Inclusion of 6 mol.% *N*-glutaryl-DOPE eliminated the aggregation, suggesting that inhibition of such aggregation may account, at least in part, for the ability of *N*-(ω-carboxy)acylamido-PEs to enhance the circulation lifetimes of these fluid phase liposomes.

One obvious correlate of the results presented in this manuscript is that large sterically stabilizing polymer additives are not necessary in all cases to generate liposomes with long circulation lifetimes. The average circulation half-time for 100 nm DSPC/*N*-glutaryl-DPPE liposomes was statistically indistinguishable ($P > 0.05$) from the circulation half-time of DSPC/PEG-DSPE liposomes in the experiments presented here. It is difficult to make direct quantitative comparisons between our results and previously published circulation half-times for other liposomes containing GM_1, hydrogenated PI, or one of several hydrophilic polymer-linked phospholipids [11–28] because of differences in animal systems or other conditions. However, it is clear that the reduction of aggregation-related uptake by the use of *N*-glutaryl-DPPE at only 10 mol.% generates a new class of long-circulating liposomes.

Depending on the desired agent and mechanism of delivery, gel-phase long-circulating liposomes may be particularly desirable. Rigid gel-phase bilayers are probably more stable in vivo because they appear to be less susceptible than more fluid liquid-crystalline bilayers to penetration by lipoproteins and lipid loss to serum components by exchange [52,53,64], although incorporation of cholesterol can increase stability [65]. Furthermore, PEG-linked phospholipids do not protect fluid-phase bilayers from some serum components that induce leakage and lipid transbilayer diffusion [67]. PEG-phospholipids may also block desired liposome-cell surface contacts that are necessary for drug delivery mediated by the binding of liposomal ligands, although coupling to the outer PEG surface may help in some cases [68]. It is possible that gel-phase long-circulating liposomes containing *N*-(ω-carboxy)acylamido-DPPEs will complement sterically stabilized liposomes and overcome some of these problems.

Acknowledgements

The authors thank Shaukat Ali for help with the synthesis of some DPPE derivatives and Aminu Saad for help with analyzing the pharmacokinetic results. We also thank Dana Devine for helpful discussions

concerning liposome opsonization and Sharma Minchey for assistance with graphics. This study was supported by The Liposome Company.

References

[1] T.M. Allen, C.B. Hansen, D.E.L. de Menezes, Adv. Drug Deliv. Rev. 16 (1995) 267–284.
[2] K.J. Hwang, in: M.J. Ostro (Ed.), Liposomes: from Biophysics to Therapeutics, Marcel Dekker, New York, 1987, pp. 109–156.
[3] G. Gregoriadis, in: G. Gregoriadis (Ed.), Liposomes as Drug Carriers: Recent Trends and Progress, Wiley, Chichester, 1988, pp. 5–18.
[4] S. Mumtaz, P.C. Ghosh, B.K. Bachhawat, Glycobiology 1 (1993) 505–515.
[5] D.D. Lasic, in: R. Lipowsky, E. Sackmann (Eds.), Structure and Dynamics of Membranes: Vol. 1A, Elsevier, Amsterdam, 1995, pp. 491–519.
[6] J.F. Scieszka, L.L. Maggiora, S.D. Wright, M.J. Cho, Pharm. Res. 8 (1991) 65–69.
[7] H.M. Patel, Crit. Rev. Ther. Drug Carrier Syst. 9 (1992) 39–90.
[8] W.M.J.M. Bogers, N. van Rooijen, D.J. Janssen, L.A. van Es, M.R. Daha, Eur. J. Immunol. 23 (1993) 433–438.
[9] W.M.J.M. Bogers, R.K. Stad, D.J. Janssen, M. Rits, H. Bazin, L.A. van Es, M.R. Daha, Eur. J. Immunol. 21 (1991) 1093–1099.
[10] D.V. Devine, K. Wong, K. Serrano, A. Chonn, P.R. Cullis, Biochim. Biophys. Acta 1191 (1994) 43–45.
[11] T.M. Allen, C. Hansen, J. Rutledge, Biochim. Biophys. Acta. 981 (1989) 27–35.
[12] A. Gabizon, D. Papahadjopoulos, Proc. Natl. Acad. Sci. U.S.A. 18 (1988) 6949–6953.
[13] T.M. Allen, A. Chonn, FEBS Lett. 223 (1987) 42–46.
[14] D. Papahadjopoulos, A. Gabizon, Ann. NY Acad. Sci. 507 (1987) 64–74.
[15] M. Haga, F. Kato, M. Yoshida, F. Kohara, Y. Kato, Chem. Pharm. Bull. 24 (1986) 2979–2988.
[16] Y. Namba, T. Sakakibara, M. Masada, F. Ito, N. Oku, Chem. Pharm Bull. 38 (1990) 1663–1666.
[17] G. Blume, G. Cevc, Biochim. Biophys. Acta 1029 (1990) 91–97.
[18] A.L. Klibanov, K. Maruyama, V.P. Torchillin, L. Huang, FEBS Lett. 268 (1990) 235–237.
[19] J. Senior, C. Delgado, D. Fisher, C. Tilcock, G. Gregoriadis, Biochim. Biophys. Acta 1062 (1991) 77–82.
[20] T.M. Allen, C. Hansen, Biochim. Biophys. Acta 1068 (1991) 133–141.
[21] I.A.J.M. Bakker-Woudenber, A.F. Lokerse, M.T. Ten Kate, J.W. Mouton, G. Storm, Biochim. Biophys. Acta 1138 (1992) 318–326.
[22] M.C. Woodle, K.K. Matthay, M.S. Newman, J.E. Hidayat, L.R. Collins, C. Redemann, F.J. Martin, D. Papahadjopulos, Biochim. Biophys. Acta 1105 (1992) 193–200.
[23] T.M. Allen, C. Hansen, F.J. Martin, C. Redemann, A. Yau-Young, Biochim. Biophys. Acta 1066 (1991) 29–36.
[24] D.D. Lasic, F.J. Martin, A. Gabizon, S.K. Huang, D. Papahadjopoulos, Biochim. Biophys. Acta 1070 (1991) 187–192.
[25] D. Papahadjopoulos, T.M. Allen, A. Gabizon, E. Mayhew, K.K. Matthay, S.K. Huang, K.-D. Lee, M.C. Woodle, D.D. Lasic, C. Redemann, F.J. Martin, Proc. Natl. Acad. Sci. USA 88 (1991) 11460–11464.
[26] V. Torchilin, M.I. Shtilman, V.S. Trubetskoy, K. Whitman, A.M. Milstein, Biochim. Biophys. Acta 1195 (1994) 181–184.
[27] V.P. Torchilin, V.G. Omelyanenko, M.I. Papisov, A.A. Bogdanov, V.S. Trubestskoy, J.N. Herron, C.A. Gentry, Biochim. Biophys. Acta 1195 (1994) 11–20.
[28] M.C. Woodle, C.M. Engbers, S. Zalipsky, Bioconj. Chem. 5 (1994) 493–496.
[29] G. Gregoriadis, J. Senior, FEBS Lett. 119 (1980) 43–46.
[30] J. Senior, G. Gregoriadis, FEBS Lett. 145 (1982) 109–114.
[31] K.J. Hwang, K.K. Luk, P. Beaumier L, Proc. Natl. Acad. Sci. U.S.A. 77 (1980) 4030–4034.
[32] R.T. Proffitt, L.E. Williams, C.A. Presant, G.W. Tin, J.A. Uliana, R.C. Gamble, J.D. Baldeschwieler, Science 220 (1983) 502–505.
[33] T.M. Allen, J.M. Everest, J. Pharmacol. Exp. Ther. 226 (1983) 539–544.
[34] R.M. Abra, C.A. Hunt, Biochim. Biophys. Acta 666 (1981) 493–503.
[35] R.M. Abra, C.A. Hunt, Res. Comm. Chem. Pathol. Pharmacol. 36 (1982) 17–31.
[36] J. Senior, J.C.W. Crawley, G. Gregoriadis, Biochim. Biophys. Acta 839 (1985) 1–8.
[37] Y.S. Park, K. Maruyama, L. Huang, Biochim. Biophys. Acta 1108 (1992) 257–260.
[38] P.L. Ahl, S.K. Bhatia, P. Roberts, A.S. Janoff, Biophys. J. 66 (1994) A176.
[39] L.D. Mayer, M.J. Hope, P.R. Cullis, Biochim. Biophys. Acta 858 (1986) 161–168.
[40] Y. Stein, G. Halperin, O. Stein, FEBS Lett. 111 (1980) 104–106.
[41] S.R. Green, W.F. Beltz, D.I. Goldberg, R.C. Pittman, J. Lipid Res. 30 (1989) 1405–1410.
[42] P.L. Ahl, Ch. Lu, W.R. Perkins, S.R. Minchey, L.T. Boni, T.F. Taraschi, A.S. Janoff, Biochim. Biophys. Acta 1195 (1994) 237–244.
[43] P.S. Chen Jr., T.Y. Toribara, H. Warner, Anal. Chem. 28 (1956) 1756–1758.
[44] L.T. Boni, S.R. Minchey, W.R. Perkins, P.L. Ahl, J.L. Slater, M.W. Tate, S.M. Gruner, A.S. Janoff, Biochim. Biophys. Acta 1146 (1993) 247–257.
[45] S.M. Selby (Ed.), Standard Mathematical Tables, CRC Press, Boca Raton, FL, p. 554.
[46] D.P. Stites, R.P.C. Rogers, in: D.P. Stites, A.I. Terr (Eds.), Basic and Clinical Immunology, Appleton and Lange, Norwalk, CT, 1991, pp. 217–262.

[47] H.J. Baker, J.R. Lindsey, S.H. Weisbroth, in: The Laboratory Rat: Volume 1, Appendix 1: Selected Normative Data, Academic Press, New York, 1979.
[48] C.S. Chong, K. Colbow, Biochim. Biophys. Acta 436 (1976) 260–282.
[49] R.L. Juliano, D. Stamp, Biochem. Biophys. Res. Commun. 63 (1975) 651–685.
[50] T.M. Allen, G.A. Austin, A. Chonn, L. Lin, K.C. Lee, Biochim. Biophys. Acta 1061 (1991) 56–64.
[51] K. Maruyama, T. Yuda, A. Okamoto, S. Kojima, A. Suginaka, M. Iwatsuru, Biochim. Biophys. Acta 1128 (1992) 44–49.
[52] J. Senior, G. Gregoriadis, K. Mitropoulos, Biochim. Biophys. Acta 760 (1983) 111–118.
[53] F. Bonte, R.L. Juliano, Chem. Phys. Lipid 40 (1986) 359–372.
[54] A. Chonn, S.C. Semple, P.R. Cullis, Biochim. Biophys. Acta 1070 (1991) 215–222.
[55] A. Chonn, S.C. Semple, P.R. Cullis, J. Biol. Chem. 267 (1992) 18759–18765.
[56] H.M. Patel, Crit. Rev. Ther. Drug Carrier Syst. 9 (1992) 39–90.
[57] D. Liu, A. Mori, L. Huang, Biochim. Biophys. Acta 1104 (1992) 95–101.
[58] D.C. Litzinger, A.M.J. Buiting, N. Rooijen, L. Huang, Biochim. Biophys. Acta 1190 (1994) 99–107.
[59] D. Liu, F. Liu, Y.K. Song, Biochim. Biophys. Acta 1235 (1995) 140–146.
[60] A. Chonn, P.R. Cullis, D.V. Devine, J. Immunol. 146 (1991) 4234–4241.
[61] J. Marjan, Z. Xie, D.V. Devine, Biochim. Biophys. Acta 1192 (1994) 35–44.
[62] R.P. Rand, V.A. Parsegian, Biochim. Biophys. Acta 988 (1989) 351–376.
[63] L.J. Lis, M. McAlister, N. Fuller, R.P. Rand, V.A. Parsegian, Biophys. J. 37 (1982) 657–666.
[64] A.R. Tall, P.H.R. Green, J. Biol. Chem. 256 (1981) 2035–2044.
[65] G.J. Kirby, J. Clarke, G. Gregoriadis, Biochem. J. 186 (1980) 591–598.
[66] P.G. Waser, U. Muller, J. Kreuter, S. Berger, K. Munz, E. Kaiser, B. Pfluger, Int. J. Pharm. 39 (1987) 213–227.
[67] P. Meers, A. Janoff, S. Ali, Biophys. J. 70 (1996) A83.
[68] C.B. Hansen, G.Y. Kao, E.H. Moase, S. Zalipsky, T.M. Allen, Biochim. Biophys. Acta 1239 (1995) 133–144.