Evidence for Phospholipid Bilayer Formation in Solid Lipid Nanoparticles Formulated with Phospholipid and Triglyceride

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Purpose. Solid lipid nanoparticles (SLN) are comprised of a high-melting point triglyceride (TG) core with a phospholipid (PL) coating. This study has investigated the possible formation of multiple PL bilayers on the TG core of SLN's as a function of increasing the PL:TG molar ratio.

Methods. Trilaurin (TL) was used as the SLN core. Dipalmitoylphosphatidylcholine (DPPC) or a mixture of DPPC and dimyristoylphosphatidylglycerol (DMPG) were used to produce neutral and negatively charged SLN's. The volume of aqueous phase associated with the PL was determined using calcein and 6-carboxyfluorescein (6-CF) as hydrophilic markers incorporated during the preparation of the SLN's.

Results. The diameter of the SLN's decreased as the molar ratio of PL to TL was increased, until a PL:TL ratio of 0.15 was reached. After this point the diameter was not affected by further increases in the molar ratio. The experimental amount of PL required to prepare SLN's was significantly higher than the theoretical amount required to form a single monolayer on the surface. The aqueous volume associated with the PL was increased with increasing PL:TL molar ratios.

Conclusions. The results obtained suggest that the formation of multiple PL bilayers is probable in SLN's prepared with a high molar ratio of PL to TL. The volume of the aqueous phase between the PL-bilayers, estimated from the amount of the hydrosoluble markers trapped in this phase, provides an indication of the relative number of bilayers at different PL:TL ratios

KEY WORDS: nanoparticles; phospholipid bilayer; markers; zeta potential; aqueous phase.

INTRODUCTION

Polymeric nanoparticles and lipid emulsions have a number of distinct advantages over liposomes for use as delivery systems for lipophilic drugs (1). The solid matrix of polymeric nanoparticles has the potential for allowing drug release over a prolonged period (2). The major disadvantages of polymeric nanoparticles are the difficulty of scaling up and the relatively slow degradation of the polymers (up to 3–4 weeks), which can cause impairment of the reticuloendothelial system (3) as well as cytotoxicity towards macrophages (4). Fat emulsions, which are known from parenteral nutrition studies to be non-toxic (5), have also been used as drug carriers (6). Because of the distribution of the active agent between the liquid oil phase and blood, the incorporated drug is released rapidly after intravenous injection (7–9).

Solid lipid nanoparticles (SLN) have advantages of both polymeric nanoparticles and fat emulsions (9,10). Unlike lipid emulsions, which have a fluid core, the solid core of SLN can prolong the release of incorporated drugs (2). The external surface of phospholipid (PL): triglyceride (TG) SLN's is analogous to that of a liposome, where modification of PL bilayers on the surface has been shown to result in significant changes in targeting and pharmacokinetics (11,12). The presence (and subsequent modification) of phospholipid bilayers on the surface of SLN's also offers the potential for controlled targeting and enhanced circulation residence times.

The objective of this study was to investigate the effect of increasing PL:TG ratios on SLN diameter and the possible formation of PL bilayers on the surface of the SLN. Our results indicate that multiple bilayer formation occurs at PL:TG ratios greater than 0.15.

MATERIALS

Trilaurin (TL), soybean oil (SB) and calcein were obtained from Sigma Chemical Company (St. Louis, MO). Dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylglycerol sodium salt (DMPG) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). 6-carboxyfluorecein (6-CF) was purchased from Eastman-Kodak (Rochester, NY). All materials were used without further purification, and all other chemicals were of analytical or USP grade.

METHODS

SLN Preparation

Mixtures of different molar ratios of PL to TL was dissolved in chloroform in a round bottom flask at room temperature. The weight of TL was constant (200 mg) in all experiments. After removal of the organic phase by rotary evaporation under vacuum (70°C), an oily lipid film was obtained on the flask wall. Aqueous solutions of calcein or 6-CF (20 ml, 1 mM calcein or 6-CF adjusted to pH 7.4 with NaOH) were added to the thin lipid film at 70°C and rotated for 5 min. The emulsion obtained (mean diameter 1–2 μm) was homogenized for 10 cycles at 70°C and 13000 psi using a high pressure homogenizer (Emulsiflex®-30, Avestin Inc., Ottawa, Canada) to produce solid lipid nanoparticles. Hot SLN's were allowed to cool to 25°C over a period of 1 hour. All other SLN and liposome preparations were made in 10 mM HEPES buffer, pH 7.4, containing 0.85% NaCl (HEPES/NaCl).

The theoretical amount of PL required was calculated on the basis of the formation of a closely packed continuous monolayer of PL with an average surface occupation for the head group of DPPC or DMPG at 25°C and pH 7.4 of 0.5 nm^2 (13–15). The mass of TL was constant (200 mg) in all preparations and the density of TL was assumed to be 1.027 g/cm^2 at room temperature (16), therefore the volume obtained with 200 mg TL could be determined. The volume ($4/3\Pi r^3$) and the surface area ($4\Pi r^2$) of one spherical particle was calculated for different diameters. From the volume of TL used and the volume of one particle the number of particles produced by 200 mg TL was determined and the total surface area of these particles calculated. Assuming an average head group surface occupation

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of 0.5 nm^2, the total number of PL molecules and subsequently the number of mol of PL required to cover this surface area was calculated. The equation for the theoretical values was

$$Y = A.^{exp}(-k.\ x)$$

where $A = 398 \pm 55$ nm and $k = 8.15 \pm 1.45$ is constant (95% confidence limits).

To determine the presence of liposomes in the SLN preparations, liposomes were produced by high pressure homogenization using DPPC or a DPPC/DMPG mixture under the same condition used for the SLN's. Rotary evaporation and hydration of phospholipid films (17) was used as a second method to produce liposomes. These liposomes were sonicated at 55°C (80 watts) using a Branson 1210R-MT sonicator, (Branson ultrasonics Co., Danbury, CT) until they had the same size distribution as the SLN's. Liposomes and SLN's or a mixture of liposomes and SLN's were centrifuged at 80,000g for 30 min. at 4°C and the absence or presence of flocculates or pellets noted.

Particle Size Measurement and Zeta Potential

SLN's were diluted to an appropriate concentration with HEPES/NaCl buffer. The mean particle size was determined using photon correlation spectroscopy (N4 Plus, Coulter Electronics Inc., Hialeah, FL.). Mean particle diameter was calculated in size distribution processor mode (SDP), using a particle count rate of between 5×10^4 and 1×10^6 counts per second. The zeta (ζ) potential was measured at different pH values in isotonic buffers using a Delsa 440SX (Coulter Electronics Inc., Hialeah, FL.).

Determination of Trapped Calcein and 6-CF in Phospholipid Bilayer

Free 6-CF or calcein was removed from the SLN preparations by gel permeation chromatography on a PD-10 column (Sephadex G-25M; Pharmacia, Sweden) using HEPES/NaCl buffer as eluent. Fractions (0.5 ml) containing SLN's were collected and analyzed as follows: Ethanol (2 ml) was added to 500 µl of calcein- or 6-CF-loaded SLN fractions. The amount of 6-CF or calcein released from the disrupted SLN was determined by spectrofluorimetry (Perkin Elmer 650-10LC spectrofluorimeter, Norwalk, CT, USA). Calcein was determined at an excitation wavelength of 491 ± 10 nm and an emission wavelength of 510 ± 5 nm. An excitation wavelength of 490 ± 10 nm and an emission wavelength of 520 ± 5 nm was used to determine 6-CF. The volume of the aqueous phase trapped between the phospholipid bilayers was calculated from the amount of 6-CF or calcein associated with the SLN's.

To determine the possibility of binding of the markers to PL, 2 ml of an aqueous solution of calcein or 6-CF was added to 2 ml SLN, and the mixture shaken for 2 hours. SLN-associated calcein or 6-CF was determined as described above after separation by gel permeation chromatography. Preliminary experiments revealed that PL, TL and ethanol had no effect on the excitation and emission spectra of either calcein or 6-CF. Standard curves were routinely prepared in the ethanol/HEPES buffer to measure SLN-incorporated calcein or 6-CF.

RESULTS AND DISCUSSION

High pressure homogenization is routinely used for the production of emulsions for iv. administration, liposomes, and SLN. Turbulence, cavitation, shearing, collision and intense mixing are among the factors responsible for size reduction. Figure 1 shows the decrease in the mean diameter of the SLN prepared by high pressure homogenization as a function of PL:TL molar ratio. A plateau was reached at a molar ratio of 0.15 for both negatively charged and neutral particles. Phospholipids energetically favor the oil/water interface. Thus, to accommodate the additional phospholipid, additional interfacial area is produced leading to smaller particle sizes. However when the radius of curvature reaches a particular low value, the phospholipids may no longer energetically favor further decreases in particle size. At this point the phospholipids form other structures, possibly including multilayers.

TL has a transition temperature (T_t) of 44°C (14) and the T_t of DPPC is 41°C (18). Therefore both lipids are present in the gel state at room temperature. Although the T_t of DMPG is 24°C at pH 7.4 (19), the effect of DMPG on the liquid-gel transition of negatively charged SLN's is negligible because of the small mol proportion used (5 mol%).

The observed amount of PL required in these experiments was much higher than the theoretical amount required to form a monolayer coverage (Figure 1). The possibility that excess PL could form liposomes or micelles was ruled out in view of the following observations:

1. SLN's floated on the top of the emulsion after ultracentrifugation for 30 min. at 4°C and 80,000 × g, but no sedimentation pellet was observed. However, a sedimentation pellet was observed after ultracentrifugation of liposomes possessing the same size range as the SLN's. A sedimentation pellet and flocculate layer was observed after ultracentrifugation when liposomes were mixed with SLN's.

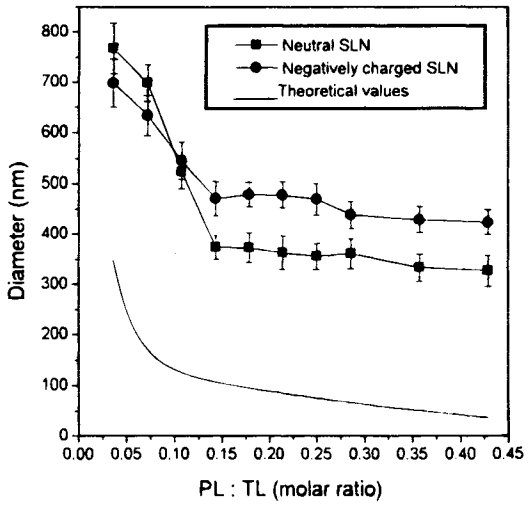

Fig. 1. The effect of the PL:TL molar ratio on the mean particle diameter of neutral and negatively charged SLN. Mean ± SD of 3 experiments. Theoretical values were calculated for neutral SLN. The experimental conditions for the determination of mean particle diameter were as follows: fluid refractive index 1.33; temperature 25°C; viscosity 0.93 centipoise; angle of measurement 90.0°; sample time 10.5 µs, and sample run time 90 sec.

2. The mean diameter of liposomes prepared using the high pressure homogenizer was 117 ± 35 nm. The particle size distribution of SLN's prepared under the same conditions did not reveal any particles with a size range appropriate for micelles or of liposomes produced by high pressure homogenization.

3. A biphasic size distribution of 84 ± 32 nm and 324 ± 39 nm was observed after mixing SLN's and liposomes.

It was therefore concluded that excess PL did not result in the formation of liposomes but was forming multiple bilayer structures around the lipid core of the SLN. Such bilayer structures occur in multilamellar liposomes (20). If this is the case for SLN's prepared with excess PL, the volume of the aqueous phase between the bilayers should increase in proportion to the number of bilayers around the SLN. This hypothesis is supported by the data in figures 2a and 2b which shows that increasing amounts of PL are associated with significant increases in the volume of the SLN-associated aqueous phase.

Figure 3 shows the associated aqueous phase volume trapped in SLN's and fluid-core nanospheres prepared using soybean oil (SB) as the fluid core. Associated aqueous phase volume was significantly greater in the liquid nanoparticles than in the SLN's, and increased with increasing PL:SB molar ratios. The incorporation of hydrophilic markers in liquid nanoparticles produced with a low amount of DPPC is indicative of the presence of inverse micelles in the liquid core (21). Using a high molar ratio of PL:SB, the associated aqueous phase volume increased as a result of the formation of liposomes. The presence of liposomes was confirmed when two size distributions of 88 ± 21 nm and 351 ± 45 nm was observed and a sedimentation pellet occurred after ultracentrifugation for 30 min. at 4°C and 80,000 × g. The existence of multiple PL bilayers on the core and trapped aqueous vesicles inside of the liquid core of fluid commercial fat emulsions has been demonstrated by Li et al. (21). The volume of aqueous phase associated with the SLN's in the present study is however considerably less than that observed by Li et al., indicating little (if any) aqueous volume trapped in the solid core of the SLN's. The exact assembly, number of layers, and the volume of aqueous phase between these layers may depend on a variety of factors such as the charge and type of PL used and the pH of the medium.

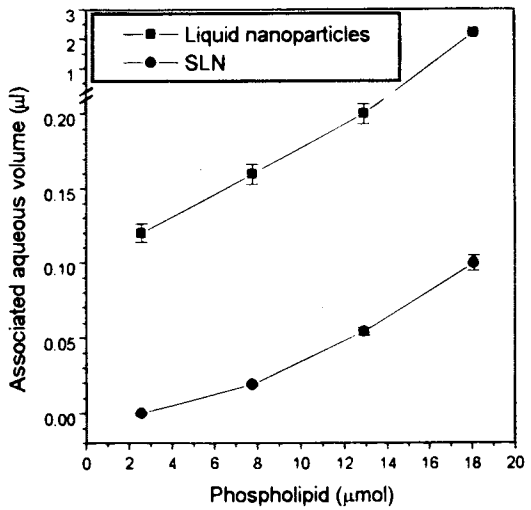

Fig. 3. Associated aqueous phase volume of neutral nanoparticles possessing a fluid (SB) or solid (TL) core. 200 mg TL or SB were used as the nanoparticle core. Mean ± SD of 3 experiments.

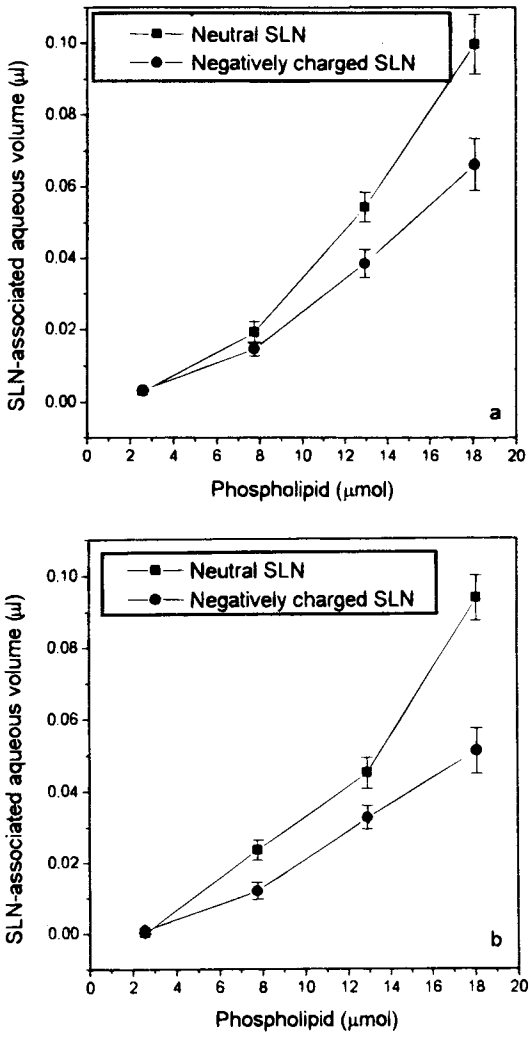

Fig. 2. Associated aqueous phase volume in SLN determined by a: 6-CF and b: calcein. Mean ± SD of 3 experiments.

Calcein and 6-CF did not associate with SLN's when mixed after preparation, and were not soluble in n-octanol. TL, DPPC and ethanol did not have any effect on the excitation and emission spectra of calcein and 6-CF. In the absence of any evidence for the chemical interaction of calcein or 6-CF with PL or TG, or of partitioning into the lipid phase of the SLN, these results indicate that a high PL:TG ratio produces SLN with multiple bilayer structures on the surface that are capable of incorporating additional marker molecules.

Table I shows that SLN's prepared with neutral phospholipid (DPPC) at pH 7.4 have a ζ potential of -4 ± 1 mV. By using a PL mixture containing a negative PL (5 mol% DMPG and 95 mol% DPPC) the negative ζ potential was increased to -13 ± 3 mV. This increase in negative ζ potential was dependent on the mol% of DMPG used to prepare the SLN's. Although there was a significant difference in particle diameter between neutral and negatively charged SLN's (Table I), increasing the DMPG content from 5 to 30 mol%, which

Table I. SLN ζ Potential as a Function of mol % DMPG[a]

Phospholipid mixture DPPC/DMPG mol%	ζ potential (mV)	Diameter (nm)
100/0	−4 ± 1	301 ± 29
95/5	−13 ± 3	378 ± 34
90/10	−26 ± 3	388 ± 29
80/20	−39 ± 4	375 ± 28
70/30	−54 ± 5	402 ± 39

Note: The experimental conditions for the determination of ζ potential were as follows: current 0.7 mA; frequency range 500 Hz; temperature 25°C; fluid refractive index 1.33; viscosity 0.93 centipoise; dielectric constant 78.3; conductivity 16.7 ms/cm; on time 2.5 sec; off time 0.5 sec, and sample run time 60 sec.

[a] SLN's were prepared with PL:TL molar ratio of 2:7 in HEPES/NaCl buffer. Mean ± SD of 3 experiments. The increase in ζ potential of DMPG-containing SLN's was linearly correlated with the mol% DMPG ($r = 0.99$, $p = 0.007$) but not with diameter ($r = 0.63$, $p = 0.36$).

resulted in a significant increase in ζ potential, did not significantly increase the particle diameter (Table I). The ζ potential of neutral and negatively charged SLN's (95:5, DPPC:DMPG) was independent of particle diameter (Table II).

Because of the presence of the PL (bilayers) on the surface of SLN, changes in the pH of the external medium will have an effect on the electric charge of these particles. Table III shows that changes in pH produce significant changes in the ζ potential of the SLN's. DPPC has a negatively charged oxygen on the phosphate group and a positively charged quaternary ammonium group. The pK of the phosphate group of DPPC is approximately 2 (22,23). At pH 3 the suppression of ionization of the phosphate group becomes significant, resulting in dominance of the positively charged choline group. Therefore the molecule will have a net positive charge resulting from the contribution of the positive charge of the quaternary ammonium of the choline group. The addition of 5 mol% DMPG, pK = 3 (24), resulted in SLN's with a negative charge over the pH range 3 to 10. The requirement for a negative charge on the particle over a wide pH range may be necessary if unwanted interactions with biological molecules are to be avoided (25).

The effect of the negative charge on the determination of the PL-associated aqueous phase volume was significant. In

Table II. SLN Preparations with 1 mM Aqueous Solution of Calcein at pH 7.4[a]

PL:TL molar ratio	Diameter (nm)		ζ potential (mV)	
	Neutral	Negative	Neutral	Negative
0.5:7	699 ± 32	635 ± 35	−4 ± 1	−13 ± 4
1:7	385 ± 24	475 ± 34	−4 ± 1	−13 ± 3
3:7	328 ± 21	386 ± 34	−4 ± 1	−13 ± 4
5:7	313 ± 22	374 ± 31	−5 ± 2	−13 ± 4
7:7	317 ± 19	368 ± 26	−5 ± 2	−14 ± 4

[a] 100% DPPC was used as PL for the neutral SLN's and a mixture of DPPC:DMPG, 95:5 mol% was used as PL for the negatively charged SLN's. Neither calcein nor 6-CF had an effect on SLN diameter and ζ potential (data not shown). Mean ± SD of 3 experiments.

Table III. Effect of pH on ζ Potential of SLN[a]

pH	Neutral SLN ζ potential (mV)	Negatively charged SLN ζ potential (mV)
3	−3 ± 2	−4 ± 2
4	−1 ± 2	−7 ± 2
7	−4 ± 2	−12 ± 3
10	−5 ± 2	−13 ± 3

[a] 100% DPPC was used as PL for the neutral SLN's and a mixture of DPPC:DMPG, 95:5 mol% was used as PL for the negatively charged SLN's. For pH's 3 and 4 citrate buffer (10 mM); pH 7 phosphate buffer (10 mM) and pH 10 carbonate-bicarbonate buffer (10 mM) were used. Mean ± SD of 3 experiments.

negatively charged SLN's the associated aqueous volume was smaller than in the neutral SLN's. At pH 7.4 the two amino groups of calcein are protonated to give a molecule with a net charge of −4 (26). Repulsion between the negative charge of the DMPG and the negative charge of calcein may therefore decrease the amount of incorporated calcein. The same phenomenon was observed with 6-CF, which is also negatively charged at pH 7.4.

Multilayered SLN's have the potential for development into an effective drug delivery system for the controlled release or targeting of lipophilic drugs via the use of cell-specific PL's (27) or via the use of antigen specific monoclonal antibodies attached to surface PLs (28).

In conclusion, this study has demonstrated that in the presence of excess PL, a multiple bilayer structure is likely to be formed around the solid lipid core of SLN's. The volume of the aqueous phase between the PL-bilayers can be estimated from the amount of the hydrosoluble markers trapped in this phase. However, the use of charged molecules and charged PLs may effect the entry of molecules into the aqueous layers, or facilitate interactions between the PL membrane and the marker molecules (29,30). The specificity of these interactions merits further investigation.

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