Insertion of poly(ethylene glycol) derivatized phospholipid into pre-formed liposomes results in prolonged in vivo circulation time

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Abstract **Transfer of $MPEG_{1900}$-DSPE from micellar phase to pre-formed liposomes imparts long in vivo circulation half-life to an otherwise rapidly cleared lipid composition. $MPEG_{1900}$-DSPE transfers efficiently and quickly in a time and temperature dependent manner. There is negligible content leakage and a strong correlation between assayed mol% $MPEG_{1900}$-DSPE, liposome diameter increase, and pharmacokinetic parameters such as distribution phase half-life. Since a biological attribute (liposome clearance rate) can be modified by the insertion process, it suggests a simple and economical way to impart site-specific targeting to a variety of liposome delivery systems. This method is also a convenient way to measure the 'brush' thickness of such conjugates directly.**

Key words: Liposome; Poly(ethylene glycol); Insertion; Critical micellar concentration; Pharmacokinetics

1. Introduction

Since the seminal report on ganglioside GM_1 [1], other specific lipids have been discovered that impart prolonged in vivo circulation time to otherwise rapidly cleared liposomes. Poly (ethylene glycol)-derivatized (pegylated) phospholipid has been especially successful in prolonging the plasma distribution phase [2,3], and increasing the therapeutic efficacy of entrapped drugs in both animal tumor models (for reviews see [4-6]) and in the clinic [7]. In this body of work, $MPEG_{1900}$-DSPE is always co-dissolved with other lipids in an organic phase prior to hydration and self-assembly into liposomes. The next generation of liposome delivery systems will use targeting molecules to achieve site specific delivery. Many laboratories are now studying how to achieve this specificity by attaching ligands such as peptides, proteins, and saccharides as 'pendants' on the end of polymer-derivatized lipids. New methods for transferring polymer-grafted lipids to liposomes are necessary because some pendant molecules may be available in minute quantities, have limited solubility and stability in solvent, or be incompatible with various stages of manufacture. In addition, it would be advantageous if a single preparation of liposome-encapsulated drug could be divided and customized with a variety of inserted targeting candidates.

Lipid monomers are known to transfer from one lipid phase to another via the aqueous phase (for review see [8]). Kanda et al. observed that a nitroxide spin-labeled diacyl ganglioside having a critical micellar concentration (CMC) of less than 20 μM could be inserted from micelles into pre-formed multilamellar vesicles [9]. We have observed that $MPEG_{1900}$-DSPE forms aqueous micellar solutions and has a CMC below 20 μM as well. Therefore, we decided to use this pegylated phospholipid as a model compound to see if it could be inserted and retained in rapidly cleared, pre-formed liposomes in sufficient quantity to achieve its biological effect, namely, prolonged in vivo circulation time. We also report on the rate of entrapped content leakage caused by the insertion process, and the use of this process to measure directly the 'brush' thickness of polymer-derivatized lipids.

2. Materials and methods

2.1. Materials

Unspecified reagents were ACS grade purity or better and obtained from J.T. Baker Inc. (Philipsburg, NJ). The 1900 molecular weight fraction of methoxypoly(ethylene glycol) was attached via a carbamate linkage to the amino headgroup of 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine ($MPEG_{1900}$-DSPE) according to [3]. Purified $MPEG_{1900}$-DSPE had no other lipid impurities and less than 5% poly(ethylene glycol) impurity as determined by thin layer chromatography. DTPA-DSPE, an indium ion chelating lipid, was synthesized according to [10]. HSPC was purchased from Natterman AG (Cologne, Germany), DPPG and DSPE from Avanti/Polar Lipids (Alabaster, AL) and CH from Croda, Inc. (Fullerton CA). ^{111}Indium chloride was purchased from Amersham, Inc. (Arlington Heights, IL) and 5,6-carboxyfluorescein (5,6-CF) was from Molecular Probes (Eugene, OR).

2.2. Liposome preparation and $MPEG_{1900}$-DSPE insertion

The lipid matrix of HSPC/CH/DPPG/DTPA-DSPE (52.4/45.4/2.0/0.2, molar ratio) was mixed in chloroform and dried under high vacuum overnight to remove residual solvent. The hydrated multilamellar vesicles were extruded repeatedly through double-stacked 0.1 μm pore size polycarbonate membranes (Costar/Nuclepore, Cambridge, MA) in a thermally jacketed, high pressure extrusion cell (Lipex, Vancouver, BC, Canada) as described in [11] to a final mean diameter range of 80–85 nm. For leakage studies, 100 mM purified 5,6-carboxyfluorescein was entrapped inside the liposomes according to [12].

Experiments were conducted by first equilibrating the liposome dispersion and a concentrated micellar $MPEG_{1900}$-DSPE dispersion to the desired temperature. The transfer reaction was started by mixing aliquots to give a final theoretical 3 mol% of $MPEG_{1900}$-DSPE to total lipid. At specified time points, the micellar material was separated from the liposome fraction by Sepharose 4B (Sigma Chemical

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Abbreviations: AUC, area under the concentration-time curve; 5,6-CF, 5,6-carboxyfluorescein; CH, cholesterol; CMC, critical micellar concentration; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol, sodium salt; DTPA-DSPE, *N*-diethylenetriaminepentaacetic acid-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, sodium salt; HSPC, hydrogenated soy 1,2-diacyl-*sn*-glycero-3-phosphocholine; $MPEG_{1900}$-DSPE, *N*-carbamyl-methoxypoly(ethylene glycol)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, sodium salt, 1900 Da methoxy poly(ethylene glycol) fraction

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Co., St. Louis, MO) column chromatography. Liposome fractions in the void volume were pooled for analyses.

2.3. Pharmacokinetics

Liposomes were loaded with ^{111}indium to measure circulating plasma levels of liposomes. Preparations were incubated with the radionuclide at room temperature for 1 h, and non-chelated radiolabel (<10%) was removed by Sephadex G-50 (Sigma Chemical Co.) column chromatography. Pharmacokinetic studies were performed using adult male Sprague-Dawley rats (205–260 g, 3 per treatment group). A 400–500 μl sample (~0.5 μCi ^{111}indium) was administered by tail vein injection. Whole blood samples of approximately 400 μl were collected from the retro-orbital sinus of anesthetized rats at selected time points. The blood samples were aliquoted into triplicate 100 μl samples and the DPMs in each sample were determined using a Beckman/Gamma 5500 gamma counter. Pharmacokinetic evaluations were performed using RSTRIP software (Micromath Inc., Salt Lake City, UT).

2.4. Analytical methods

Mean liposome diameter was determined by dynamic laser light scattering (Coulter N4, Hialeah, FL). Net increases in mean particle diameter were calculated by subtracting the pre-incubation diameter from the liposome mean diameter measured after Sepharose 4B clean-up. Absorbance measurements were made with a U-2000 spectrophotometer (Hitachi Instrument Co., San Jose, CA). Lipid phosphorus was determined according to the method of [13]. $MPEG_{1900}$-DSPE, HSPC, and CH concentration were determined using the HPLC method of [14] with modifications to be described elsewhere (C. Mendez, in preparation).

3. Results and discussion

Dispersing $MPEG_{1900}$-DSPE at concentrations as high as 20 mM produced a clear, pearlescent micellar solution. We determined the CMC of $MPEG_{1900}$-DSPE by serial dilution of a micellar solution of assayed phosphorus concentration into saline, and monitoring micelle turbidity at 240 nm (Fig. 1). Below the critical concentration, only monomers were present and light scattering was at a constant background level. Turbidity appeared and grew as the concentration of micelles increased. Regression analysis of the intersection point of scattering and background indicates the CMC for $MPEG_{1900}$-DSPE is 5.8 μM (2.2–10.8 μM, 95% confidence interval range).

The significant water solubility of $MPEG_{1900}$-DSPE suggested to us that, while hitherto this lipid had been co-dissolved with other matrix lipids in organic solvent prior to hydration, it might be possible to insert it into pre-formed liposomes. Functional insertion would presumably increase plasma circulation time of the liposomes in proportion to the final concentration of $MPEG_{1900}$-DSPE. To test this hypothesis, we prepared pre-formed liposomes containing 2 mol% DPPG to ensure rapid clearance in the absence of inserted, functional $MPEG_{1900}$-DSPE ($t_{1/2}$ <1 h, [2,15]).

The main endothermic phase transition (T_c) of HSPC is approximately 58°C (data not shown), so we prepared a two-factor, uniform shell design experimental matrix to exam-

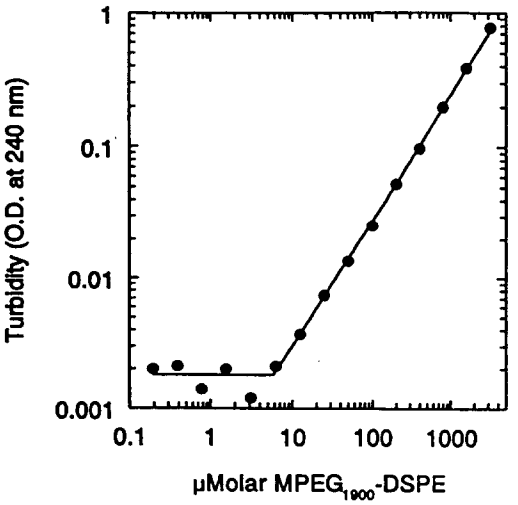

Fig. 1. Turbidometric analysis of $MPEG_{1900}$-DSPE CMC.

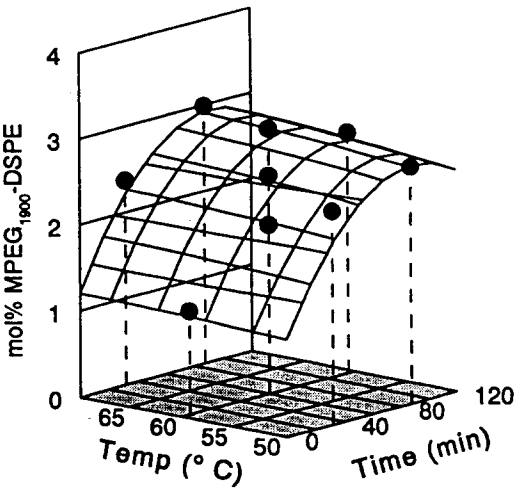

Fig. 2. Uniform shell design of time and temperature dependence of $MPEG_{1900}$-DSPE incorporation into ~80 nm diameter pre-formed HSPC/CH/DPPG/DTPA-DSPE (52.4/45.4/2.0/0.2, molar ratio) liposomes. Molar percentage assayed after incubation and column chromatography removal of non-inserted lipid. 5 min at 20°C had 0.9 mol% inserted.

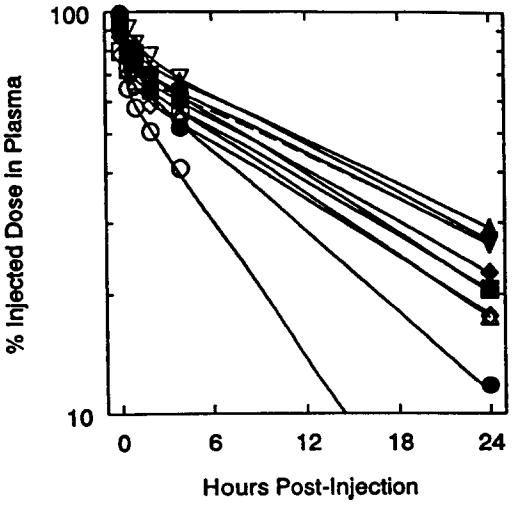

Fig. 3. Rat plasma pharmacokinetics (^{111}indium label) of preparations in Fig. 2. 0.5 μCi liposomal ^{111}indium was given by tail vein to treatment groups (n=3 males). Liposome preparations are as in Fig. 2. The dashed line is a positive control 5.5 mol% formulation prepared by co-dissolving $MPEG_{1900}$-DSPE in organic solvent with other lipids. Error bars are removed for clarity. mol% inserted measured by HPLC (○) 0.9%, (●) 1.2%, (□) 2.0%, (■) 2.4%, (△) 2.5%, (▲) 2.5%, (◇) 2.7%, (◆) 2.8%, (▽) 3.0%, (▼) 3.1%.

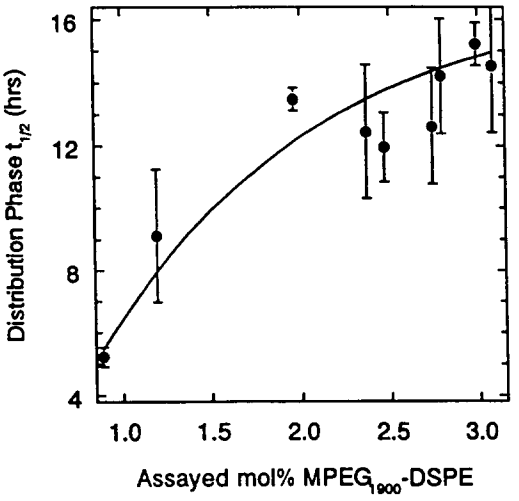

Fig. 4. Distribution phase half-life as a function of mol% $MPEG_{1900}$-DSPE of the pharmacokinetic profiles of Fig. 3. Error bars are standard deviation.

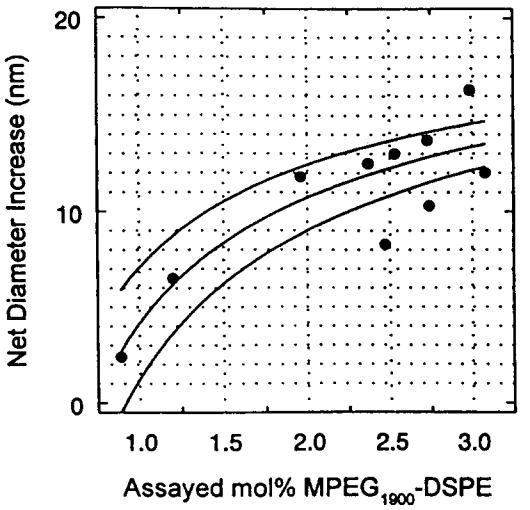

Fig. 5. Liposome diameter increase as measured by dynamic light scattering as a function of assayed $MPEG_{1900}$-DSPE. Line is fit to the flexible brush model presented in [18] with 95% confidence intervals of the estimate.

ine insertion below (50°C), at (60°C), and above (70°C) the T_c as a function of time. After co-incubation of liposomes and micelles for the specified time and temperature, the liposome fractions were collected by chromatography and analyzed for mol% $MPEG_{1900}$-DSPE (Fig. 2), changes in liposome diameter, and plasma circulation time. We also co-incubated liposomes and micelles for 5 min at 20°C as a control, fully expecting virtually no insertion of $MPEG_{1900}$-DSPE. To our surprise, this preparation was assayed at 0.9 mol% $MPEG_{1900}$-DSPE. Insertion proceeded rapidly over the studied temperature range (Fig. 2), also with about 1 mol% insertion after 5 min at 60°C and nearly reached the expected theoretical maximum (3 mol%) in about 1 h. Thus, virtually complete integration of $MPEG_{1900}$-DSPE into the liposome matrix can be achieved under these conditions.

For liposome matrices containing pegylated phospholipids, extrapolation of Silvius and Zuckerman's data suggests an in vitro half-life of exchange rate of $MPEG_{1900}$-DSPE *out* of the bilayer to be approximately 70 h [16]. Thus, if $MPEG_{1900}$-DSPE is properly inserted under our conditions, long circulation times would be expected. This hypothesis was confirmed in vivo (Fig. 3). The statistical significance of the correlation between assayed $MPEG_{1900}$-DSPE content, plasma mean residence time, plasma distribution phase half-life, area under the curve, and net increase in liposome diameter was analyzed by the Pearson product moment coefficient [17] (Table 1). The correlation between chemical, physical, and pharmacokinetic properties of the preparations is highly significant. For instance, the relationship between distribution phase half-life and mol% $MPEG_{1900}$-DSPE (Fig. 4) shows an increasingly long circulation time with increasing molar percentage. Since 80 nm liposomes made by the extrusion method are unilamellar [11] and the $MPEG_{1900}$-DSPE structure suggests negligible flip-flop from outer to inner bilayer leaflet, the density on the outer leaflet is essentially twice the assayed value.

Invariably, there was a net increase in liposome diameter following incubation and clean-up which correlated very strongly with assayed mol% $MPEG_{1900}$-DSPE and all pharmacokinetic parameters (Table 1). We attribute this increase to the formation of the flexible, polymeric 'brush'. The net diameter increase as a function of assayed $MPEG_{1900}$-DSPE content was fit to the 'flexible brush' model equation [18] (Fig. 5). Assuming that $MPEG_{1900}$-DSPE density on the outer bilayer is twice the assayed total, we estimate the brush thickness at 5 mol% surface density to be 6.1 nm (5.4–6.8 nm, 95% confidence interval). Our direct measurement, slightly larger than the 5 nm estimate inferred from zeta potential measurements [19], and from force-repulsive measurements on giant liposomes [20], may be due to different buffer conditions and lipid composition..

The significant insertion of $MPEG_{1900}$-DSPE after only 5 min at 20°C was unexpected. Since the diameter increase correlated well with insertion of $MPEG_{1900}$-DSPE, we explored the insertion phenomenon over a broader range of temperature and incubation time using particle size measurement as a rapid and convenient analytical tool (Fig. 6). For liposomes of this composition, the rate and extent of diameter increase (and inferred $MPEG_{1900}$-DSPE insertion) was proportional to tem-

Table 1
Pearson correlation coefficient (P value in parentheses) of chemical, physical and pharmacokinetic properties of liposomes with inserted $MPEG_{1900}$-DSPE

	Net nm increase	mol%	MRT	$T_{1/2}$
mol%	0.838 (0.005)			
MRT	0.948 (<0.001)	0.910 (0.001)		
$T_{1/2}$	0.948 (<0.001)	0.910 (0.001)	1.000 (<0.001)	
AUC	0.976 (<0.001)	0.876 (0.002)	0.972 (<0.001)	0.974 (<0.001)

MRT, mean residence time; $T_{1/2}$, distribution phase half-life.

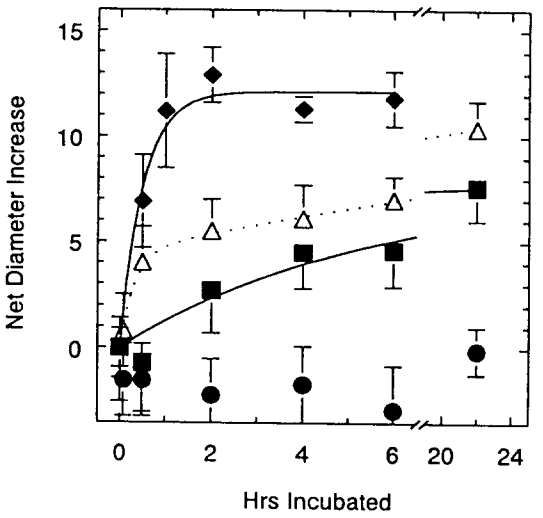

Fig. 6. Kinetics of liposome diameter increase as a function of incubation temperature. Pre-formed liposome composition and size as in Fig. 1. (●) 2°C, (■) 20°C, (△) 37°C, (◆) 60°C. The 60°C sample was incubated for only 6 h because overnight incubation resulted in significant sample degradation.

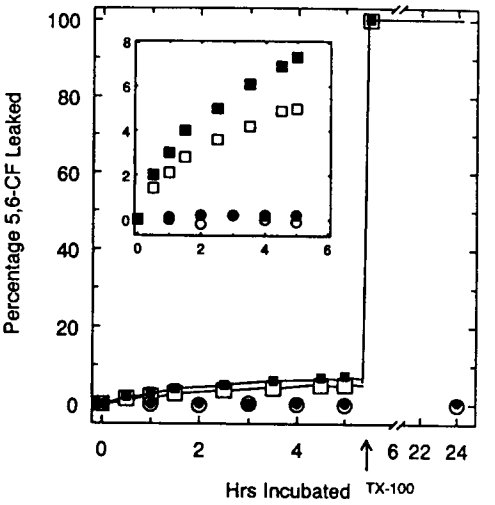

Fig. 7. Kinetics of entrapped 100 mM 5,6-CF leakage in the continuous presence or absence of 3 mol% $MPEG_{1900}$-DSPE initially in micelles. Pre-formed liposome composition and size as in Fig. 1. Inset is an enlargement of the first 5 h. Triton X-100 is added at the arrow to completely lyse the vesicles for complete leakage. (○) 37°C+(no $MPEG_{1900}$-DSPE), (●) 37°C+($MPEG_{1900}$-DSPE), (□) 60°C+(no $MPEG_{1900}$-DSPE), (■) 60°C + ($MPEG_{1900}$-DSPE).

perature. Only at 2°C was there no significant increase in diameter over 24 h.

The asymmetric addition of lipid might be expected to cause membrane defects resulting in leakage. We studied leakage at 37°C and 60°C with and without inserted $MPEG_{1900}$-DSPE by encapsulating highly concentrated and fluorescence-quenched 5,6-CF (Fig. 7). Saline or $MPEG_{1900}$-DSPE micelles in saline were added at time zero, and aliquots were removed at specified time points for determining the increase of fluorescence due to leaked 5,6-CF. Triton X-100 (Sigma Chemical Co.) was added to samples at 6 h post addition to determine 100% leakage. Although Fig. 6 indicates a considerable size increase at 37°C due to insertion of $MPEG_{1900}$-DSPE, there was essentially no dye leakage even after 24 h continuous incubation. There was less than 8% dye leakage after 5 h at 60°C in the presence of $MPEG_{1900}$-DSPE, only a one-third increase over leakage in its absence (Fig. 7, inset). Thus, the insertion process results in little if any leakage during and after the procedure, suggesting that contents are likely to remain entrapped during preparation and storage periods.

$MPEG_{1900}$-DSPE insertion is a mild and efficient procedure. The insertion rate is temperature dependent, but orders of magnitude faster than the exchange rate out of the bilayer [16] resulting in the transfer of long in vivo circulation properties to pre-formed liposomes. Since functional transfer of pegylated lipids is feasible, it may be a useful method by which custom libraries of targeted delivery systems can be created from a base formulation of liposome-entrapped drug and a collection of ligand-polymer-lipid conjugates. Future work will explore the intricate relationships between acceptor liposome composition, polymer chain length, lipid anchor, and physicochemical properties of the targeting ligand.

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