New Drug Delivery Nanosystem Combining Liposomal and Dendrimeric Technology (Liposomal *Locked-In* Dendrimers) for Cancer Therapy

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ABSTRACT: Liposomal locked-in dendrimers (LLDs), the combination of liposomes and dendrimers in one formulation, represents a relatively new term in the drug carrier technology. LLDs undergone appropriate physicochemical investigation can merge the benefits of liposomal and dendrimeric nanocarriers. In this study generation 1 and 2 hydroxy-terminated dendrimers were synthesized and were then "locked" in liposomes consisting of DOPC/DPPG. The anticancer drug doxorubicin (Dox) was loaded into pure liposomes or LLDs and the final products were subjected to lyophilization. The loading of Dox as well as its *in vitro* release rate from all systems was determined and the interaction of liposomes with dendrimers was assessed by thermal analysis and fluorescence spectroscopy. The results were very promising in terms of drug encapsulation and release rate, factors that can alter the therapeutic profile of a drug with low therapeutic index such as Dox. Physicochemical methods revealed a strong, generation dependent, interaction between liposomes and dendrimers that probably is the basis for the higher loading and slower drug release from the LLDs comparing to pure liposomes. © 2010 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 99:3561–3571, 2010

Keywords: liposome; dendrimer; doxorubicin; *in vitro* release; differential scanning calorimetry; fluorescence spectroscopy

INTRODUCTION

Since the late 1960s, interest in methods of drug delivery has focused on the creation of new modifications of established drugs with the objective of getting a drug into the patient in the simplest possible way. The proper choice of delivery system can overcome problems relating to solubility, can regulate bioavailability and can therefore improve the overall ADME profile (*A*bsorption, bio*D*istribution, *M*etabolism, and *E*xcretion) of a candidate drug.[1]

The effectiveness of a drug can generally be improved in cases where there is need of controlled release in the bloodstream. This is particularly important in the case of the treatment of certain diseases, cancer therapy, for example, in which the administration of low molecular weight cytostatic drugs by themselves can cause severe side effects due to their poor biodistribution, whereas controlled delivery can greatly improve their therapeutic profile. In this respect, drug delivery systems based on nanoscale materials have the potential for minimum release prior to reaching the target site and selective accumulation at the desired locations *in vivo* due to the enhanced permeation and retention (EPR) effect.[2]

Polymers and liposomes represent two of the most thoroughly studied categories of nanoparticles with

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potential application as carriers of bioactive molecules. Liposomes, which constitute the earliest used category of nanocarriers, are able to encapsulate either lipophilic or hydrophilic drugs in their lipidic chains or aqueous interior, respectively. Research has proven that they are able to ameliorate the pharmacokinetic and pharmacological profile of many drugs[3–5] giving way to the appearance of several liposomal formulations in the market. The application of liposomes, though, is limited because of their thermodynamic instability giving rise to phenomena such as aggregation, fusion, or drug leakage upon storage. However, these problems have been overcome to a large extent due to freeze drying.[6,7]

Dendrimers, a so-called 4th new architectural class of polymers, represent a much newer category of drug delivery vehicles.[8–10] The dendritic macromolecular structure is well-defined and consists of a central core, branching units, and terminal functional groups which can be further chemically modified. Due to their precise architecture, dendrimers possess an advantage over other generally polydisperse nanoparticles and this allows for greater control over their pharmacodynamic profile, while as vehicles for drug delivery they can be used either for encapsulation of bioactive compounds or for their covalent or noncovalent attachment at the periphery. They also offer other potential advantages such as prolongation of drug circulation time, protection of a drug from its surroundings, increase in drug stability (and possibly effectiveness), and the ability to target diseased tissue.[11–14]

Although the first attempt to incorporate a drug into dendrimers was done in 1989[15], it was only in 2001 that a combination between dendrimers and liposomes and the study of the interactions of the components took place for the first time.[16] Liposomal locked-in dendrimers (LLDs) technology—liposomes incorporating dendrimers—is a relatively new term in the drug delivery literature. Locked-in dendrimers may be viewed as a dendrimer-based class of modulatory liposomal controlled release systems (MLCRS) leading to high entrapment and modification of the release profile of bioactive molecules from liposomal vesicles.[1] It has been established that liposomal formulations of certain anticancer agents are extremely sensitive to the drug release rates, with the slowest releasing systems exhibiting the best efficacy profiles.[17,18] Therefore, for liposomal formulations, it is very important to control the drug release rate.

The milestone for the creation of the liposomal locked in dendrimer concept was the work by Khopade et al.[19] In that study, cationic PAMAM dendrimers were incorporated in the aqueous interior of liposomes in order to increase the encapsulation efficiency for the acidic anticancer drug methotrexate. The loading of the drug indeed increased proportionally to dendrimer generation while the leakage of the drug from the system decreased. The results from this work were impressive, though little data on the physicochemical interactions between the components were given. An analogous study was made by Papagiannaros et al.[20] in which a PAMAM G4–doxorubicin (Dox) complex was formed prior to encapsulation in liposomes and the results seem promising in terms of drug release and cytotoxic activity against cancer cell lines. The immobilization of anionic liposomes with PAMAM G4 has been explored using FT-IR, X-ray diffraction, and SPR[21] and the results revealed that PAMAM G4 dendrimers may be used to fabricate porous carrier films on the liposome surface in which ions or small molecules can be released from liposomes and can diffuse through the PAMAM layers. Another study by ^{31}P NMR and AFM, using neutral liposomes and lipid bilayers interacting with PAMAM G7 dendrimers gave similar results which were attributed to the formation of lipid–dendrimer aggregates.[22,23]

A very important method for studying interactions in complex systems such as LLDs is differential scanning calorimetry (DSC) and in recent years our groups have published several reports of thermal analysis data for the interaction of dendrimers with model lipid membranes or liposomes.[24–26] Despite the numerous methods applied for dendrimer–lipid interactions there is certainly a gap in the literature concerning the physicochemical characterization of LLDs.[1]

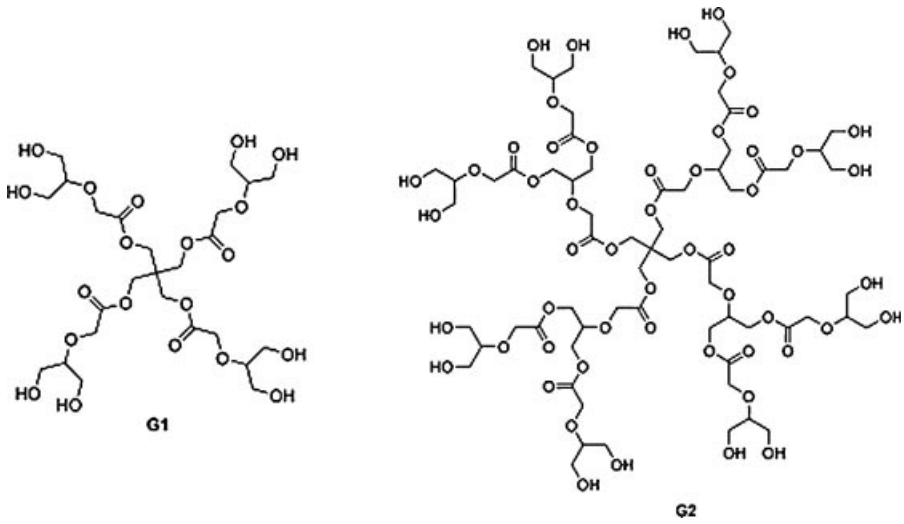
In this study new synthetic generation 1 and 2 polyether–polyester dendrimers (PG1&PG2) were incorporated into liposomes consisting of DOPC/DPPG. The anticancer drug Dox was loaded into pure liposomes or LLDs and the final products were subjected to lyophilization. The loading of Dox as well as its *in vitro* release rate from all systems was determined and the interaction of liposomes with dendrimers was assessed by thermal analysis and fluorescence spectroscopy.

MATERIALS AND METHODS

PG1&PG2 Dendrimer Synthesis

We have synthesized two new hydroxy-terminated dendrimers (Scheme 1) containing an aliphatic polyether–polyester backbone. Both bear branches composed of glycerol and acetic acid monomers. The choice of building blocks was based on a requirement for biocompatible, biodegradable, and water soluble compounds.

Following a divergent strategy we prepared dendrimers G1 and G2. Using the pentaerythritol



Scheme 1. Structure of G1 and G2 PG dendrimer.

molecule as the core, esterification with 2-[1,3–bis(benzyloxy)propan-2-yloxy]acetic acid gave G1-Bn. Removal of the benzyl groups by hydrogenolysis led to G1-OH. Following the same procedure, we also prepared G2-OH (Scheme 2).

We chose to use the benzyl ether moiety as protective group for its additional ability to serve as an NMR "tag" for the characterization of the growing molecule.

General Procedures

All reactions requiring dry or inert conditions were carried out in flame-dried glassware under an atmosphere of argon. Pd/C (10%, w/w) was purchased from Sigma Aldrich (St Louis, MO, USA), *N*,*N*′-dicyclohexylcarbodiimide (DCC) from Merck (Darmstadt, Germany). 4-(dimethylamino)*pyridinium p*-toluenesulfonate (DPTS) was prepared according to the literature.[27] Solvents were dried under argon by conventional methods. (THF distilled over sodium benzophenone and DMF over molecular sieve 40A.) Reactions were monitored by TLC (Merck Kieselgel 60 F254). After aqueous work-up of reactions mixtures, organic solutions were routinely dried over anhydrous sodium sulfate. Column chromatography was carried out on Kieselgel 60 (particle size 40–63 μm) as supplied by Merck. Size exclusion chromatography (SEC) was performed using Bio-Beads SX1 Beads 200-400Mesh from Bio-Rad (California, USA). HR NMR experiments were acquired to Varian 600 and 300 MHz spectrometers at 25°C. Compounds were dissolved in $CDCl_3$ or D_2O. The 2D ^{1}H-^{1}H DQF-COSY, ^{1}H-^{13}C edited-HSQC, and ^{1}H-^{13}C HMBC experiments assisted structure characterization. Experimental data were processed using VNMR routines. Chemical shifts (δ) are reported in ppm while spectra were referenced by the standard experimental setup. Elemental analyses were performed at the National Hellenic Research Foundation in Athens using a Perkin Elmer (Massachusetts, USA) PE2400 II analyzer. MS analyses were performed using a TSQ 7000 Finnigan MAT instrument operating in ESI mode.

Synthesis of 1,3-Bis(Benzyloxy)-2-Propanol (1)

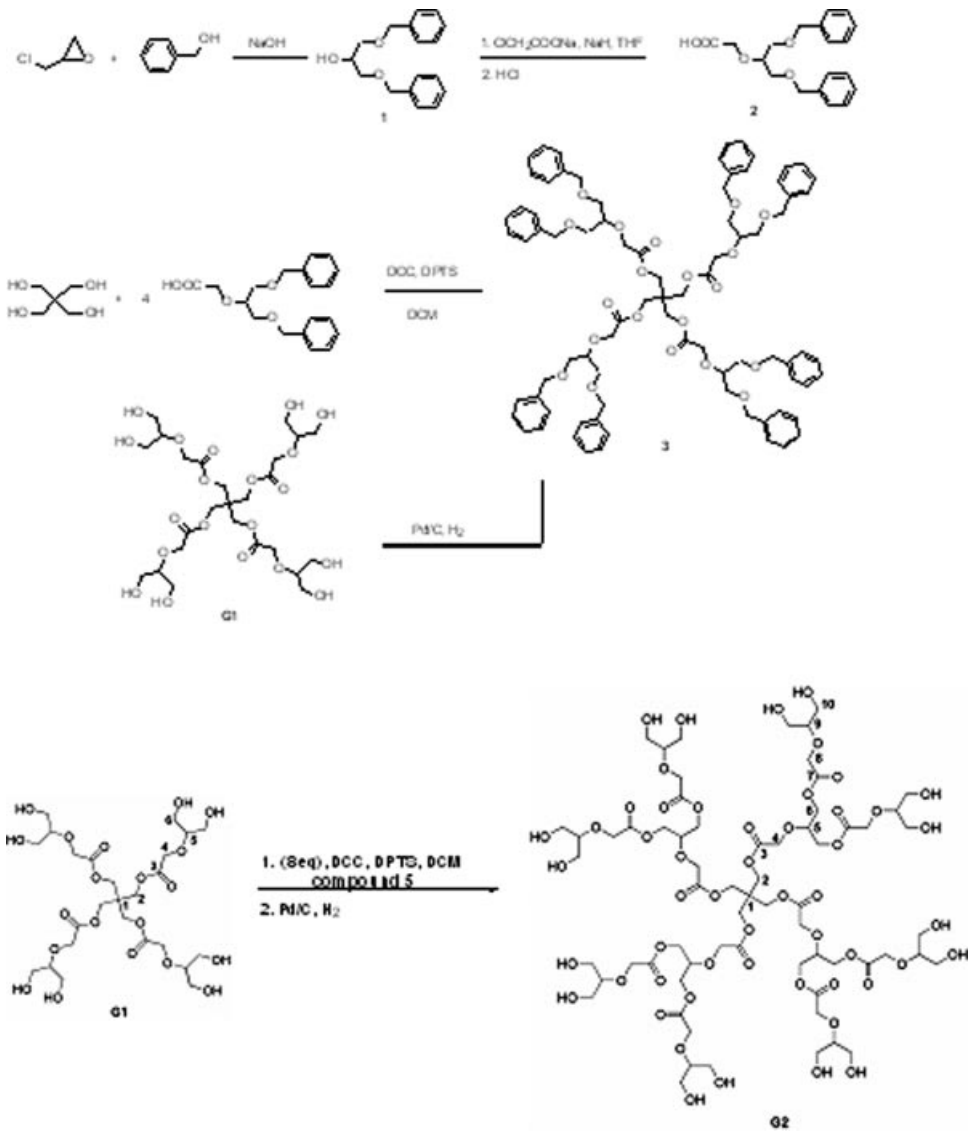
1,3-bis(benzyloxy)-2-propanol was prepared by the reaction of 2 equiv. of benzyl alcohol with epichlorohydrin according to a literature procedure.[28]

Synthesis of 2-{2-(Benzyloxy)-1-[(Benzyloxy)Methyl] Ethoxy}Acetic Acid (2)

To a suspension of NaH (5.2 g, 130 mmol, washed with toluene) in 120 mL dry THF, were added slowly 27.2 g of compound 1,3-bis(benzyloxy)-2-propanol (100 mmol). Dry sodium α-chloroacetate (12.5 g, 108 mmol) was added and the solution then refluxed for 48 h. After removal of organic solvent, the solid residue was dissolved in hot water. The aqueous solution was extracted with toluene and hexane. The aqueous phase was acidified with conc. aq. HCl and then extracted with CH_2Cl_2. After removal of organic solvent 25.7 g of the product were obtained as a yellow liquid (yield 78%).[29] ^{1}H-NMR ($CDCl_3$): δ 3.56 (d, $J = 5.4$ Hz, 4H, CH–CH_2), 3.79 (q, $J = 5.4$ Hz, 1H, CH), 4.31 (s, 2H, CH_2–C=O), 4.57 (s, 4H, benzyl-CH_2), 7.37 (m, 10H, arom.), ^{13}C NMR ($CDCl_3$): δ 68.45, 69.67, 73.46, 79.21, 127.68, 127.88, 128.39, 136.92, 172.8 (C=O).

Synthesis of G1-Bn (3)

9.9 g (30 mmol) of 2-{2-(benzyloxy)-1-[(benzyloxy)-methyl]ethoxy}acetic acid (**2**), 5.1 g (5.1 mmol) of pentaerythritol, and 1.76 g (6 mmol) DPTS were



Scheme 2. Divergent synthesis of G1 and G2 PG dendrimer.

dissolved in 50 mL of dry CH_2Cl_2. The mixture was flushed with argon and 6.2 g (30 mmol) DCC was added. The reaction mixture was stirred for 72 h at room temperature under an argon atmosphere. After filtration of urea, extraction with CH_2Cl_2, washing with water and drying over Na_2SO_4, an oily residue was obtained which after purification by silica gel column chromatography (hexane/ethyl acetate: 70/30) gave the product as an oil in a yield of 4.6 g (60%). ^{1}H-NMR ($CDCl_3$): δ 3.64 (d, $J = 9.6$ Hz, 16H, CH_2-6), 3.85 (q, $J = 9.6$ Hz, 4H, CH-5), 4.08 (s, 8H, CH_2-2), 4.39 (s, 8H, CH_2-4), 4.54 (s, 16 H, benzyl-CH_2), 7.34 (m, 40H, arom.), ^{13}C NMR ($CDCl_3$): δ 42.0 (C1), 62.1(C2), 67.7(C4), 70.6 (C6), 73.4 (C7benzyl), 78.7(C5), 127.7, 128.4, 138.1(arom.),170.2 (C3, C=O), ESI *m/z*: 1408.9 ($MH + Na^+$), (theory: 1385.6 MH). Anal. Calcd for $C_{81}H_{92}O_{20}$: C, 70.21; H, 6.69. Found: C, 70.18; H 6.57.

Synthesis of G1

To a solution of 0.2 g (0.144 mmol) of G1-Bn in a mixture of 3 mL CH_2Cl_2 and 8 mL EtOH was added Pd/C (10%, w/w). The flask was first evacuated and then filled with H_2 and the reaction mixture stirred for 4 h at RT. After completion of the reaction, the catalyst was removed by filtration through Celite and washed with EtOH. Evaporation of the filtrate gave 0.07 g (73%) of the desired product as a colorless viscous oil.

^{1}H NMR (D_2O) δ: 3.42 (s, 8H, CH_2-2), 3.45 (m, 4H, CH-5), 3.48 ((dd $J = 11.8$, 5.6 Hz), 8H, CH_2-6), 3.55 ((dd $J = 11.8$, 3.8 Hz), 8H, CH_2-6), 4.07 (s, 8H, CH_2-4); ^{13}C NMR (D_2O) δ: 45.3 (C1), 60.6 (C6), 60.9 (C2), 67.6 (C4), 81.5 (C5), 176.2 (C3) ESI *m/z*: 687.5 ($M + Na^+$), (theory: 664.2 M). Anal. Calcd for $C_{25}H_{44}O_{20}$: C, 45.18; H, 6.67. Found: C, 45.00; H 6.45.

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Synthesis of G2-Bn (5)

G2-Bn was obtained following a procedure similar to that described for compound G1-Bn. 0.390 g (0.58 mmol) of G1, 2.29 g (5.1 mmol) of 2-{2-(benzyloxy)-1-[(benzyloxy)methyl]ethoxy}acetic acid (**2**) and 0.733 g (6 mmol) DPTS were dissolved in 22 mL of dry THF and (1.74 mL) dry DMF. The mixture was flushed with argon and 1.44 g (30 mmol) DCC was added. The reaction mixture was stirred for 10 days at room temperature under an argon atmosphere. After filtration of urea and concentration to remove solvent, an oily residue was obtained which was purified by SEC (CH_2Cl_2) in a yield of 40% (0.73 g).

^{1}H NMR ($CDCl_3$) δ: 3.61 ((dd, $J = 14$ Hz, 4.5 Hz), 32H, CH2-10), 3.7 ((q, $J = 4.8$ Hz), 4H, CH-5), 3.82 (m, 8H, CH-9), 4.04 (s, 8H, CH2-2), (4.04-4.23) (m, 16H, CH2-6), 4.29 (s, 8H, CH2-4), 4.33 (s, 16H, CH2-8), 4.5 (br.s, 32H, CH2-benzyl), (7.25–7.31) (m, 80H, ar-H); ^{13}C NMR ($CDCl_3$) δ: 42.1 (C1), 62.0 (C2), 62.8 (C6), 67.7 (C4), 67.8 (C8), 70.6 (C10), 73.4 (C11), 75.8 (C5), 78.7 (C9), 127.5, 128.4 (phenyl C13-C15), 138 (phenyl C12), 170.1 (C3), 170.4 (C7) ESI *m/z*: 3.200.4 ($M + K^+$), 3.161.4, (theory: 3.161.3 M).

Anal. Calcd for $C_{177}H_{204}O_{52}$: C, 67.20; H, 6.50. Found: C, 66.9; H 6.35.

Synthesis of G2

G2-OH was obtained following a similar procedure to that used for compound **4**. To a solution of 0.5 g (0.158 mmol) of G2-Bn in a mixture of 5 mL THF and 5 mL MeOH was added Pd/C (10%, w/w). The flask was first evacuated and then filled with 50 psi of H_2 and the reaction mixture stirred for 20 h at room temperature. After completion of the reaction, the catalyst was removed by filtration through Celite and washed with MeOH and THF. Evaporation of the filtrate gave 0.245 g (yield 90%) of the desired product as a colorless viscous oil. ^{1}H-NMR (D_2O) δ: 3.47 (s, 8H, CH_2-2), 3.62-3.51 (m, 60H, CH-5,9, CH_2-6,10), 4.21 (s, 24H, CH_2-4,8); ^{13}C NMR (D_2O) δ: 42.1 (C1), 60.5 (C6, C10), 60.9 (C2), 66.8 (C4, C8), 81.5 (C5, C9), 174.7(C3, C7). ESI *m/z*: 1744.5 ($M + Na^+$), (theory: 1721.52 M). Anal. Calcd for $C_{65}H_{108}O_{52}$: C, 45.35; H, 6.32. Found: C, 45.55; H 6.45.

Pure Liposome and Liposomal Locked in Dendrimer Preparation

The liposomes prepared in this study consisted of DOPC and DPPG at a 10:0.6 ratio (the lipid system from now on will be referred to as just DOPC). In the case of LLDs, dendrimeric solutions in methanol were mixed with the lipid solutions. The initial dendrimer/lipid molar ratio was 0.1. $(NH_4)_2SO_4$ 150 mM, pH 5.5, with 150 mM sucrose as a cryoprotectant was added and the mixture was vortexed until the induction of a homogenous emulsion. MLV preparation was made using the reverse phase evaporation method (REV).[30] Sonication was applied to afford SUVs with reduced P.I. and the extraliposomal pH was changed to 7.5 through gel permeation chromatography using a Sephadex G75 column with PBS 10 mM pH 7.5/150 mM sucrose as a mobile phase.

Dox was loaded to pure liposomes or LLDs by incubation in room temperature for 1 h. Unentrapped Dox was removed by gel permeation chromatography (Sephadex G75).

Freeze Drying of Liposomal Suspensions

Free or Dox-loaded liposomes were frozen at −80°C overnight and were subjected to lyophilization in order to overcome stability issues concerning liposomal suspensions.[31] The lyophilization was achieved using a freeze drier (TELSTAR Cryodos-50, Terrassa, Spain) under the following conditions: condenser temperature from −50°C, vacuum 8.2×10^{-2} mb). Reconstitution was made by adding the appropriate amount of HPLC-grade water.

Characterization of Free and Doxorubicin-Loaded Liposomes

The hydrodynamic diameter of empty and Dox-loaded liposomes was measured by light scattering. Size and ζ-potential of liposomes are the parameters that indicate their physical stability. 100 μL of the liposomal suspension was 30-fold diluted in HPLC-grade water (pH 5.6–5.7) immediately after preparation or after reconstitution and *z*-average mean and ζ-potential of the empty and Dox-loaded liposomes were measured. Samples were scattered (633 nm) at 90°, and measurements were made at 25°C in a photon correlation spectrometer (Zetasizer 3000 HS, Malvern Instruments, Malvern, UK) and analyzed by the CONTIN method (MALVERN software).

Dendrimer and lipid quantification was done by HPTLC-FID (Iatroscan)[32] with chloroform/methanol/water 60:20:3.2 (v:v) as a mobile phase.

The incorporation of Dox into liposomes was determined by UV spectrometry (UV-1700, UV–Visible Spectrophotometer, SHIMADZU, Pharmaspec, Kyoto, Japan) at wavelength 480 nm after the addition of methanol to the liposomal suspension, with the aid of a Dox calibration curve in methanol. Pure methanol was used as blank.

In Vitro Release Studies

Dox-loaded Liposomal suspensions were placed in 12000 MWCO 25 mm width dialysis sacks (Sigma Aldrich, St Louis, MO, USA). Dialysis sacks were inserted in RPMI 5% medium in shaking water bath (Selecta, Barcelona, Spain) set at 37°C. Aliquots of samples (1 mL) were taken from the external solution at specific time points and that volume was replaced with RPMI incubated at 37°C. Dox concentrations

were measured with UV spectrometry after the addition of 2 mL HPLC-grade water. As reference sample RPMI at 37°C, diluted threefold with HPLC-grade water was used. The cumulative percentage of drug release was calculated and plotted versus time using the equation:

$$\% \text{ Released Dox} = \frac{[\text{Dox}]_{\text{released}}}{[\text{Dox}]_{\text{initial}}}$$

Membrane Fluidity Measurements

The change of fluidity of the lipid bilayer due to increasing dendrimer incorporation in the membrane was measured using a steady-state fluorescence polarization technique. Two different fluorescent probes were used: 1,6-diphenyl-1,3,5-hexatriene (DPH), an apolar molecule which is incorporated into the hydrophobic region of the liposome bilayer with its long axis parallel to the acyl chains, and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), which is anchored at the surface of the liposome bilayer in contact with the water due to its positively charged amino groups. Since DPH probe is incorporated deeper into the lipid bilayer than TMA-DPH, the use of both probes in the same lipid membrane allows for the comparison of membrane order at different depths of the bilayer. Measurements were made using a Perkin Elmer luminescence spectrometer LS-50B equipped for fluorescence polarization measurement. Three hundred micromolar liposomal suspension was added to the cuvette followed by the addition of 1 μM DPH (in tetrahydrofuran) or TMA-DPH (in methanol). The sample was stirred well and incubated in the dark at room temperature for 20 min. The cuvette holder was temperature controlled by water thermostat (MLW-U) with 37°C. The readings were taken at intervals of 2 s. The fluorescence anisotropy values (r) of the samples were calculated by the fluorescence data manager program FL WinLab The Perkin-Elmer corporation, Version 3.00 using the following equation:

$$r = \frac{(I_{VV} - GI_{VH})}{(I_{VV} + 2GI_{VH})}$$

where I_{VV} and I_{VH} are the vertical and horizontal fluorescence intensities, respectively, to the vertical polarization of the excitation light beam. The factor $G = I_{HV}/I_{HH}$ (grating correction factor) corrects the polarizing effects of the monochromator. The excitation wavelengths were 348 and 340 nm the fluorescence emission was measured at 426 and 430 nm nm for DPH and TMA-DPH, respectively.

Thermal Analysis Measurements

The DSC method was used for the stability characterization of the DOPC lipid bilayers incorporating dendrimers. The measurements were performed with a TA Instruments DSC 2920. The lipid bilayer samples where prepared by hydration of dry lipid/dendrimer mixtures at appropriate ratios (0%, 3%, 10%, 20% of dendrimer content) in excess of HPLC-grade water and were placed in stainless steel pressure-resistant 60 μL pans which were sealed. Empty pan was used as the reference. Two cooling-heating cycles were performed from 25 to −50°C at 2°C/min scanning rate. The second heating run was taken into account.

The raw data were worked out with the software "THESEUS"[33] dedicated for handling raw calorimetric data. Briefly, the output signal in mW units was divided by the sample lipid mass and by the heating rate to be converted into apparent heat capacity, C_P^{app} in $kJ\,K^{-1}\,mol^{-1}$ units. The trace of C_P^{app} was finally scaled with respect to the baseline to obtain the excess (with respect to the low temperature lipids state) specific heat, $C_P^{ex}(T)$. The area underlying the recorded peaks, so treated, directly corresponds to the lipid phase transitions relevant enthalpy in $kJ\,mol^{-1}$ units. Errors were evaluated on the basis of at least three replicas.

Statistical Analysis

Results are shown as mean ± SD of $n = 3$ independent experiments. To analyze differences in variables before and after freeze drying the paired Student's t-test was used. Comparison between different groups was done using One-way ANOVA followed by Tukey multiple comparison test when equal variances were assumed and Dunnett's C multiple comparison test when equal variances were not assumed. In order to assess the correlations between the pairs of the variables parameters of the regression line were estimated together with the regression coefficient r. The no correlation hypothesis was rejected on the $p = 0.05$ significance level. p-Values <0.05 were considered statistically significant. Statistical analysis was done using SPSS 14.0.

RESULTS AND DISCUSSION

Characterization of Free and Dox-Loaded Liposomes

The physicochemical characteristics of free and Dox-loaded liposomes and LLDs before freeze drying or after reconstitution can be seen in Table 1. PG1 incorporation in DOPC liposomes did not affect their size, z-potential or P.I. Respective data were obtained for PG2 LLDs, except the case of vesicular size that was augmented from 70 to 96.7 nm. This fact could be due to bigger dendrimer size and higher encapsulated dendrimer/lipid ratio in the case of PG2 leading to higher level pressure of the aqueous interior of the liposome and consequently to larger vesicles.[34] Dox

Table 1. Physicochemical Characteristics and Component Ratios of Final Nanosystems

Nanosystem	Size (nm)	SD	PI	SD	ζ-Potential (mV)	SD	Dox/Lipid	SD	PG/Lipid	SD	Lyophilization
DOPC	70	4.4	0.273	0.012	−24.4	5.3					Before
	71.2	1.6	0.272	0.021	−30	1.8					After
DOPC/DOX	73.2	0.4	0.308	0.029	−22.8	4.6	0.19	0.02			Before
	106.5	10.7	0.386	0.028	−26.1	6.9					After
DOPC/PG1	62.3	2.6	0.271	0.01	−19	0.1					Before
	67.9	1.1	0.246	0.006	−22	0.7					After
DOPC/PG1/DOX	63.9	2.8	0.274	0.016	−19.1	1.3	0.24	0.02	0.21	0.03	Before
	112.6	15.6	0.369	0.038	−17.8	3.2					After
DOPC/PG2	96.7	14.2	0.298	0.129	−32.5	18.9					Before
	96	4.2	0.285	0.054	−29.4	5.7					After
DOPC/PG2/DOX	93.8	16.6	0.316	0.043	−19.6	1.6	0.28	0.03	0.31	0.12	Before
	109.6	20.1	0.382	0.037	−19.1	5.2					After

incorporation in pure liposomes or LLDs did not affect any of the physicochemical characteristics of the empty systems. Reconstitution after freeze drying was successful in all cases, with Dox-loaded vesicles presenting size increase and higher P.I. index, as seen elsewhere in the literature.[35] This could be explained by a slight leakage of Dox during freeze drying due to thermomechanical stress during freezing stage on the membrane affecting its permeability.[36] As reported in the literature[37] extraliposomal Dox may induce aggregation of fusion of negatively charged vesicles leading to higher mean diameter and P.I. values. In our case the slight increase of the parameters above should be due to partial vesicular fusion. In the case of aggregated particles, according to Fonseca et al., the extraliposomal Dox can be removed by chromatography or cation exchange resins and the process becomes reversible. In our case there was no reversibility when removing the extraliposomal Dox, which furthermore accounted to <5%. From these remarks it is concluded that the leakage of Dox during freeze drying leads to fusion of the lipidic vesicles while probably, an amount of free Dox gets reencapsulated inside the fused vesicles during the process.

Dendrimer locking into liposomes was almost quantitative, as measured by HPTLC-FID. Although there was significant phospholipid loss during production steps, more than 90% of initial dendrimer was entrapped in the liposomal vesicle leading to a dendrimer/lipid ratio of 0.21 ± 0.03 and 0.31 ± 0.12 for PG1 and PG2 LLDs, respectively (see Tab. 1). Freeze drying of LLDs did not affect dendrimer entrapment, as more than 97% of dendrimers remained in the vesicle as measured by HPTLC-FID after gel permeation chromatography.

Dox loading to either pure liposomes or LLDs using ammonium sulphate gradient was in the range of 95% as reported in the literature.[38] Interestingly, Dox/lipid ratio was found to be higher when comparing PG1 LLDs to pure liposomes (0.24 ± 0.02 against 0.19 ± 0.02) and even higher in the case of PG2 LLDs (0.28 ± 0.03). These results can be explained by the size difference between PG1 and PG2, with PG2 bearing more chemical groups and steric space able to interact with bioactives like Dox in a stronger manner. These results confirm the findings of Khopade et al.[19] In that work, methotrexate/lipid ratio increased by increasing PAMAM dendrimer generation, reaching a plateau at generation 5. This fact was explained either by the basicity caused by PAMAM dendrimers establishing a pH gradient, or by the interaction of final –NH2 groups of PAMAM with –COOH of methotrexate. In our case, since PG dendrimers are uncharged, interactions with Dox should involve hydrogen bonding and hydrophobic interactions.

Freeze drying did not affect Dox entrapment, as is reported in the literature.[39] More than 95% of Dox remained in the vesicle, as measured by UV–Vis after gel permeation chromatography following reconstitution.

In Vitro Release Studies

The entrapment of dendrimers in the liposome affected significantly the *in vitro* release rate of encapsulated Dox. Although there was fast leakage during the first hour of the experiment for both pure liposomes and LLDs ($32.6 \pm 1.5\%$ for DOPC and $19.7 \pm 5.4\%$ and $18.7 \pm 1.9\%$ for DOPC/PG1 and DOPC/PG2, respectively) the release of Dox from LLDs after that time point was significantly lowered comparing to pure liposomes. At 96 h the cumulative release for pure liposomes was $74.6 \pm 7.8\%$ while for DOPC/PG1 it was $32.2 \pm 1\%$ and for DOPC/PG2 it was $27.9 \pm 2.8\%$ (see Fig. 1). From the data obtained it is obvious that the interaction of dendrimers with Dox created the appropriate force for the latter to be maintained in the interior of the liposome. These findings are in accordance with Khopade et al.[19] who found significant lowering of methotrexate release from LLDs compared with pure liposomes. Khopade

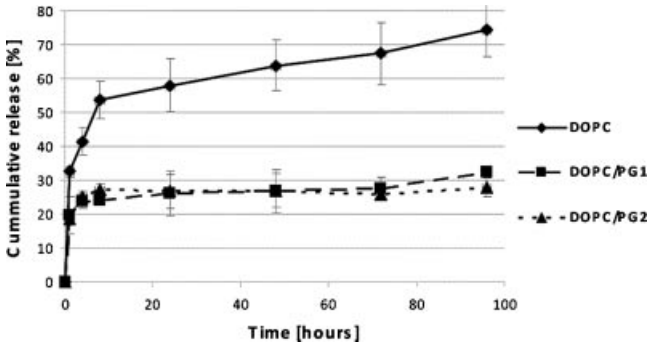

Figure 1. *In vitro* release of Dox-loaded pure liposomes or LLDs in RPMI 5%.

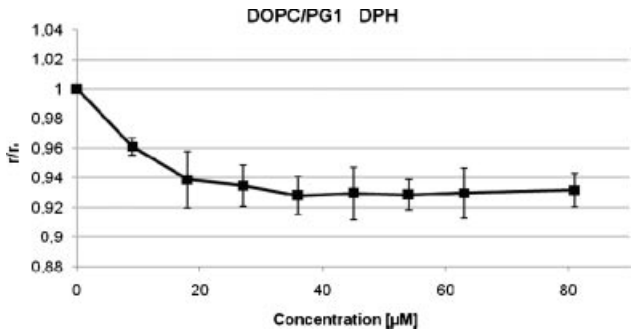

Figure 2. Fluorescence anisotropy of DPH probe incorporated in DOPC liposomes with increasing concentrations of PG1 dendrimer.

et al. also noticed dependence of release rate on dendrimer generation, comparing generations 2, 3, and 4. In our case the difference between generations 1 and 2 was not significant and this fact should be attributed to the open structure of small generation dendrimers (below generation 3).[40,41] Such open structure could not provide the appropriate dendrimer conformation for encapsulation of relatively big molecules such as Dox. We may thus assume that there is no internalization of Dox in the dendrimer cavity but more likely the formation of Dox–dendrimer network, meaning Dox molecules surrounded by and interacting with dendrimer molecules. In this kind of network the size of dendrimer and the dendrimer to lipid molar ratio would be less significant than the conformation of generation 1 and 2 dendrimers that should be similar for both dendrimers due to their similar chemistry and is affected by the greater mobility of dendrimer molecules at 37°C compared to 25°C that the Dox loading process takes place. The fast release of Dox from LLDs during the first hour should be due to the liquid crystalline structure of DOPC membranes at 37°C. Liposomes in liquid crystalline state bear lipid chains in gauche conformation and in a state of great mobility, permitting Dox to cross the membrane easily.[42] Thus, probably, during the first hour of the experiment the Dox fraction in the interior of the liposome that does not interact with dendrimers leaks out of the vesicle leading to high release rate. From then on the release rate diminishes as the interliposomal Dox inside the liposome is in dendrimer "bound" form.

Membrane Fluidity Measurements

Fluorescence spectroscopy was applied in order to estimate the interaction of PG1 and PG2 dendrimers with DOPC liposomes. The polar head groups of phosphatidyl choline did not seem to interact significantly with either dendrimer as TMA-DPH anisotropy values did not change significantly upon dendrimer incorporation (data not shown). PG1 exhibited a significant interaction with the apolar part of the membrane, inducing a concentration-dependent fluidization of the membrane (Fig. 2). The DPH anisotropy decreased until a PG1/DOPC ratio of 0.09 and then remained constant, meaning that higher dendrimer concentration did not induce more fluid membrane. In the case of PG2 (that is significantly bigger compared to PG1) the data were very scattered probably due to the induction of membrane phase separation (see thermal analysis chapter). Thus clear anisotropy conclusions cannot be obtained for the DOPC/PG2 system (Fig. 3).

Thermal Analysis Measurements

The incorporation of PG1 and PG2 dendrimers into the DOPC lipid bilayers affected significantly the thermotropic behavior of the membrane lowering the T_m, as can be seen in Figures 4 and 5. On the other hand, the ΔH of the transition was not affected by the incorporation of the dendrimers (all enthalpies were in the order of $36 \pm 2\,\mathrm{kJ\,mol^{-1}}$) leading to the conclusion that the destabilization effect induced by dendrimer incorporation was predominantly of entropic nature. This is in line with the lack of interactions of the lipid polar groups with the dendrimers

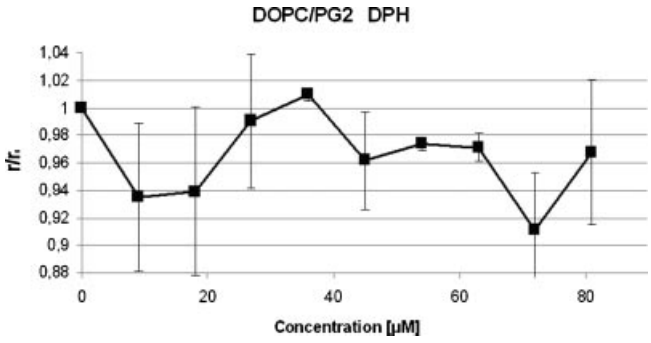

Figure 3. Fluorescence anisotropy of DPH probe incorporated in DOPC liposomes with increasing concentrations of PG2 dendrimer.

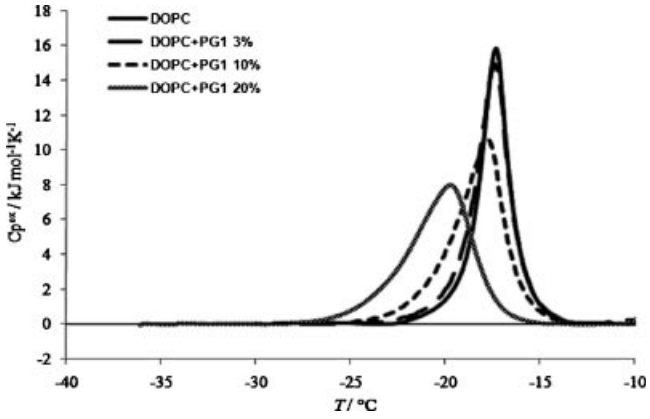

Figure 4. DSC thermograms of DOPC lipid bilayers in the presence of increasing concentrations of PG1 dendrimer.

mentioned above (see fluorescence spectroscopy). These destabilizing effects are much more pronounced in the case of PG2 which permits the system to form more than one phase (see Fig. 5) with different stability and order parameters. Similar destabilizing effects concerning the thermal interaction between dendrimers and liposomes have been documented in the literature.[24,25] This tendency, in fact, may prevent a clear measurement with the fluorescence anisotropy method (see Fig. 3). The lowering of the T_m of the membrane due to incorporated dendrimers leads to the conclusion that PG1 and, especially, PG2 induce a more fluid lipid bilayer, that reflects a higher mixing affinity of the dendrimers in the liquid crystalline phase.[43,44] This is favorable in the case of DOPC liposomes, since vesicular incorporation of dendrimers and Dox as well as *in vivo* administration of the final product take place with vesicles in the liquid crystalline phase.

Although lipid bilayers and liposomes are formed in different regions on the DOPC/DPPG/water/dendrimer phase diagram, one may nonetheless advance the

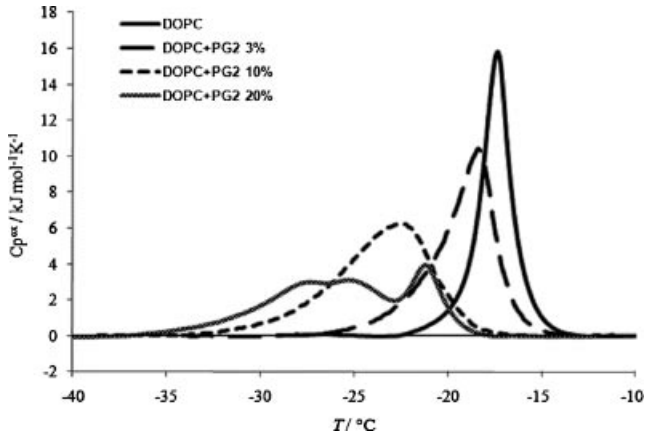

Figure 5. DSC thermograms of DOPC lipid bilayers in the presence of increasing concentrations of PG2 dendrimer.

hypothesis that the high flexibility (without compromise the enthalpic interactions) allow more phases to be hosted within the outer envelope of the liposomes in the presence of dendrimers. Addition of Dox could imply further possibilities of molecular arrangements pushing the system to a more stable thermodynamic state (including the migration of some Dox+ dendrimer complexes toward the liposomal aqueous core). This is maybe is on the basis of the higher Dox encapsulation efficiency of DOPC/PG2 system.

CONCLUSION

Despite the numerous advantages of dendrimers and liposomes, a combination of them has appeared very few times in the literature. LLDs could be considered as an efficient class of MLCRS with a great potential for carrying a high drug load and modifying the drug release rate from the system. The modification of the above parameters may increase the therapeutic index profile of the carried bioactive substance.

In this work liposomes and new polyether–polyester dendrimers were combined to afford a novel lyophilized LLD system incorporating the potent anticancer drug Dox. The LLDs exhibited high drug loads and slower *in vitro* release rate compared to pure liposomes. In order to study the physicochemical interactions between lipidic components and dendrimers, fluorescence spectroscopy and thermal analysis were applied. The combination of those two techniques revealed a strong, concentration dependent, interaction between the lipidic chains of DOPC and PG1 or PG2 dendrimers. On the other hand, the interaction of the polar head groups of the lipids with both dendrimers was found to be not significant. Furthermore the interaction between lipids and dendrimers is of entropic nature, as derived from thermal analysis results, leading to the conclusion that probably there is no bond formation between lipids and dendrimers but the interactions are of steric nature and are favored in the interior of the acyl chains. PG2 dendrimer at high concentrations induces a clear phase separation of the lipid bilayer due to its larger size, probably leading to higher encapsulation percentage into the more thermodynamically favorable aqueous interior of a unilammelar liposomal vesicle.

The loading of Dox to the liposomal locked in dendrimers through pH gradient method was highly successful. Apart from the protonation of Dox shifting the equilibrium of free to encapsulated Dox to the encapsulated form, in the systems we studied there was a second force, incorporated dendrimers, which increased the antracycline's loading. Probably the complex formed by dendrimers and Dox while crossing the membrane finds a more thermodynamically

stable location at the interior of the liposome leading to high drug loading and slower release compared to pure liposomes. The interaction between Dox and LLDs is the subject of a forthcoming publication by our group.

To summarize, LLDs seem like a promising system for the delivery of bioactive molecules. In the case of a drug with low therapeutic index such as Dox, high drug to carrier ratios and minimum release in the bloodstream prior to reaching the target tissue are of utmost importance and LLDs seem to be able to help in both directions. Care has to be taken, though, to the greatest consideration of physicochemical interactions of such complicated systems in order to rationally design stable, safe, and efficient drugs.

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