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Liposomes as a carrier for gentamicin delivery: Development and evaluation of the physicochemical properties

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ABSTRACT

The physicochemical properties of liposomal formulations containing gentamicin were investigated. A sustained release of gentamicin from liposomes was observed at both 4 °C and 37 °C in phosphate buffered saline. The distribution of the mean diameters of these liposomal formulations, evaluated by dynamic light scattering (DLS) over a 48 h time period, was bimodal with large polydispersity index values, i.e., ≥0.6. Incorporation of 5- or 16-doxylstearic acids (5- or 16-DSL) into the liposomes allowed the use of EPR spectroscopy to study the fluidity, order parameter, and phase behavior of the phospholipid bilayers in response to the compositions, temperature and time. While, our results revealed that gentamicin disturbs the packing and fluidizes the phospholipid chains, it did not seem to alter the nature of the microdomains at the polar interface of the bilayers. Simulation of the EPR spectra of 5-DSL containing liposomes revealed (1) the heterogeneous nature of the liposomal domains at the polar interfacial region, and (2) that encapsulation of gentamicin neither significantly alters the dynamic properties of the existing domains, nor induces the phase repartition of the liposomes within a 48 h time course.

1. Introduction

Gentamicin is an effective broad-spectrum antibiotic that exhibits rapid concentration-dependent antimicrobial efficacy to various Gram-negative organisms. However, bacterial barriers and adverse effects such as nephrotoxicity and neurotoxicity limit its daily dosage (McKinnon and Davis, 2004). Efforts have been made to determine its optimal therapeutic regimens in order to increase its overall efficacy while minimizing drug toxicity.

Liposomal drug delivery systems have the distinct advantage of being both nontoxic and biodegradable because they are mainly composed of phospholipids and phospholipid derivatives (Schiffelers et al., 2001a). Over the years, significant breakthroughs have been achieved in liposomal formulations for drug delivery applications (Barenholz, 2001) and a few liposomal drugs, that are mainly antifungal and antitumor, have been approved for clinical use (Lian and Ho, 2001). The encapsulation of aminoglycosides into liposomes significantly improves their therapeutic index by altering their pharmacokinetics and pharmacodynamics, thus enhancing the accumulation of the drug at the infection sites (Xiong et al., 1999). In spite of the numerous advantages of using liposomes as carriers to deliver gentamicin over the free form of the drug, *in vitro* studies of liposome-encapsulated aminoglycosides have been mainly focused on the elucidation of the relationship between liposome composition and the efficacy of the inhibition of bacterial growth (Schiffelers et al., 2001a). Recently, extensive studies focused on characterizing the physical properties of liposomes, such as the measurement of vesicle size and their population distributions, phase structures and dynamics, entrapped volume, as well as their structural flexibility and packing rigidity, have been conducted to address the pharmacokinetic issues of liposome drug delivery (Hyslop et al., 1990; Marsh, 2001; Rappolt et al., 2003; Small, 1984). However, detailed information at the molecular level concerning how the structural characteristics of lipid bilayers

Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phophocholine; PBS, phosphate buffered saline; EPR, electron paramagnetic resonance; chol, cholesterol; 5-DSL, 5-doxylstearic acid; 16-DSL, 16-doxylstearic acid; SL, spin label; SL-Gentamicin, spin labeled gentamicin; RES, reticuloendothelial system; PC, phosphocholine; PS, phosphaidylserine; PEG, polyethylene glycol; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; EPC, egg phosphocholine; CHEMS, cholesteryl-hemisuccinate; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]; DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine; DLPS, dilauroylphosphatidylserine.

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affect the physical stability and therapeutic efficacy of liposomal formulations is lacking. In this study, we report a detailed evaluation of the stability of DPPC liposome–gentamicin assemblies in relation to their biophysical properties and chemical characteristics of liposomes.

We utilized electron paramagnetic resonance (EPR) spectroscopy to provide structural and dynamic information on liposomal systems. EPR spectroscopy is extensively used to detect stable free radicals, such as the nitroxide ($NO^{\bullet}$-containing) radicals and their derivatives (Plonka and Elas, 2002; Swartz et al., 2004), that have been incorporated either into the phospholipid bilayers or in the aqueous core compartment of the liposome. The change in the EPR spectral line-shapes of the free radicals as a function of the liposomal composition, temperature and time reveals precise information on the local (re-) organization in the conformation and orientation of the phospholipids (Swartz et al., 2004). For example, the EPR spectrum of DPPC-5-doxyl stearic acid vesicles will change from three relatively broad peaks to three sharp peaks at the main transition temperature of pure DPPC (T_m = 41.1 °C) where the vesicles change from the gel phase to the liquid crystalline phase (Roche et al., 2006).

The aim of the present study was to evaluate the important factors associated with the *in vitro* stability of gentamicin-containing DPPC liposomes. We first assessed the encapsulation efficiency of the liposomal formulations along with the effect of gentamicin on the mean diameters and the polydispersity index of the liposomes. We also sought to determine the phase behavior of phospholipids as a function of composition and temperature. We also investigated the changes in fluidity of the phospholipid chains at both the polar interface and the central region of the bilayers at 37 °C over a 48 h time period to obtain insight on the structural integrity of DPPC/chol/gentamicin liposomes. Finally, we elucidated the liposomal stability at 37 °C paying particular attention to the effect of gentamicin on the molecular dynamics and the characteristics of the microdomains.

2. Materials and methods

2.1. Chemicals

The spin-labeled stearic acids, 5-doxylstearic acid (5-DSL) and 16-doxylstearic acid (16-DSL) were purchased from Sigma–Aldrich (St. Louis, MO, USA), along with ascorbic acid and cholesterol. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was obtained from Northern Lipids Inc. (Vancouver, BC, Canada). Gentamicin sulfate was obtained from TMD Chemicals Inc. (Gibbstown, NJ, USA). Spin labeled gentamicin (SL-gentamicin) was synthesized in our facility. Sucrose was from Caledon Laboratories Ltd. (Georgetown, ON, Canada).

2.2. Liposome preparation

DPPC liposomes were prepared using the dehydration–rehydration method as previously described (Mugabe et al., 2006). In brief, this involved mixing 40 mg of DPPC with 10 mg of cholesterol (2:1 mole ratio) and dissolving the mixture in a minimum amount of chloroform. A thin phospholipid film was formed on the wall of a round bottom flask by removing the organic solvent using a rotary evaporator (Brinkman R205) equipped with a vacuum controller and water bath set at 50 °C. A stream of dry nitrogen was passed over the film to remove residual traces of the solvent. The dried lipid film was next hydrated by adding 2 ml of a 25 mg/ml sucrose solution at 50 °C. To reduce the size of the vesicles formed, the solution (20 mg/ml) was sonicated (Sonic Dismembrator Model 500) at 4 °C for 5 min using the following settings: frequency = 20 kHz, wave amplitude = 50% with pulse on/pulse off = 40 s/20 s. The mixture was then centrifuged at 400 × *g* (IEC MicroMax RF) for 10 min at 4 °C. The resulting supernatant, containing relatively small-sized DPPC vesicles was lyophilized. The resulting powder was prepared just prior to rehydrating in 2 ml of PBS (pH 7.4) and incubating at 50 °C for 1.5 h. This method of preparing liposomes was modified from that of Kirby and Gregoriadis (1984). Final suspensions of all liposomal formulations, with a lipid concentration of 15.7 ± 0.3 mg/ml measured by UV–vis (Stewart, 1980), were adjusted to pH 6.2 as indicated in Section 2.8.2.

2.3. Preparation of spin labeled gentamicin

Gentamicin sulfate (pH 4.5) was converted to its basic form by passing it through an anionic ion-exchange column (Amerblite IRA 400, Aldrich) followed by lyophilization. It was then reacted with 5,5,7,7-tetramethyl-1-oxa-6-azaspiro[2.5]oct-6-yloxy in a molar ratio of 1:1 using dimethylformamide (DMF) as the solvent (Rapoport et al., 1999). The solution was then agitated for 18 h at 37 °C. The DMF was removed under vacuum and the solid material was dissolved in a minimum amount of distilled water. The solution was lyophilized after its pH was adjusted to 4.5 with 1 M H_2SO_4, and the final product, which is a water soluble compound, was purified by washing off the organic component with several ml of hexane. The SL-gentamicin was characterized by EPR spectroscopy as previously described (Rapoport et al., 1999). It was found that approximately 5% of the gentamicin was spin labeled. This was estimated by comparing the normalized intensity of the low field EPR line of a known quantity of the SL-gentamicin to that of a known quantity of 5,5,7,7-tetramethyl-1-oxa-6-azaspiro[2,5]oct-6-yloxy.

2.4. Encapsulation efficiency

Encapsulation efficiency (EE%), defined as the ratio of the amount of active compound remaining inside the liposomes to the initial amount of SL-gentamicin introduced, was calculated. The concentration of SL-gentamicin encapsulated in the liposomes was estimated with the agar-well diffusion method using a laboratory strain of *Staphylococcus aureus* (ATCC 29213) previously described by Mugabe et al. (2006). Detailed description of the procedure can be found in Section 2.6.

2.5. Liposomal size measurements by dynamic light scattering (DLS)

The mean diameters and size distribution of vesicles were determined by photon correlation spectroscopy using a NICOMP 270 Submicron Particle Size Analyzer (Santa Barbara, CA, USA) operating at 23 °C. Aliquots, removed at various time intervals from a sample incubated at 37 °C, were used for measurement. Instrument parameters used to collect the data were: λ = 632.8 nm; a scattering angle of 90°; automatic channel width selection, and a total data acquisition time of 20 min. The Nicomp multimodal mode was selected because the Chi-square value was larger than 3.0.

2.6. Microbiological assay

The concentrations of antibiotics incorporated into liposomal vesicles were measured by agar diffusion assay using laboratory strains of *S. aureus* (ATCC 29213) as indicator organism for gentamicin. Briefly, we used an overnight culture of the organisms in Mueller–Hinton broth to prepare a bacterial solution equivalent to a McFarland 0.5 (1.5×10^8 bacterial/ml). The bacterial suspension in warm (50 °C) Muller–Hinton agar was then poured into a sterile

steel plate (440 mm × 340 mm) and left to solidify for 1 h at room temperature. Wells of 5 mm diameter, made with a well puncher, were filled with 25 μl of samples or standard solutions. The plate was covered and incubated for 18 h at 37 °C. We then measured the inhibition zones and, the average of duplicate measurements was used in data analysis. A standard curve was constructed with known concentrations of free gentamicin (0.0039–8 mg/ml) and was utilized to calculate concentrations of the entrapped antibiotics that were released from the liposomes by 0.2% Triton X-100. The minimum detection limit of the assay for gentamicin was 7.8 ng/ml.

2.7. Drug release kinetics

The liposomes used to study the kinetic release were prepared as described in Section 2.2, with the exception that SL-gentamicin dissolved in PBS buffer was added to the supernatant containing the relatively small-sized DPPC vesicles (1:1, w/w; lipid to drug). The mixture was lyophilized and rehydrated as described in Section 2.2. After the incubation period, the suspension was centrifuged (at a relative centrifugal force = 13,400 × *g* for 15 min at 4 °C). The supernatant which contained the unencapsulated SL-gentamicin was discarded and the pellet was washed twice with PBS. The pellet was then resuspended in 2 ml of PBS buffer containing ascorbic acid (Asc; 25 μmol). The concentration of the ascorbic acid used does not induce an osmotic difference between the inner and outer side of the bilayers. Aliquots of the final liposomal suspension were transferred to Eppendorf tubes and incubated in a water bath at 4 °C and 37 °C, respectively. Samples were then removed at various times during the 48 h time period. The amount of gentamicin released from the liposome was determined by both microbiological assay and EPR spectroscopy at 4 °C and 37 °C as described below.

2.8. EPR measurements

The liposomal samples were transferred to a melting capillary tube (i.d. = 1.5 mm) which was subsequently inserted into a standard 4 mm i.d., quartz EPR tube centered in the TE_{102} rectangular cavity of a Varian E9 spectrometer operating at 9 GHz. The EPR cavity was fitted with a Dewar insert for use in the variable temperature experiments. The temperature of the N_2 gas flowing through the Dewar insert was monitored and controlled (between 20 °C and 55 °C) with a Lakeshore model 330 temperature controller (accuracy ± 0.5 °C). First harmonic in-phase EPR spectra were recorded at a microwave power of 1–3 mW, 100 kHz modulation frequency, and a 1–2 G modulation amplitude over a 100 G scan range after the sample had reached thermal equilibrium. The EPR spectra (6 scans) were digitized using the spectrometer's built-in microcomputer and the spectral acquisition software, EPRWare (Scientific Software Service, Bloomington, IL).

2.8.1. Drug release EPR measurements

The EPR spectra of liposomal formulations consisting of DPPC/chol/SL-gentamicin were recorded at different time intervals using the spectrometer settings described above. Ascorbic acid, present in the PBS used to resuspend the liposomes, reduces nitroxide radicals to the EPR-silent hydroxylamines. Due to its charge, ascorbic acid does not readily cross the intact liposomal bilayers. Hence only those spin probes in the external volume, i.e., those released from liposomes can be reduced (Honzak et al., 2000). This reduction is reflected in a decrease in the intensity of the EPR spectrum of the nitroxide radical. The intensity of the EPR spectrum was quantified by double integration of the signals for the samples taken at the different time intervals (I_t). These intensities were divided by that determined for the initial sample at $t = 0$ and expressed as a percentage. Addition of Triton X-100 to the aliquot of the liposomes leads to the complete release of the entrapped contents into the solution. The complete loss of the EPR signal suggested that a sufficient amount of ascorbic acid was added to reduce the nitroxide radicals over the entire time course.

2.8.2. Determination of the fluidity and the order parameter (S) of liposomal systems by EPR spectroscopy

The liposomes used in the fluidity and order parameter studies were prepared from a mixture of DPPC, cholesterol and 5-DSL or 16-DSL with a 100:50:1 molar ratio. Unlabeled gentamicin sulfate was used when making the drug-encapsulated liposomes. Since the protonated form of gentamicin has the optimum antibacterial activity, the pH of gentamicin-containing liposomes was maintained at 6.2 (Gamazo et al., 2004).

The EPR spectrum of the nitroxyl ring in 5-DSL and 16-DSL is sensitive to the local host environment. For 5-DSL, $A_{||}$ and $A_{\perp}$, the hyperfine (hf) splitting tensors parallel and perpendicular with respect to the applied magnetic field direction, were estimated by the separation in gauss of the outermost ($2A_{max}$) and innermost ($2A_{min}$) peaks of the EPR spectra obtained at temperatures ranging from 20 °C to 55 °C (Fig. 1a). They are indicators of the rotational motional freedom of the phospholipids' acyl chain parallel and perpendicular to the external magnetic field. A large value of $2A_{max}$ represents a decrease in fluidity. The order parameter (S) represents the time averaged angular deviation of the stearic acid chain from its average orientation in the lipid bilayers. It usually shows a decreasing gradient along the phospholipid bilayers. Ordered phases such as the gel or ordered liquid crystal phase, are characterized by values of S that approach 1 while the more fluid phases are characterized by S values that are significantly less than 1. The S values were estimated by the following equation:

$$S = \frac{2(A_{||} - A_{\perp})}{2A_{zz} - (A_{xx} + A_{yy})} \times \frac{a'_0}{a_0} \quad (1)$$

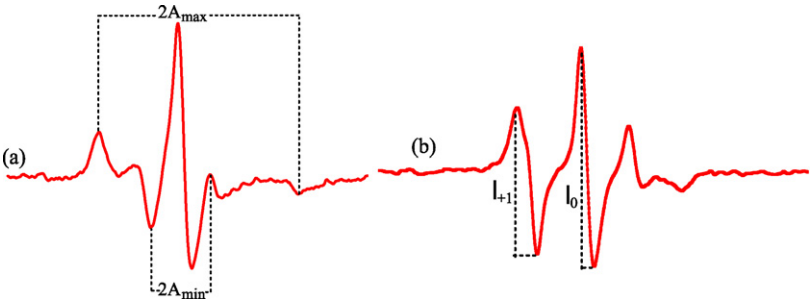

Fig. 1. A typical EPR spectrum for (a) 5-DSL containing liposomes and (b) 16-DSL containing liposomes. The parameters used to obtain information on bilayer fluidity, namely A_{max}, A_{min}, I_{+1} and I_0 are indicated.

where a_0 is the isotropic hf splitting constant which is estimated by

$$a_0 = \frac{A_{\parallel} + 2A_{\perp}}{3} \quad (2)$$

and a'_0 is the anisotropic hf splitting constant characteristic of the spin probe used. An estimate of a'_0 is obtained from the following equation:

$$a'_0 = \frac{A_{xx} + A_{yy} + A_{zz}}{3} \quad (3)$$

From the components of the hyperfine splitting tensor for *n*-doxyl, A_{xx} = 6.3 G, A_{yy} = 5.8 G, and A_{zz} = 33.6 G (Tetik and Korkmaz, 1998), a'_0 is estimated to be 15.2 G.

In considering the high mobility of hydrocarbon chains at elevated temperature, the ratio of I_{+1}/I_0, the intensity of peaks at low and central field, respectively (Fig. 1b) was employed to characterize the fluidity of hydrocarbon chains in the central regions of bilayers containing 16-DSL (Szabo et al., 2004).

2.9. EPR spectral simulation

EPR spectra recorded at 37 °C for the 5-DSL containing DPPC, DPPC/chol, and DPPC/chol/gentamicin liposomes were estimated using the EPRsim program (version 4.99, 2005) (Štrancar et al., 2000). Spectral simulation allows us to extract a number of parameters, but *S*, τ, *d*, and p_a were of particular interest in this study. Optimization was performed using Simplex hybridized with the Genetic procedure until the best fit was obtained.

2.10. Statistical analysis

The data are presented as the mean ± S.D. of three independent experiments. The significance was determined by the Student's *t*-test at 0.05 level.

3. Results

3.1. Encapsulation efficiency

There was approximately 25.7 ± 1.0% of the initial amount of SL-gentamicin used encapsulated into the DPPC/chol vesicles.

3.2. Liposomal size

We measured the mean diameter (*D*) of DPPC/chol/SL-gentamicin vesicles in order to determine the effect of SL-gentamicin sulfate on the physical stability of the drug–liposome system over a 48 h time period. For the purpose of comparison, we also determined the mean diameters of pure DPPC and DPPC/chol liposomes. A bimodal size distribution was generally observed for the three formulations over the entire time course (Fig. 2). The mean diameters of all liposomal vesicles in the first and also the most highly populated mode (>93%) was between 12 nm and 80 nm (Table 1). It is noteworthy that all the vesicles prepared in this study showed relatively large polydispersity index (PI) values (>0.6), which implies a heterogeneous distribution of vesicles of many different diameters. We did not observe a significant difference in the mean diameters ($P > 0.05$) of the liposomes prepared in this study. In addition, incubation of the liposomes of all three compositions causes an increase in the average diameter with time.

Table 1
Liposomal mean diameters (*D* in nm) and polydispersity index (PI) of three DPPC-based liposomal formulations at 25 °C measured at 0, 24 and 48 h

Incubation time (h)	DPPC		DPPC/chol		DPPC/chol/Gn	
	D	PI	*D*	PI	*D*	PI
0	38.7 ± 7.8	0.69	32.3 ± 15.6	0.77	12.2 ± 2.2	0.62
24	35.3 ± 10.2	0.66	55.3 ± 17.5	>1	37.5 ± 9.2	0.60
48	59.0 ± 11.0	0.66	79.8 ± 12.1	0.64	47.3 ± 3.4	0.60

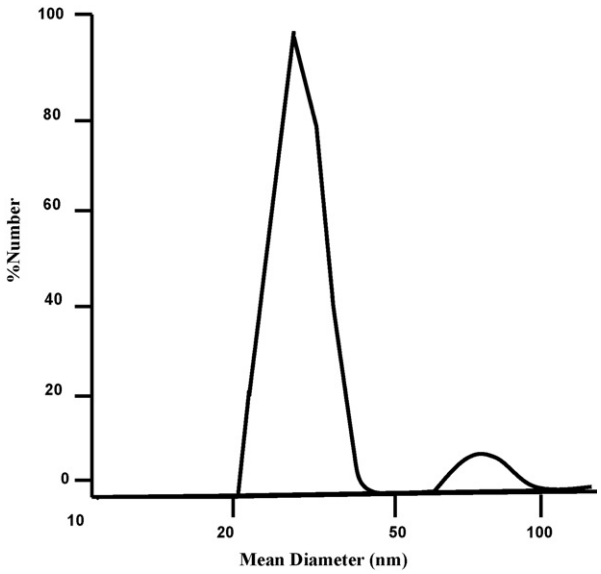

Fig. 2. A typical plot of the % population distribution versus the hydrodynamic diameter (nm) for spin labeled gentamicin encapsulated in DPPC/chol liposomes. The plots displayed typical bimodal distribution in that the mean diameters of each population differ significantly.

3.3. Kinetic release rate of gentamicin from liposomes

The release of SL-gentamicin from DPPC/chol liposomes at 4 °C and 37 °C over a 48 h time period, was monitored by both EPR spectroscopy and an antimicrobial assay (Fig. 3). It should be noted that the % retention of SL-gentamicin is in reference to the SL-gentamicin that was incorporated inside the liposomes (≈10 mg) at the zero time point. The encapsulated SL-gentamicin, determined at 4 °C, is quickly decreased to 94.0% (±2.27, assay) and 90.0% (±5.53, EPR), respectively, during the first 6 h of incubation and then steadily drops down to 88.0% (±4.50, assay) and 83.1% (±12.82,

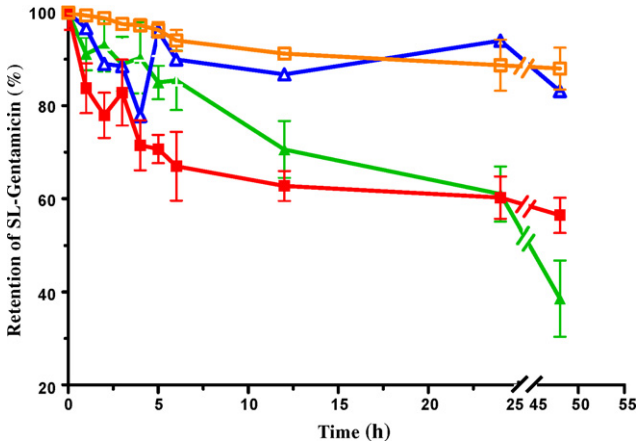

Fig. 3. The kinetic profiles for the release of SL-gentamicin from DPPC/chol liposomes at 4 °C and 37 °C as determined by EPR spectroscopy (▲, 37 °C and △, 4 °C) and microbiological assay (■, 37 °C and □, 4 °C) over a 48-h period. Each data point is the mean of 3 parallels ± S.D.

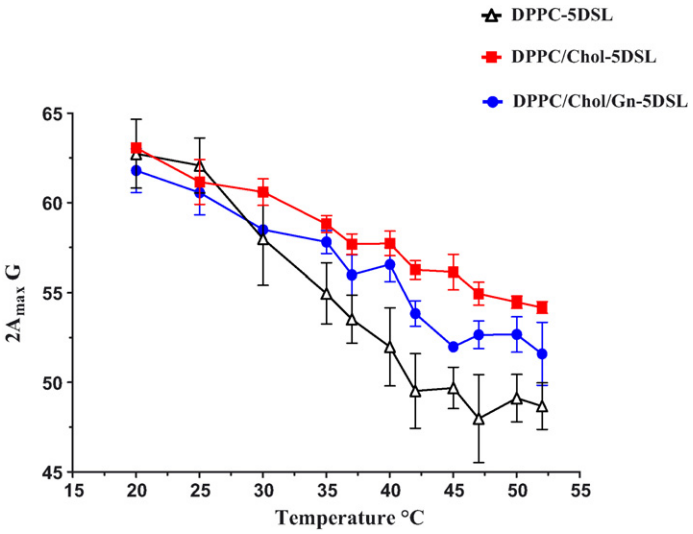

Fig. 4. The plot of the $2A_{max}$ values versus temperature for pure DPPC (Δ), DPPC/chol (■) and DPPC/chol/gentamicin (●) liposomes. $2A_{max}$ represents the value in gauss between the outermost extrema observed in the EPR spectrum of 5-DSL containing DPPC liposomes. The values are representative of lipid bilayer fluidity in the region of the phosphate head groups. Each data point is the mean of 3 parallels ± S.D.

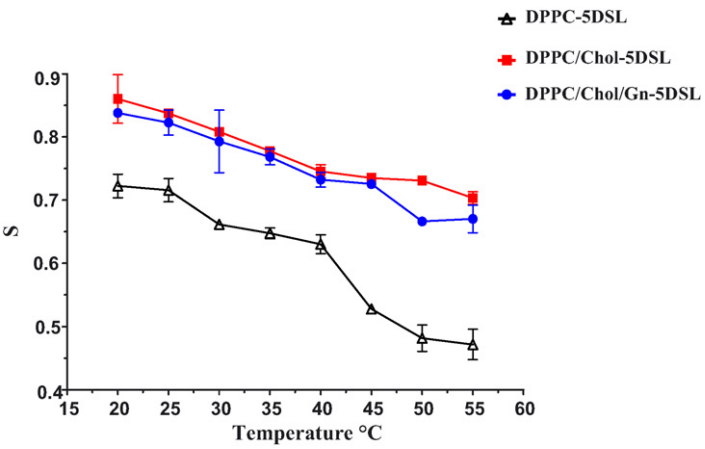

Fig. 5. The plot of the order parameter (S) versus temperature for pure DPPC (Δ), DPPC/chol (■) and DPPC/chol/gentamicin (●) liposomes. S was calculated using Eq. (1) and the parameters extracted from the EPR spectrum of 5-DSL containing DPPC liposomes. Each data point is the mean of 3 parallels ± S.D.

EPR), respectively, between the 6 h and 48 h mark. At 37 °C, the liposomes retain 67.0% (±6.38, assay) and 85.5% (±7.38, EPR) of the SL-gentamicin after incubation for 6 h and the retention gradually falls to 56.5% (±3.74, assay) and 38.6% (±8.19, EPR) after 48 h. The % retention obtained by the two methods was not significantly different. The microbiological results for the liposomal formulations measured at 37 °C show first-order kinetic drug release behavior whereby a substantial amount of gentamicin initially escapes from the liposomes over a short period of time at which point the release plateaus remaining virtually constant until the end of the time course. The other kinetic profiles measured at two temperatures by EPR and at 4 °C by microbiological assay showed a gradual decrease in the liposomal retention of the SL-gentamicin over 48 h.

3.4. Dynamic properties and microdomains

3.4.1. Effect of temperature on fluidity and order parameters (S)

The effect of composition and temperature on the fluidity of the lipid assembly and phospholipid packing were investigated by monitoring the variations in: (a) $2A_{max}$ and $2A_{min}$ of the EPR spectrum of 5-DSL containing liposomes, and (b) the intensity of the $m_I = +1$ (I_{+1}) and $m_I = 0$ (I_0) EPR transitions for the 16-DSL containing liposomes. The $2A_{max}$ values determined from the EPR spectra of the liposomal formulations recorded between 20 °C and 52 °C are presented in Fig. 4. In addition, the order parameters (S) were calculated by substituting the values of $2A_{max}$ and $2A_{min}$ for $A_{||}$ and $A_{\perp}$, in Eq. (1) (Fig. 5). Finally, a plot of I_{+1}/I_0 versus temperature was prepared for the 16-DSL containing liposomes (Fig. 6). Despite the fact our interest lies in gentamicin containing liposomes, the thermal behavior of empty DPPC liposomes and DPPC/chol liposomes (1:2 molar ratio) were also examined for the purpose of comparison. We found that the $2A_{max}$ and S values decreased with increasing temperature. The incorporation of cholesterol, such that the lipid:chol mole ratio is 2:1, causes a significant increase in the $2A_{max}$ and S values. More specifically, the difference between the $2A_{max}$ values for DPPC/chol and DPPC is the largest at 52 °C, i.e., 5.5 G. Similarly S increases by 0.14 at $T < 40$ °C and by 0.23 at $T = 55$ °C when cholesterol is incorporated into the liposomes. The

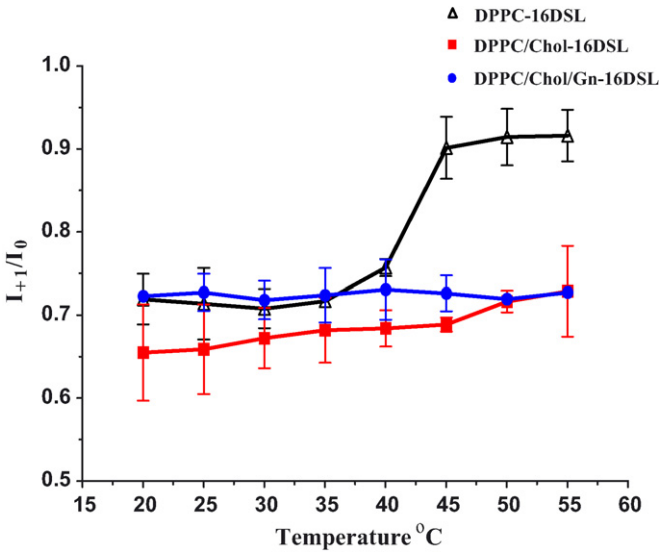

Fig. 6. Plots of I_{+1}/I_0 versus temperature for pure DPPC (Δ), DPPC/chol (■) and DPPC/chol/gentamicin (●) liposomes. The ratio of the intensity of the peak at low (I_{+1}) field to that at center field (I_0) for 16-DSL containing liposomes is indicative of the fluidity of hydrocarbon chains in the central regions of bilayers. Each data point is the mean of 3 parallels ± S.D.

encapsulation of gentamicin into the aqueous core compartment of DPPC/chol/5-DSL liposomes causes a decrease in the $2A_{max}$ values, as shown in Fig. 4, throughout the temperature range studied. The average decrease in the $2A_{max}$ values of approximately 1.2 G for temperatures between 20 °C and 40 °C and of 2.4 G for temperatures between 40 °C and 52 °C, indicates that the gentamicin encapsulation weakens ($P < 0.05$) the rigidity conferred by the cholesterol. However, the S values are only slightly affected by the addition of gentamicin at $T < 50$ °C, whereas S decreases significantly ($P < 0.05$) at $T > 50$ °C. The value of I_{+1}/I_0 between 20 °C and 45 °C for the DPPC/chol/gentamicin liposomes is approximately 0.07–0.03 units higher than that of the DPPC/chol liposomes indicating an increase in fluidity. On average, this represents an 8.2% increase when we take into consideration the experimental uncertainty associated with the I_{+1}/I_0 values (approximately ±0.034). This effect started to diminish and completely disappeared at 55 °C. Because the increase in the I_{+1}/I_0 value is not statistically significant ($P > 0.05$) we are

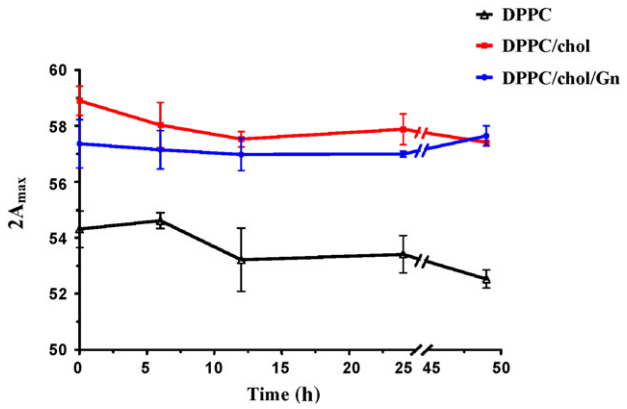

Fig. 7. Plots of $2A_{max}$ versus time for pure DPPC (△), DPPC/chol (■) and DPPC/chol/gentamicin (●) liposomes containing 5-DSL obtained at 37 °C. Each plot is an indicator of the fluidity fluctuations at the polar phosphate head group region of the bilayer during 48 h. Each data point is the mean of 3 parallels ± S.D.

unable to say with certainty that gentamicin fluidizes the phospholipid chains mid-way into the bilayer.

3.4.2. Effect of time on the fluidity

The variation of the fluidity at 37 °C of the three liposomal formulations, i.e., DPPC, DPPC/chol and DPPC/chol/gentamicin, containing 5- or 16-DSL was determined by monitoring the $2A_{max}$ and I_{+1}/I_0 values as a function of time (Figs. 7 and 8). Firstly, a dramatic change in fluidity at the polar head group of pure DPPC/5-DSL vesicles (Fig. 7) was detected during the first 6–12 h as the $\Delta 2A_{max}$ increases about 1.5 G ($P < 0.05$). On the other hand, the change in I_{+1}/I_0 values, associated with the reorganization of the central region of the bilayer, occurred during the initial 6 h; the I_{+1}/I_0 values decreased from 0.78 ($t = 0$) to 0.68 ($t = 6$ h) (Fig. 8). The variation in the values of both $2A_{max}$ and I_{+1}/I_0 decreased considerably for the remainder of the time course. Addition of cholesterol resulted in a significant increase in the $2A_{max}$ values indicating an increase in the rigidity of the vesicles. In addition, the $2A_{max}$ values steadily decreased from 58.9 G at $t = 0$ h to 57.5 G at $t = 12$ h at which point the values reached a plateau (Fig. 7). A similar trend was also observed for the gentamicin containing liposomes where the $2A_{max}$ values decreased by 0.3 G in going from 0 to 12 h and plateaued at a value of approximately 57.0 G (Fig. 7). The variation in

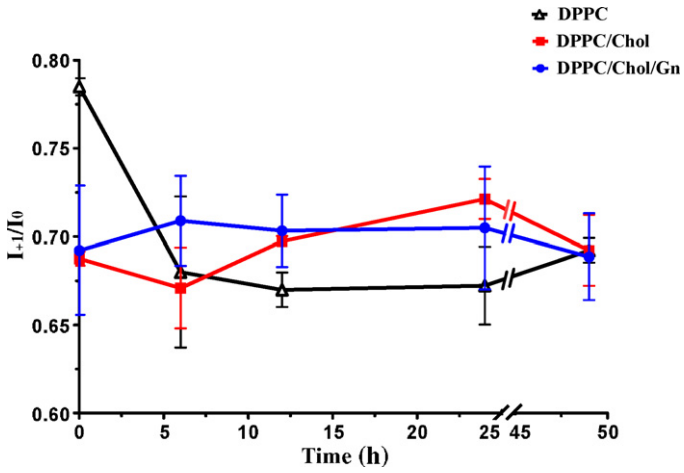

Fig. 8. Plots of I_{+1}/I_0 versus time for pure DPPC (△), DPPC/chol (■) and DPPC/chol/gentamicin (●) liposomes containing 16-DSL obtained at 37 °C. Each plot is a reflection of the fluidity fluctuations in the central region of the bilayer during 48 h. Each data point is the mean of 3 parallels ± S.D.

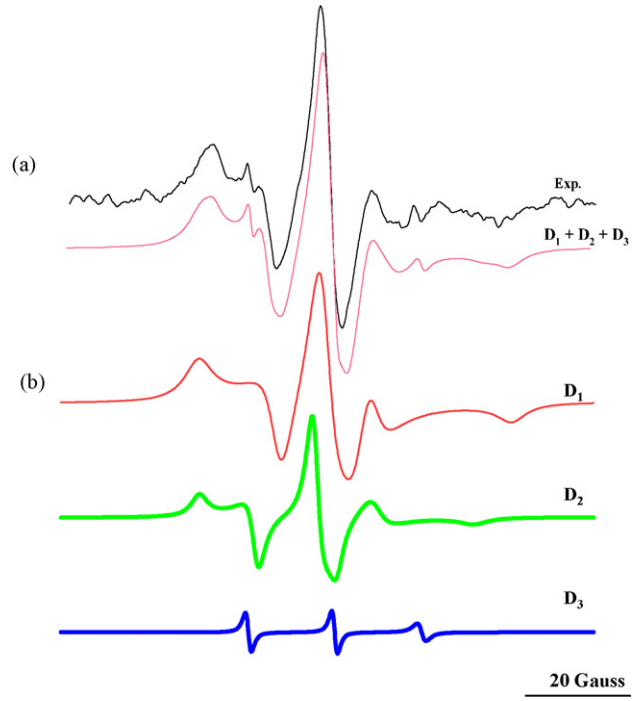

Fig. 9. (a) The experimental EPR spectrum of empty DPPC/5-DSL liposomes recorded at 37 °C (top), and the simulated EPR spectrum using EPRsim (bottom). (b) the simulated EPR spectra of the domains, D_1, D_2 and D_3.

the motional activity of the phospholipid chain in the central region of the bilayer was not statistically significant for the DPPC/chol and DPPC/chol/gentamicin liposomal compositions.

3.4.3. Domain characteristics

The best fit simulations of the EPR spectra, recorded at 37 °C, of 5-DSL containing liposomes made of DPPC, DPPC/chol and DPPC/chol/gentamicin were obtained by assuming the coexistence of three domains, i.e., D_1, D_2, and D_3. To better illustrate this, the simulated spectra of the three domains (D_1, D_2, and D_3) are shown in Fig. 9; included is the experimental EPR spectrum of the DPPC/5-DSL liposomes recorded at 37 °C. The dynamic parameters used to fit the experimental spectrum, namely the order parameter (S), correlation time (τ in ns), domain weight (d) and polarity correction factor (p_a), for each of the three domains of the DPPC, DPPC/chol and DPPC/chol/gentamicin liposomes are summarized in Fig. 10.

The fact that the vesicles are made of three domains suggests that they are heterogeneous in nature. A closer examination of the parameters (S, τ, d and p_a, Fig. 10), gives us information about the dynamic properties of each domain found in the polar phosphate head group region. In DPPC liposomes, the predominant domain, D_1 ($S = 0.72 \pm 0.01$, $\tau = 0.23 \pm 0.08$, $d = 0.81$) represents the restricted fast motional domain and D_2 ($S = 0.49 \pm 0.01$, $\tau = 0.76 \pm 0.1$, $d = 0.18$) is an intermediate ordered slow motion domain; the contribution from D_3 is negligible because its population only accounts for 0.01 ± 0.01. The addition of cholesterol (DPPC:chol = 2:1) significantly modified the physical properties ($P < 0.05$) of empty DPPC vesicles by (1) increasing the order parameter of D_1 ($S_{DPPC} = 0.72$, $S_{DPPC/chol} = 0.75$), D_2 ($S_{DPPC} = 0.49$, $S_{DPPC/chol} = 0.56$) and D_3 ($S_{DPPC} = 0.17$, $S_{DPPC/chol} = 1.00$), (2) causing a decrease in the domain size of D_1 ($d_{DPPC} = 0.81$, $d_{DPPC/chol} = 0.75$), (3) replacing D_3 of DPPC with a new more ordered and populated domain ($S_{DPPC} = 0.17$, $S_{DPPC/chol} = 1.00$) possessing a different rotational motion ($\tau_{DPPC} = 0.19$, $\tau_{DPPC/chol} = 0.12$). Gentamicin does not

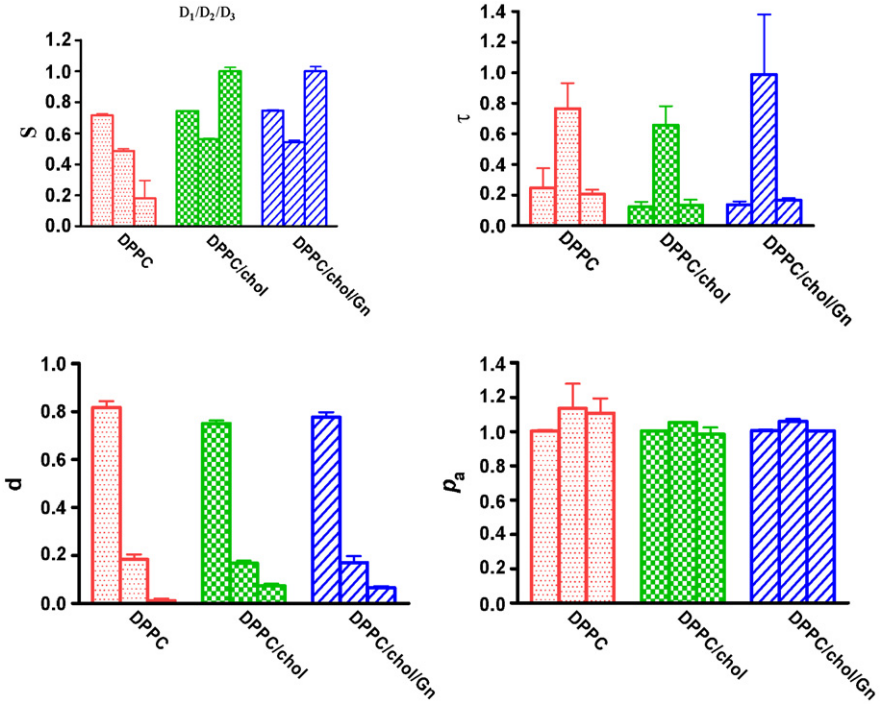

Fig. 10. Domains and their dynamic parameters, including order parameter (S), correlation time (ns), polarity correction factor (p_a), and domain size (d), extracted with the aid of spectral simulation for 5-DSL containing liposomes made of DPPC, DPPC/chol and DPPC/chol/gentamicin at 37 °C using EPRsim. Results are the mean of three individual parallels ± S.D.

appear to have a significant effect on the domain properties of DPPC/chol liposomes.

4. Discussion

Caution must be exercised when comparing the % encapsulation efficiency of liposomal formulations because they are influenced by factors such as vesicle type, pH, method of preparation, liposome composition, the ratio of lipid to drug and the physicochemical properties of liposomes (Puglisi et al., 1995). Although the DPPC/chol/SL-gentamicin liposomes were prepared in an identical fashion to the DPPC/chol/gentamicin liposomes studied by Mugabe et al. (2006), the encapsulation efficiency of 25.7% found for the DPPC/chol/SL-gentamicin liposomes is slightly lower than that reported for the DPPC/chol/gentamicin, i.e., 33%. This small difference may be due to the presence of the spin label.

It is known that the mean diameters of liposomes are also subject to various factors which include the preparation protocol, the liposomal compositions, the lamellarity, and the detection techniques to name a few. The dynamic light scattering measurements carried out on the liposomal formulations prepared in the present study showed a bimodal size distribution with the mean diameter of the most populated mode being between 12 nm and 0 nm. From a drug delivery point of view, the advantages of smaller sized liposomes, usually <100 nm, include prolonged opsonisation and slow uptake by the RES. In fact, enhanced accumulation and extravasations of smaller sized liposomes has been reported (Sharma and Sharma, 1997).

The large polydispersity index values observed indicates that there are probably large sized vesicles formed in the liposomal suspension, and we assumed that it was associated with the sonication procedure. It is stressed by Pereira-Lachataignerais et al. (2006) that the increase in the sonication power and the time applied will generally decrease the size and polydispersity index. However, the large particles may also result from the formation of large lipid aggregates.

In general, the addition of gentamicin has no significant effect on the mean diameters of liposomes relative to that of the DPPC or DPPC/chol liposomes ($P > 0.05$). There are a few studies involving gentamicin containing PC liposomes that have appeared previously. For example, Mugabe et al. found that the mean diameter is 205.4 ± 28.1 nm for DPPC/chol/gentamicin liposomes prepared by the dehydration–rehydration method (Mugabe et al., 2006). Lutwyche et al. used DLS to examine the mean diameters of gentamicin encapsulated in DPPC/chol (55:45 molar ratio) liposomes processed by extrusion and found that the typical size was 134 ± 28 nm (Lutwyche et al., 1998). In another report, the mean sizes of liposomes prepared with hydrogenated PC/chol/gentamicin using a molar ratio of 7:7:2 were measured by photon correlation spectroscopy and found to be 15.1 ± 8.7 μm (Cabanes et al., 1998).

Incubation of the DPPC, DPPC/chol and DPPC/chol/SL-gentamicin liposomes causes an increase in their average diameter with time (Table 1). Such an increase may result from the physical instability of the liposomes. Due to the neutral charge of DPPC lipids, smaller vesicles could readily unite with their nearest neighbors and subsequently aggregate or fuse into larger ones. Some factors that could induce structural transformations, for example, the formation of rod-like vesicles or the distortion of the shape of the vesicles, and an increase in lamellarity (Were et al., 2003) are thought to provoke aggregation and form clusters with a broad size distribution (Berger et al., 2001). Lentz et al. (1987) indicated that aggregation is one of the ways for a thermodynamically unstable system to dissipate the excess surface energy resulting from the packing defects. In the case of the DPPC/chol/SL-gentamicin liposomes there is the added possibility that the liposomes increase in size because of an osmotic effect due to gentamicin.

The temperature-dependent release profiles of gentamicin through the symmetric-acyl chains of DPPC (Fig. 3) are in agreement with those of other water-soluble compounds studied (El

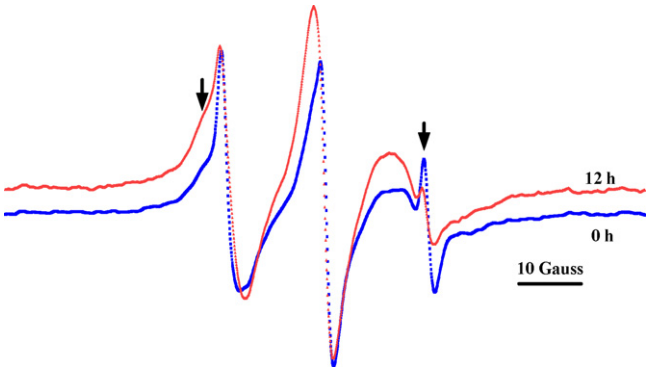

Fig. 11. Experimental EPR spectra of empty DPPC/16-DSL liposomes obtained at 37 °C after incubation for 0 and 12 h, respectively. It is clear that the 16-DSL is found in a domain that restricts its motion (left arrow) as well as a domain that allows more mobility (right arrow). The redistribution of the 16-DSL within the domains is reflected in the change in the spectra as a function of time.

Jastimi et al., 1999; Ginalska et al., 2005; Hays et al., 2001; Xiang and Anderson, 2006). The temperature-induced variation in the kinetic rate observed in this study is believed to arise from the difference in the structure of the phospholipid's subgel and ripple gel phase. One generally accepted interpretation correlates the solute permeability with the physical state of the boundary domains of the liposome's gel and liquid crystalline phases (Papahadjopoulos et al., 1973). In fact, Cruzeirohansson and Mouritsen suggested that leakage is associated with the disordered area of the membrane. They stressed that although the temperature will induce the intermediate permeable area of the phospholipid membrane to grow, the magnitude of the overall permeability is not controlled by the temperature (Cruzeirohansson and Mouritsen, 1988). However, it is important to note that there are certainly other factors, such as: (a) the kinetic energy of the solute, (b) the interactions between solute molecules and the phospholipid molecules, and (c) the physical state of the solute, involved in the mechanism of moving the water soluble compound across the bilayer (El Jastimi et al., 1999; Ginalska et al., 2005; Li et al., 1998; Xiang and Anderson, 2006). Mugabe et al. (2006) observed a similar *in vitro* kinetic profile, monitored by microbiological assay at 37 °C, for DPPC/chol/gentamicin liposomes with mean diameters of 205.4 ± 28.1 nm, but the percentage of the gentamicin release plateaued at approximately 82% within 48 h instead of 56.5% observed in the present study. This discrepancy is thought to be due to the small size of the vesicles used in our study as high surface constraint will result in distorted packing between the phospholipid molecules, especially at the phosphate head groups. The distortion in molecular alignment is believed to play an important role in facilitating the molecular diffusion across the bilayers.

To better understand the modulation effects of cholesterol and gentamicin on the dynamic properties of the domains within the liposomes, the fluidity profiles, domain structures and the dynamic parameters (S and τ) were extracted from the EPR spectra of 5-DSL containing liposomes. Cholesterol is known to restrict the motions of the phospholipid chains and to improve ordering of the membrane bilayers. However, the encapsulation of gentamicin into liposomes perturbs the packing and rigidity of the phospholipid–cholesterol assembly predominantly at the polar interface. The impact of gentamicin on the fluidity and order parameter at the interfacial region are temperature-dependent with the greatest changes occurring at $T > T_m$. Although the molecular basis of this behavior is not well understood, Ramsammy and Kaloyanides indicated the possibility of the formation of hydrogen bonds between the amino groups of gentamicin and the ester O–C=O groups of phospholipids (Ramsammy and Kaloyanides, 1988). In addition, the position and number of NH_2, NH_3^+ and OH functionalities in the aminoglycosides will determine their binding strength to their targets (Corzana et al., 2007).

Moreover, the effect of polysaccharides on phospholipid packing has been elaborated by many researchers. This is pertinent because gentamicin is considered a polysaccharide. On one hand, Nagase et al. investigated the ability of trisaccharides to lower the transition temperature of a DPPC/water suspension measured by DSC. They found that the saccharide molecules interrupt the phospholipid packing by interacting with the phosphate groups of DPPC via hydrogen bonds when the water content is less than 20 wt% based on the "water replacement hypothesis" (Nagase et al., 1997). On the other hand, results of the DSC analysis of pure DPPC in a fully hydrated state with trehalose showed that sugar rings can increase the spacing between the head groups so as to shift the T_m to a higher value (Crowe and Crowe, 1991; Ohtake et al., 2004). It is certain from our fluidity results and order parameters using 5-DSL containing liposomes that the impact of gentamicin on the phospholipid packing occurs because it localizes inside the bilayer somewhere around C-5. Because the increase in the I_{+1}/I_0 value is not statistically significant ($P > 0.05$) we are unable to say with certainty that gentamicin fluidizes the phospholipid chains mid-way into the bilayer. The results involving 16-DSL, would suggest the hydrophilic nature of gentamicin does not allow it to penetrate easily into the mid section of the bilayer and so the phospholipids remain relatively unperturbed. In other words the disturbance is too weak to be detected by EPR spectroscopy.

Regarding the fluidity-time profiles shown by the averaged values of $2A_{max}$ (Fig. 7) and I_{+1}/I_0 (Fig. 8) of the liposomes at 37 °C over 48 h, it was found that the changes in the fluidity of the phospholipid chains is independent of the time for liposomes containing cholesterol whereas for the pure DPPC liposomes the fluidity was found to change with time. The transmembrane movement of the gentamicin molecule and the amount of gentamicin retained were not related to the change in fluidity because we observe the release of gentamicin even though the fluidity remained constant over 48 h.

The comparison of our results to other studies is limited because there is little information available about the change in fluidity of artificial membranes with time from the drug delivery perspective.

The importance of determining the change in fluidity of the liposomes with time is that it can significantly modify the physical stability of the vesicles which in turn can be used to develop

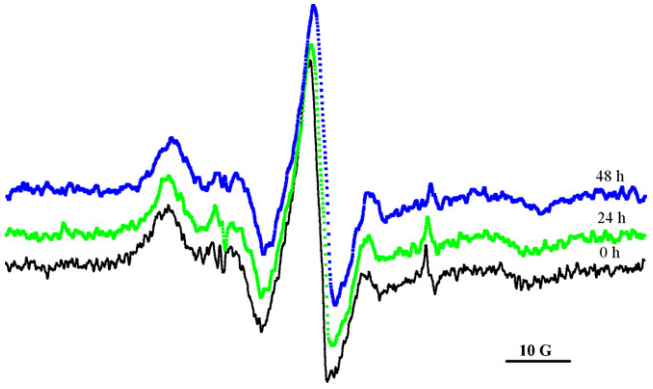

Fig. 12. Experimental EPR spectra of DPPC/chol/gentamicin/16-DSL liposomes obtained at 37 °C after incubation for 0, 24 and 48 h, respectively. It is clear that the fraction of the fluid domain is decreasing over time. The line-with of the central peak remains unchanged during the 48 h time period.

fluid liposomes improving the efficiency of liposomal-drug delivery and controlling the drug bioavailability. For instance, Schiffelers et al. (2001b) investigated the therapeutic efficacy of EPC/PEG-DSPE liposome-encapsulated gentamicin in fluid form. Their results indicated that fast local release of gentamicin from the fluid liposomes maintained a higher concentration of the drug at the infectious sites contributing to a superior therapeutic efficacy compared to a rigid liposome (PEG-DSPE) (Schiffelers et al., 2001b). Beaulac et al. also showed that the fluid tobramycin containing liposomes (DPPC:DMPG) promoted the interaction between liposomes and bacterial cell walls of *P. aeruginosa* resulting in improved bactericidal activity, in contrast to rigid liposomes (Beaulac et al., 1996). Furthermore, van Kuijk-Meuwissen et al. evaluated the skin penetration ability of the rigid (DSPC:CHEMS) and fluid bilayers (DLPC:CHEMS) using confocal laser scanning microscopy. They found that the rigid liposomes could not penetrate as deeply as the fluid ones and barely released their contents until incubated for 6 h. Therefore they concluded that the thermodynamic state of the liposomes strongly influences their skin penetration and solute molecule release (van Kuijk-Meuwissen et al., 1998).

From the EPR results we were able to conclude that the bilayer is made up of three domains. Similar observations of the domain complexity were reported for a variety of other liposomal compositions. For instance, Vrhovnik et al. studied the membrane heterogeneity and the coexistence of different domains in MLV of DPPC:chol (30 mol% of chol) liposomes (Vrhovnik et al., 1998), while Šentjurc et al. characterized the domains and their dynamic characteristics within model systems for plasma membrane (OPP/cholesterol) systems using EPR spectral simulation (Šentjurc et al., 2002).

Our simulation results (Fig. 10) indicate that the heterogeneity of the lipid bilayers is not dependent on the addition of cholesterol and gentamicin. The presence of cholesterol induces the formation of L_0 domain and increases the overall ordering of the phospholipid chains. The highly ordered domain possessing a high degree of rotational mobility is assigned to the liquid-ordered phase (L_0) reported for cholesterol containing liposomes because L_0 is a fluid-like, highly ordered phospholipid assembly (Gliss et al., 1999). It was suggested that cholesterol forms hydrogen bonds with the choline group and that the rigid steroid moiety can restrict the chain mobility up to the C12 position (Berger et al., 2001; Were et al., 2003). The parameters (S, τ, d) determined from the EPR spectra of DPPC/chol liposomes with and without gentamicin were not statistically different (Fig. 10). This implies that gentamicin has a negligible impact on the domain properties of phospholipids at physiological temperature.

It is known that the SUV have a propensity to aggregate and fuse over time. Thus, the effect of gentamicin on the phase transformation or phase separation of the liposomes was examined. By examining the line-shape of the EPR spectra obtained at 37 °C, we concluded that phase reorganization did not occur at the interface region within a 48 h time period for all three liposomes. However, we did observe phase repartitioning as a function of time for the pure DPPC liposomes in the interior of the bilayers (Fig. 11), and this was speculated to arise from either (1) fusion of the DPPC vesicles; or (2) the formation of pores in the interior of lipid bilayers. The fusion of the SUV liposomes is generally reflected by the narrowing of the central EPR signal, as indicated by Beni et al., in response to the formation of the monolayer resulting from lipid exchange and fusion (Beni et al., 2006). Because we do not observe variations in the central peak width with time, repartitioning is most likely due to the disappearance of the pores in the bilayers interior, rather than via intervesicle interactions such as membrane fusion or coalescence. The addition of cholesterol dramatically reduces the fraction of the fluid domain. The escape of gentamicin from the DPPC/chol bilayers did not induce further domain repartition examined by 16-DSL (Fig. 12). Based on the abovementioned observations, it is thus concluded that the liposomes maintain their integrity during the 48 h time period. Taking into account the fact that the mean diameter of the liposomes generally increase with time, it is believed that the gentamicin containing liposomes are microscopically stable, but underwent aggregation during the 48 h time course as a result of interliposomal interaction. The above observation is encouraging because the thermodynamic stability of the gentamicin containing liposomes under physiological conditions is one of the prerequisites for efficient drug delivery.

5. Conclusion

In this study, we determined the physicochemical properties of liposomal formulations containing gentamicin. We paid particular attention to the physical stability of the liposome–gentamicin vesicles *in vitro*. The results revealed that the smaller sized gentamicin containing liposomes exhibit a continuous release of its content. In addition their sizes were found to increase over the 48 h time period. In general, the addition of gentamicin fluidizes the bilayer close to the polar interface, and this effect is most noticeable in the liquid crystalline phase. DPPC/chol/gentamicin liposomes possess multiple domains at 37 °C. The domain properties remain unchanged during the 48 h time course. We conclude that gentamicin containing liposomes maintain their microscopic stability as fusion and coalescence is not triggered during the observation time.

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