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Antimicrobial effectiveness of liposomal polymyxin B against resistant Gram-negative bacterial strains

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Abstract

Polymyxin B is a polycationic antibiotic effective in the treatment of Gram-negative bacterial infections. Systemic use of polymyxin B has been limited due to its toxicity, most notably nephrotoxicity, ototoxicity, and neuromuscular blockade. Entrapment of antibiotics in liposomes is known to enhance their antimicrobial activities while minimizing their toxic effects. In the present study, polymyxin B was incorporated into liposomes composed of either 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and cholesterol (Chol) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and Chol. The entrapment efficiency of sonicated liposomes containing DPPC/Chol ($32.1 \pm 2.43\%$) was six-fold higher than that of liposomes containing POPC/Chol ($5.35 \pm 0.32\%$). On the other hand, the entrapment efficiency of extruded DPPC/Chol liposomes ($3.23 \pm 0.46\%$) was about 30% less than that of liposomes composed of POPC/Chol ($5.10 \pm 0.37\%$). Incubation of extruded DPPC/Chol liposomes containing polymyxin B in serum at 37 °C resulted in a complete release of the antibiotic into the supernatant after 3 h as compared to 6 h in the case of POPC/Chol liposomes. Spontaneous release of polymyxin B from DPPC/Chol liposomes incubated in saline was significantly higher (66%) than that from POPC/Chol liposomes (24%) after 48 h at 37 °C. With respect to the antimicrobial activities of the liposomal polymyxin B formulations, the MICs of sonicated DPPC/Chol liposomes against Gram-negative strains were generally lower when compared to free polymyxin B. Immunocytochemistry and electron transmission microscopic studies revealed that the penetration of polymyxin B into a resistant strain of *Pseudomonas aeruginosa* was higher following its administration as a liposomal formulation as compared to its conventional form. The combination of free drug and plain liposomes had an antibacterial activity similar to that of free antibiotic. These data suggest that incorporation of polymyxin B in liposomes could be useful in the management of Gram-negative infections induced by these microorganisms.

Keywords: Polymyxin B; Liposomes; Gram-negative bacteria; Antibacterial; Antibiotic

1. Introduction

The polymyxins (polymyxin B and colistin) are polypeptide antibiotics isolated from *Bacillus polymyxa* and known to have potent bactericidal activity against a broad range of Gram-negative bacteria (Arnold et al., 2007; Horton and Pankey, 1982). In general, the polymyxins exert their bactericidal activity by binding to acidic phospholipids and lipopolysaccharides of bacterial cell membranes, resulting in leakage of intracellular components, leading to cell death (Arnold et al., 2007; Cardoso et al., 2007; Clausell et al., 2007). They also have antiendotoxin activity (Arnold et al., 2007; Clausell et al., 2007), but concerns arising from adverse effects have restricted their use almost exclusively for the treatment of Gram-negative bacilli infections that are resistant to other preferred antimicrobial agents or in intolerant patients to the preferred antimicrobials (Falagas and Kasiakou, 2006; Lee et al., 2006). Resistance in Gram-negative strains towards polycationic antibiotics, whether mutational or adaptive, is common (Macfarlane et al., 1999; Moskowitz et al., 2004; Kwon and Lu, 2006). Since polymyxin B can interact with the outer membrane of *Pseudomonas aeruginosa*, possible mechanisms of resistance include cell impermeability and alterations in cell surface (reduction in LPS, outer membrane proteins, and divalent cation levels) (Tsubery et al., 2002; McPhee et al., 2003; Kwon and Lu, 2006). These alterations decrease the binding sites on the cell surface, ultimately reducing cell wall disruption of structure

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and penetration of polymyxin B into the cytoplasm of the bacteria.

Entrapment of antimicrobial agents in liposomes has been shown to enhance their therapeutic effectiveness (Omri et al., 1994; Beaulac et al., 1996; McAllister et al., 1999) while diminishing or completely abolishing their toxic effects. Liposomes are well suited as vehicles for delivering antimicrobial agents because they usually provide a sustained drug release effect; minimized drug toxicity; and increased overall drug efficacy. In addition, liposomes protect the incorporated drug from premature immunological and enzymatic attacks (Omri et al., 2002).

The present study was undertaken to evaluate: (i) the entrapment efficiencies of DPPC/Chol and POPC/Chol liposomal formulations prepared by two different methods; (ii) the kinetic release profiles of polymyxin B from liposomal formulations; (iii) the *in vitro* antimicrobial activity of the liposomal formulation with the highest polymyxin B content against several Gram-negative bacterial strains, and (iv) the uptake of polymyxin B administered either as a free (conventional form) or liposomal formulation by a resistant *P. aeruginosa* bacterial strain.

2. Materials and methods

2.1. Chemicals

Polymyxin B and Triton X-100 were purchased from the Sigma Chemical Co. (St Louis, MO, USA). 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), cholesterol (Chol), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). All products for the immunocytochemistry studies were purchased from Cedarlane laboratories (Burlington, ON, Canada).

2.2. Bacterial strains

Bordetella bronchiseptica (ATCC 4617, ATCC 10580), *P. aeruginosa* (ATCC 25619, ATCC 27853, and clinical isolates *PA*-1, *PA*-3, *PA*-5, *PA*-8, *PA*-11, *PA*-12, *PA*-10145, *PA*-13572, *PA*-M13639, *PA*-M13640, *PA*-M13641, *PA*-48912, *PA*-48913), *Escherichia coli* (ATCC 25922, ATCC 700973), *Klebsiella pneumoniae* (ATCC 700603), *Acinetobacter lwoffii* (ATCC 17925) and *Acinetobacter baumannii* (ATCC 19606) were purchased from PML Microbiologicals (Mississauga, ON, Canada) or obtained from the Clinical Microbiology Laboratory of Memorial Hospital (Sudbury, ON, Canada). These organisms were stored at −70 °C in trypticase soy broth (PML Microbiologicals) supplemented with 10% (v/v) glycerol. For experimentation, these strains were inoculated onto BBL Mueller Hinton II agar plates (BD) and incubated for 18 h at 37 °C.

2.3. Isolation of serum

Whole blood was collected from anesthetized normal rats by cardiac puncture. Blood samples were allowed to clot at room temperature for 20 min and then centrifuged (1000 × *g*, 20 min, and 4 °C). The sera were pooled and frozen at −70 °C until used.

2.4. Preparation of liposomal polymyxin B formulations

Polymyxin B liposomes were prepared from a lipid mixture (90 μmoles) of either DPPC or POPC and Chol in a molar ratio of 2:1 by the methods described previously with slight modifications (Omri et al., 2002). In brief, the chloroform used to dissolve the lipids was removed under vacuum at 51 °C using a rotary evaporator (Buchi-Rotavapor R205, Brinkmann, Toronto, ON, Canada). To the thin dry lipid film, 6 ml of an aqueous solution of polymyxin B at a concentration of 10 mg/ml were added. This polymyxin B concentration was appropriate to produce optimal drug incorporation (data not shown). The lipid suspensions, submerged in an ice-bath, were either sonicated for 5 min in an ultrasonic bath (Sonic Dismembrator Model 500, Fischer Scientific, USA) or extruded twice through a double-stacked 100 nm pore-size polycarbonate membranes (Nucleopore Corp. Pleasanton, CA, USA) in an extruder (Lipex Biomembranes Inc., Vancouver, BC, Canada). Sonicated samples were then freeze-dried overnight for preservation (Labconco model 77540, USA). Dehydrated liposomes were rehydrated in saline above the phase transition temperature of lipids for 2 h and unencapsulated drug was washed off twice by centrifugation. The dehydration of sonicated liposomes did not affect the entrapment values of the formulations (data not shown). For all experiments, the size of sonicated or extruded liposomal suspensions was determined with the use of a coulter N4SD particle-size analyzer (Coulter Electronics of Canada, Burlington, ON, Canada) and was found to have a mean diameter of 343 ± 28 nm for sonicated liposomes and 172.5 ± 22 nm for extruded liposomes. The content of polymyxin B entrapped in liposomes was measured by a microbiological assay as described below after disrupting the lipid membranes by 0.2% (v/v) of Triton X-100.

2.5. Microbiological assay for the measurement of polymyxin B

The concentrations of polymyxin B were measured by a microbiological assay employing *B. bronchiseptica* (ATCC 4617) as an indicator organism. This organism was grown overnight in trypticase soy broth at 37 °C, and bacterial suspensions were adjusted with saline to contain an equivalence of a McFarland standard of 0.5 to ensure dense confluent growth on the plates. A total of 2 ml of diluted culture was added to 175 ml of autoclaved molten antibiotic medium #9 (PML Microbiologicals), which had been cooled to 56 °C and mixed gently by inversion to distribute the inoculum. The seeded medium was then poured into glass plates (260 by 200 mm) on a horizontal level surface and left to harden for 30 min at room temperature. The wells in the plates were made by a puncher device (4-mm) in the agar and filled with 25 μl samples or standards, two wells each. The glass plates were covered and incubated overnight (24 h) at 37 °C. Duplicate zone inhibition diameters were averaged and compared with a series of standards. Standard curves were made with known quantities of free polymyxin B added to

saline or normal pooled rat sera depending on the sample to be assayed. The concentrations of unknown samples were obtained by extrapolation from the zones of inhibition of standards by linear regression analysis of standards. The range of linearity for polymyxin B was from 0.76 to 100 μg/ml with a correlation coefficient of at least 0.99. The minimum detection limit of the assay was 0.76 μg/ml.

2.6. Entrapment efficiency

Entrapment efficiency was calculated as the percentage of polymyxin B incorporated into liposomes relative to the initial total amount of drug in solution.

2.7. Release kinetics of liposome-entrapped polymyxin B

The release kinetics of liposome-entrapped polymyxin B was monitored as previously described (Omri et al., 1995). In short, saline or pooled normal rat sera were supplemented with liposomal polymyxin B at a concentration of 100 μg/ml and incubated at 37 °C with mild agitation. At 0 (after incubation of liposomes in sera or saline), 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 24 and 48 h after the addition of liposomal polymyxin B, aliquots of the saline or serum mixtures were removed and centrifuged; polymyxin B present in the supernatant was assayed by the microbiological assay as described above. The amount of polymyxin B released was expressed as a percentage of the antibiotic concentration retained at 0 h.

2.8. Measurement of antimicrobial activity

The minimum inhibitory concentrations (MICs) of free or liposomal polymyxin B were determined by the agar dilution method as previously described (Omri et al., 1995). Bacterial inocula were prepared from an overnight culture in Mueller Hinton II agar (BD) and adjusted to contain an equivalence of a 0.5 McFarland standard with saline. The adjusted inocula were then delivered onto Mueller Hinton II agar (BD) plates containing two-fold serial dilutions of antibiotics, using the Replianalyzer system (Oxoid Inc., Nepean, ON, Canada). Free or liposomal polymyxin B, free polymyxin B plus empty liposomes, or empty liposomes alone were tested simultaneously against all bacterial strains. The lowest concentration of antibiotic that prevented the appearance of a visible growth within the inoculation area after 18 h at 37 °C was defined as the MIC.

2.9. Liposomal polymyxin B penetration by immunocytochemistry

To determine the ability of free or liposomal polymyxin B to interact with bacterial cells, the immunogold technique was applied as previously described (Halwani et al., 2007). Briefly, a polymyxin B-resistant clinical strain of *P. aeruginosa* PA-M13641-1 was incubated with free polymyxin B (64 μg/ml; sub-MIC) or with liposomal polymyxin B (16 μg/ml; sub-MIC). Bacteria in the absence of polymyxin B were used as the negative control. Samples were incubated for intervals of 0, 4, 8 and 16 h at 37 °C. At the prescribed intervals, samples were centrifuged at 200 × *g* in order to remove polymyxin B present in the supernatant. The pellets were then pre-fixed in glutaraldehyde (0.5%) for 30 min at room temperature, washed twice with PBS, and encapsulated in 2% Mueller-Hinton II Agar which then were cut into 1 mm cubes. Several pieces of each sample were embedded in gelatin capsules filled with Spurr resin (epoxy resin) and polymerized overnight at 60 °C. Ultra-thin sections (70–90 nm) were then collected onto uncoated 300-mesh nickel grids and previewed under a Philips 400 T TEM. Selected samples were then prepared for immunogold labeling using monoclonal antibody to polymyxin B and colloidal gold (10 nm) coupled to protein A/G (Sigma–Aldrich). Control samples contained PBS instead of anti-polymyxin B antibodies. Samples were analysed using a JEOL STEM (2011) transmission electron microscope, and images were captured with a Gatan Ultrascan digital camera.

Table 1
Entrapment efficiency (%) of polymyxin B into liposomes composed of either DPPC/Chol or POPC/Chol

Method of preparation	Liposomal formulation	
	DPPC/Chol	POPC/Chol
Sonication	32.1 ± 2.43[a,c]	5.35 ± 0.32
Extrusion	3.23 ± 0.46[b]	5.10 ± 0.37

Liposomal formulations were prepared either by sonication or extrusion procedures as described in Section 2. Entrapment efficiency was calculated as the percentage of drug incorporated in liposomes relative to the initial total amount of drug in solution. Polymyxin B was measured after lysis of the vesicles with 0.2% (v/v) Triton X-100 by microbiological assay. Values are mean ± standard errors of the mean obtained from three independent experiments in duplicate.

[a] $P \leq 0.05$ was considered significant when comparing the entrapment efficiency of liposomes formed by sonication (DPPC/Chol *versus* POPC/Chol).

[b] $P \leq 0.05$ was considered significant when comparing the entrapment efficiency of liposomes formed by extrusion (DPPC/Chol *versus* POPC/Chol).

[c] $P \leq 0.05$ was considered significant when comparing the entrapment efficiency of liposomes composed of DPPC/Chol or POPC/Chol (sonication *versus* extrusion).

2.10. Data analysis

The results were expressed as mean ± S.E.M. obtained from three separate experiments. Comparisons were made by paired Student's *t*-test, and a *P*-value of 0.05 or less was considered significant.

3. Results

3.1. Entrapment efficiency

The entrapment efficiency of polymyxin B in sonicated or extruded liposomes composed of DPPC/Chol or POPC/Chol is shown in Table 1. Sonicated DPPC/Chol liposomes had significantly greater ($P < 0.05$) encapsulation efficiency (32.1 ± 2.43%) than sonicated POPC/Chol liposomes (5.35 ± 0.32%), extruded DPPC/Chol liposomes (3.23 ± 0.46) or extruded POPC/Chol (5.12 ± 0.34%). In contrast, POPC/Chol liposomes prepared by the extrusion method had higher encapsulation efficiency than DPPC/Chol liposomes.

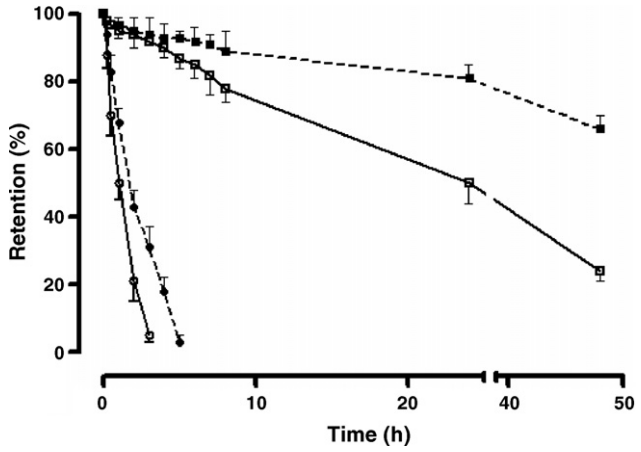

Fig. 1. Liposomal polymyxin B retention in DPPC/Chol or POPC/Chol liposomes in normal rat sera or saline within 48 h of incubation at 37 °C. Liposomal polymyxin B retention in DPPC/Chol (no color) or POPC/Chol (filled color) liposomes in normal rat sera (circle) or saline (square) within 48 h of incubation at 37 °C. Each point represents the mean ± S.E.M. of three separate experiments. Chol: cholesterol; DPPC: 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; POPC: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine.

3.2. Release kinetics of polymyxin B from liposomes

Fig. 1 depicts the release profile of polymyxin B from liposomes incubated either in saline or serum at 37 °C over a 48 h time period. The rate of release of polymyxin B in the presence of serum was much greater from the DPPC/Chol liposomes (100% within 3 h) as compared to POPC/Chol liposomes (95% within 6 h). Similarly, in the presence of saline, about 24% and 66% of polymyxin B was released in 48 h from POPC/Chol liposomes and DPPC/Chol liposomes, respectively.

3.3. Bacterial susceptibilities

Since encapsulation of polymyxin B in liposomes composed of DPPC/Chol yielded higher entrapment values, the antimicrobial effectiveness of the DPPC/Chol formulation was tested against a broad range of Gram-negative bacterial strains, including clinical isolates. The minimum inhibitory concentrations of free polymyxin B and the DPPC/Chol liposomal polymyxin B formulations against Gram-negative bacterial strains are shown in Table 2. The MICs of DPPC/Chol liposomal polymyxin B was reduced for all strains studied. DPPC/Chol liposomal polymyxin B displayed lower MICs (up to five times the dilutions) when

Table 2
In-vitro activities of free polymyxin B (PmB), free PmB plus empty DPPC liposomes (EL), and DPPC/Chol liposomally-encapsulated PmB preparations against different strains of bacteria

Gram-negative strains		Free PmB	Free PmB + EL	Liposomal PmB
B. bronchiseptica	ATCC4617	0.5	0.5	0.125
	ATCC 10580	0.5	0.5	0.125
E. coli	ATCC 25922	0.5	0.5	0.0313
	ATCC 700973	0.5	0.5	0.0625
K. pneumoniae	ATCC 700603	0.5	0.5	0.0313
A. lwoffii	ATCC 17925	0.5	0.5	0.0625
A. baumannii	ATCC 19606	1	1	0.0625
P. aeruginosa	ATCC 27853	2	2	0.125
	ATCC 25619	2	2	0.0625
P. aeruginosa	PA-1	1	1	0.0625
Clinical isolates	PA-3	2	2	0.125
	PA-5	2	2	0.0625
	PA-8	1	1	0.0625
	PA-11	2	2	0.125
	PA-12	1	1	0.0625
	PA-10145	1	1	0.125
	PA-13572	1	1	0.0625
	PA-M1369-1	1	1	0.0625
	PA-M1369-2	>512	>512	128
	PA-M13640	2	2	0.0625
	PA-M13641-1	512	512	32
	PA-M13641-2	1	1	0.0625
	PA-M26250	1	1	0.0625
	PA-48912-1	2	2	0.125
	PA-48912-2	2	2	0.125
	PA-48913	0.5	0.5	0.0313

Minimum inhibitory concentration was determined by a standard agar dilution method. Two-fold dilutions for each drug formulation were prepared. The plates were incubated for 18 h at 37 °C and then read. The MIC was recorded to be the lowest concentration of the drug that prevented visible growth and expressed in μg/ml.

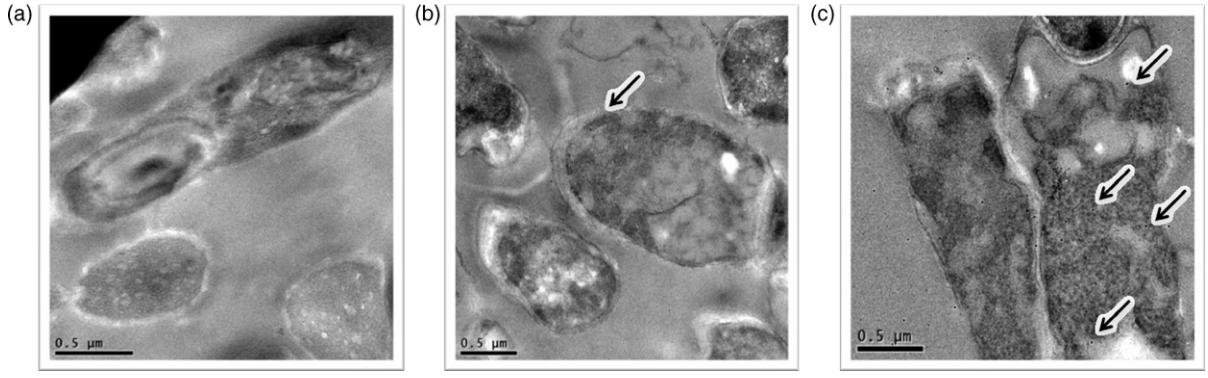

Fig. 2. Interaction of free polymyxin B and liposomal polymyxin B with bacterial membrane using immunocytochemistry. The clinical strain of *P. aeruginosa* M13641-1 was incubated (a) without polymyxin B; (b) free polymyxin B (immunolabelled drug indicated by arrow); (c) liposome-entrapped polymyxin B (immunolabelled drug indicated by arrow). The colloidal gold particles (10 nm size) are indicative of polymyxin B within bacteria. Samples were analyzed using a JEOL STEM (2011) transmission electron microscope, and images were captured with a Gatan Ultrascan digital camera.

compared to free polymyxin B. Two clinical isolates of *P. aeruginosa* displayed resistance to polymyxin B (≥512 μg/ml); the resistance to polymyxin B was lower when the antibiotic was delivered in liposomes (32 and 128 μg/ml). The antimicrobial activity of a combination of free polymyxin B and empty liposomes (i.e. without encapsulation) was identical to that of free polymyxin B alone (Table 2).

3.4. Determination of liposomal polymyxin B penetration

In comparison with the free polymyxin B (64 μg/ml) after 12 h, the liposomal formulation (16 μg/ml) delivered higher amounts of immunogold-labeled polymyxin B into the resistant strain PA-M13641-1 (Fig. 2). Samples incubated without treatment served as a negative control with no labeling.

4. Discussion

Polymyxin B is a cationic polypeptide antibiotic effective in the treatment of Gram-negative bacterial infections. Strains of *A. baumannii*, *P. aeruginosa*, and *K. pneumoniae* have emerged as common causes of hospital acquired infections in critically-ill patients and exhibit resistance to almost all available antibiotics except polymyxins (Falagas and Bliziotis, 2007). Its clinical use, however, is limited due to its toxic effects, the most important being nephrotoxicity, ototoxicity, and neuromuscular blockade (Falagas and Kasiakou, 2007). Incorporation of antibiotics in liposomes is known to enhance their antibacterial activities while minimizing their toxic effects (Omri et al., 1994; Beaulac et al., 1996; McAllister et al., 1999; Sharma et al., 2006; Ali et al., 2007).

Results presented in this study demonstrated that DPPC/Chol liposomes prepared by sonication had a greater entrapment efficiency of polymyxin B than those prepared by extrusion. The difference observed between the two methods of liposomal preparation is probably attributed to the smaller diameter size and hence reduced entrapment volume of the extruded liposomes. In contrast, POPC/Chol liposomes had similar entrapment efficiencies (Table 1), regardless of the method of preparation and this may be attributed to the fluidity state of the liposomal lipid. It is known that the entrapment efficiency of liposomes depends on several factors including the phase transition temperature, which is a function of the types of phospholipids (Lawrence et al., 1993; Kulkarni et al., 1995). For example, the transition temperature for POPC is −2 °C; preparation of POPC liposomes at 51 °C increases the fluidity of the bilayer and consequently its permeability.

In addition to the transition temperatures, the differences between DPPC/Chol and POPC/Chol prepared by sonication may be attributed to the lipid composition. It has been recognized that drug encapsulation by liposomes increases with increasing the chain length of the phospholipids' fatty acid (Anderson and Omri, 2004; Liang et al., 2005). Also, DPPC with saturated alkyl chains give rigid vesicle bilayers while POPC is composed of unsaturated hydrocarbon chains, which increase the fluidity of bilayer membranes. In another study, Lawrence et al. (1993) prepared three types (neutral, positive and negative) of liposomal polymyxin B by lipid hydration containing either hydrogenated egg phosphatidylcholine, hydrogenated egg phosphatidylcholine and stearylamine or hydrogenated egg phosphatidylcholine and dicetyl phosphate (charge:neutral lipid molar ratio 1:7, total lipid concentration 14.6 μmol). These authors found that the drug encapsulation increased in the order of: neutral (8.1 ± 2.3%) = positive (10.3 ± 5.2%) < negative (17.7 ± 0.7%). The differences observed among these studies are attributed to differences in lipid composition and the various methods of preparation (Kirby and Gregoriadis, 1984; Ravaoarinoro and Toma, 1993; Kulkarni et al., 1995; Omri et al., 1995).

For a liposomal formulation to be used clinically, it must be stable in the systemic circulation. When liposomes enter the circulation, serum components such as lipoproteins, albumin, immunoglobulins, and phospholipases destabilize liposomes (Bonte and Juliano, 1986; Simoes et al., 2004). Destabilization of liposomes results in perturbation of liposomal structural integrity and permeability properties with leakage of the entrapped agents. In our study, total release of encapsulated polymyxin B occurred from DPPC/Chol liposomes within 3 h in contrast to 6 h from POPC/Chol liposomes (Fig. 1). The difference in retention characteristics is probably related to the difference between the lipid phase behaviour of the two phospholipids. More precisely, POPC, having an unsaturated acyl group (oleoyl, 18:1), should be more fluid and have fewer packing defects. The oleoyl side chain would allow for better packing due to its greater conformational freedom imparted by the double bond present in the chain. Moreover, the presence of cholesterol in the membrane would serve to "fill in the gaps" and provide further stability and decreased permeability. This property of cholesterol increases the rigidity of POPC bilayers when compared to that of DPPC bilayers, enabling the antibiotic to diffuse slowly. Indeed, spontaneous release of polymyxin B is faster from DPPC/Chol liposomes (66% after 48 h) compared to POPC/Chol liposomes (24% after 48 h). The faster rate of polymyxin B release from DPPC/Chol liposomes would make this formulation more feasible for use in the treatment of infections.

P. aeruginosa resistance to the common antibiotic is a complex phenomenon brought by adaptive or mutational responses to the cell environment (Kenna et al., 2007; Woodford and Ellington, 2007). Cell surface alterations allow the bacterium to reduce its interactions with the antibiotic thus making it resistant (Hasegawa et al., 1997; Rouveix, 2007). Liposomal formulations have been studied extensively for their display of reduction in cellular resistance by overcoming cellular permeability (Mugabe et al., 2006; Halwani et al., 2007). The interaction of our liposomal formulation with *P. aeruginosa* and its uptake to the cytoplasm was examined by immunocytochemistry studies (Fig. 2). Liposome-entrapped polymyxin B at a concentration of 16 μg/ml polymyxin B, when compared to 64 μg/ml free antibiotic, resulted in an increased penetration of the antibiotic within bacterial cells. The lower MIC's of polymyxin B in resistant cells treated with the liposomal formulation (Table 2) and higher levels of the antibiotic within bacterial cells are evidence to suggest that delivery of polymyxin B as a

liposomal formulation was able to overcome the permeability and cell surface alterations responsible for the development of bacterial resistance.

Our results indicated that the encapsulation of polymyxin B in DPPC/Chol liposomes generally enhanced its antibacterial activity against several strains of Gram-negative bacteria. The antimicrobial activity of a combination of free polymyxin B and empty liposomes being identical to that of free drug alone are evidence to suggest that polymyxin B can be delivered to the microorganisms more effectively when the antibiotic is encapsulated in liposomes. Other investigators have also reported that the enhanced antimicrobial activity exerted by liposomal aminoglycosides is attributed to the fusional interaction between membrane phospholipids of liposomes and bacterial cells (Omri and Ravaoarinoro, 1996; Halwani et al., 2007).

In conclusion, these data suggest that the incorporation of polymyxin B into sonicated DPPC/Chol liposomes enhanced its *in vitro* activity against most of bacterial strains studied. Future experiments will assess the therapeutic effectiveness of liposomal polymyxin B against resistant strains of Gram-negative bacteria in experimental infection models.

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