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A COMPARISON OF THE *in vitro* ANTIMICROBIAL ACTIVITY OF LIPOSOMES CONTAINING MEROPENEM AND GENTAMICIN

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Abstract: The antimicrobial activity of eight cationic, two neutral and three anionic liposome compositions containing meropenem and gentamicin was tested *in vitro* in broth and serum medium. The cationic formulations showed better antibacterial efficacy against both Gram-positive and Gram-negative bacteria than the anionic and neutral ones, regardless of the encapsulated drug. The most effective formulations were the cationic PC/DOPE/DOTAP 3:4:3 and PC/Chol/DOTAP 3:4:3, as the MICs with meropenem were 2 to 4 times lower than those of the free drug.

Key words: Liposomes, Meropenem, Gentamicin, Bacteria

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Abbreviations used: Chol – cholesterol; DDAB – dimethyldioctadecylammonium-bromide salt; DMPG – 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol; DOPE – 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOTAP – 1,2-dioleoyloxy-3-trimethyl ammonium-propane; DPPC – 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; GE – gentamicin; MEM – meropenem; MHB – Mueller Hinton II Broth; MLVs – the multilamellar vesicles; MIC – minimum inhibitory concentration; MPS – the mononuclear phagocytic system; NBS – normal bovine serum; NCCLS – National Committee for Clinical Laboratory Standards; OPA – o-phthaldialdehyde; PBS – phosphate-buffered solution; PC – phosphatidylcholine, PI–- phosphatidyl inositol; SA – octadecylamine; ULVs – unilamellar vesicles.

INTRODUCTION

Liposomes are widely used as universal carriers of chemical substances in the cosmetics and pharmaceuticals industries. Liposomal forms of the anti-tumour drugs doxorubicin and vincristine [1, 2] and the antifungal amphotericin B (AmBisome) [3, 4] are already successfully used in veterinary and human healthcare. Intensive research is being carried out on liposomal formulations of antibiotics to increase their pharmacokinetic properties and antimicrobial activity [5, 6]. Liposomes have significant effect as antibiotic carriers, improving drug distribution and decreasing drugs' toxic properties [7-11].

The pharmacokinetics and antibacterial activity of liposomal antibiotics can be modified by several means. The different properties of liposomes depend on their size and lipid composition (charge and fluidity). After intravenous administration, multilamellar vesicles (MLVs) are opsonised by plasma proteins and very quickly taken up by the mononuclear phagocytic system (MPS) [6, 10]. By contrast, unilamellar vesicles (ULVs) are small and able to avoid fast uptake by the MPS, which increases their systemic circulation time. This may permit specific targeting to the intended tissues [6, 10]. Smaller vesicles also show better accumulation in infected tissue and organs because of their ability to leave the vascular compartment at the inflammatory site by means of increased capillary permeability [5, 9]. ULVs can be successfully used in the treatment of non-MPS tissue infections. The most promising results regarding toxicity decrease, plasma half-life prolongation, and retention enhancement at infected sites were shown by liposomal formulations of aminoglycosides [10, 12, 13], fluoroquinolones [9], polymyxin B [7, 14] and antitubercular drugs [15-17]. Variations in the size, surface charge, fluidity and lipid formulations of liposome vesicles make targeted delivery of encapsulated antibiotics possible.

Thanks to their ability to accumulate in the mononuclear phagocyte system (MPS), liposomal vesicles give increases in the bactericidal activity of drugs against such intracellular pathogens as *Mycobacterium* spp. [5, 15-18], *Salmonella* spp. [11, 19-21], *Francisella tularensis* [22] and *Listeria monocytogenes* [23]. Proteoliposomes and cationic liposomes were investigated for their potential targeting ability to the bacterial biofilms produced by *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Staphylococcus epidermidis*, and oral bacteria [24-26]. Fluid liposomal drug formulations were developed to increase the bactericidal efficacy of antibiotics by promoting effective interaction between the bacteria and liposomes. Compared with the free drug, various liposomal forms of fluroquinolones and aminoglycosides yielded reductions in MIC for Gram-positive and Gram-negative bacteria [27-31]. Tobramycin encapsulated in Fluidosomes® showed efficient *in vitro* antimicrobial activity against *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Escherichia coli* and *Stenotrophomonas maltophilia* strains [28]. Sachetelli demonstrated that the antibiotics in Fluidosomes® were able to overcome

bacterial resistance related to the permeability barrier and enzymatic hydrolysis by a fusion process between the liposomes and bacterial membranes [32].
There are many benefits to using liposomes as antibiotic carriers, and there are ongoing studies to find new liposomal forms of drugs. This study was designed to construct and characterise various liposomal formulations of meropenem and gentamicin. One objective of this study was to determine the most efficient drug entrapment related to the lipid composition, fluidity, and surface charge of the liposomes. The second aim was to evaluate the *in vitro* antimicrobial activity of the prepared liposomes against Gram-negative and Gram-positive bacteria in two different media: serum and broth.

MATERIALS AND METHODS

Chemicals

Meropenem (MEM) was obtained from Astra Zeneca UK Ltd. (Macclesfield, UK). Gentamicin (GE) was obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). 1,2-dioleoyloxy-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), phosphatidylcholine (PC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG) were purchased from Northern Lipids Inc. (Vancouver, Canada). Octadecylamine (SA) was obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and cholesterol (Chol) from E. Merck (Darmstadt, Germany). HPLC solvents were supplied by J.T. Baker (Deventer, Holland). Dimethyldioctadecylammonium-bromide salt (DDAB) was obtained from Avanti Polar Lipids (Alabaster, USA). Phosphatidyl inositol (PI) was purchased from Lipids Products (Redhill, Great Britain). Phthaldialdehyde (OPA) was obtained from Fluka Chemie GmbH (Buchs, Germany).

Encapsulation of antibiotics in liposomes

The lipid compositions of the various liposomes used in the experiments are presented in Tab. 1. Appropriate amounts of lipids dissolved in chloroform (10 mg/ml) were mixed in a 100-ml round-bottom flask. By evaporating the organic solvent at 40ºC, a thin film of dry lipid was formed on the inner wall of the flask. The residual solvent was removed under a high vacuum applied for at least 1 h. The dry lipid films (with 30 mg of total lipid) were hydrated by adding 1 ml of an antibiotic solution (35 mg in phosphate-buffered solution (PBS), pH 7.2-7.4). Hydration was performed at a temperature above the phase transition temperature of the main liposome lipid (20ºC for PC, 50ºC for DPPC), and was facilitated by adding two 5-mm glass beads and vortexing the liposomal suspension. To enhance drug encapsulation capacity, the FAT procedure was performed by freezing the multilamellar vesicles (MLVs) in liquid nitrogen and thawing the samples in a water bath (6-7 cycles) at the same temperature that was employed in the formation of the MLVs. Unilamellar liposomes (ULVs)

were prepared by extrusion (10x) through two stacked polycarbonate filters of 100-nm pore size (Nucleopore, Whatman) at 20ºC or at 50ºC on a Thermobarrel Extruder (Lipex Biomembranes, Vancouver, BC, Canada). The mean vesicle size was between 107 and 152 nm. Unencapsulated drug was removed from the liposomes on a Sephadex® G-50 fine (1 x 20 cm) column equilibrated with PBS. The opalescent portions (3.1-3.4 ml) of the liposome fractions were collected, and the drug and lipid concentrations were then determined. The liposome size (multimodal analysis, volume weighted) and Zeta potential were routinely determined on a Zetasizer 5000 (Malvern Instruments Ltd., Malvern, UK). The lipid concentration was determined colorimetrically with ammonium ferrothiocyanate [33].

The quantification of meropenem in liposomes was performed via the HPLC method as described elsewhere [34]. The gentamicin concentration within the liposomal suspension was determined spectrofluorimetrically based on a method developed in our laboratory. Briefly, the liposomal suspension containing gentamicin was diluted 10 times in water and then a 30-µl aliquot was placed in a glass tube, followed by 600 µl of methanol. The resulting mixture was then vortexed and, finally, 970 µl of water was added to the tube. After intensive stirring, 400 µl of the OPA reagent and 500 µl of methanol were placed in the tube, and the intensive stirring was repeated for 1 min. The control sample was prepared in the same way, but instead of the liposomal suspension, the buffer used for liposome preparation was utilised. The relative fluorescence of the test samples was measured on a Kontron SMF 25 fluorophotometer (Kontron Instruments, Italy) at an excitation wavelength of 340 nm and an emission of 455 nm. The amount of gentamicin was then read from a standard curve previously prepared using 5 different amounts of gentamicin (1-5 µg) with the same procedure as in the case of the test samples. All the samples were prepared in triplicate.

In the case of the liposomes composed of lipids bearing amino groups (DOPE, SA), the lipids were first extracted by a modified Bligh-Dyer extraction procedure. The preparation of the OPA reagent was performed according to the following procedure: 0.2 g of phthaldialdehyde was dissolved in 1 ml of methanol and the solution was then mixed with 19 ml of a 0.4 M boric buffer (pH 10.4); then 0.4 ml of mercaptoacetic acid was added and the pH was adjusted to 10.4 with a potassium hydroxide solution. This reagent was protected from light and used within 3 days of preparation.

Drug leakage into the culture medium

To determine drug leakage into the culture medium, PC/DOPE/DOTAP 3:4:3 and PC/Chol/DOTAP 3:4:3, the best liposomal compositions of the drug-bearing liposomes, were prepared as described above from 20 mg of total lipids. After liposome extrusion and free drug removal, liposomes at a concentration of 10 mg of total lipid/ml were gently mixed with broth medium in a volume ratio of 1:10, and incubated at 37ºC for 24 h. To determine the level of drug leakage,

200 µl samples were taken 0, 1, 3, 5, 20 and 24 hours after the start of incubation. Then the free drug was separated from the liposomes using micro sephadex G-50 fine columns. The quantification of meropenem and gentamicin was carried out as described above.

Bacterial strains

Four bacterial strains from the American Type Culture Collection were used: *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 700601 and *Escherichia coli* ATCC 25922 as representative Gram-negative strains and *Staphylococcus aureus* ATCC 29213 as a Gram-positive strain. The bacteria were stored at -70ºC in Trypticase Soy Broth (Becton Dickinson and Company, Cockeysville, MD, USA) supplemented with 20% glycerol.

Determination of bacterial susceptibility

The antimicrobial activity of the liposome-encapsulated antibiotic was compared with that of the free drug by determining the MICs of the bacterial strains. The MIC tests were performed via a broth microdilution method according to the NCCLS standards [35]. Serial two-fold dilutions from 0.015 µg/ml to 64 µg/ml of the free and the liposomal drug in phosphate buffer solution, pH 7.4, were prepared. Immediately after preparation, 100 µl of each of the 13 drug concentrations were placed into microtitre wells. For the experiments, bacterial strains were inoculated onto blood agar plates, incubated for 18 h at 35ºC, and then diluted in PBS to an optical density equal to the McFarland number, 0.5. 10-µl amounts of bacterial culture diluted 1:10 (approximate concentration: 10^7 cells/ml) were added to the microtitre wells containing the drug solution and incubated at 35ºC. After 2 h, 100 µl of cation-adjusted Mueller Hinton II Broth (MHB) (Becton Dickinson and Company, Cockeysville, MD, USA) or 100 µl of normal bovine serum (NBS) at a final concentration of 25% (NSB/MHB) were added to each well. The final concentration of micro-organisms was $5x10^5$ cfu/ml. The plates were then incubated for 18 h at 35ºC. MIC was defined as the lowest concentration of drug at which no visible growth of bacteria was observed after 18 h. The positive controls (growth) were bacteria in broth (serum) and bacteria with empty liposomes in broth (serum). The negative controls (sterility) were uninoculated broth, and each of the drug/liposome dilutions in broth. A control consisting of a bacterial culture with empty liposomes and free drug was also used, but no differences from the growth with the free drug alone were observed. Each assay was repeated three times, and then three additional times on a different day to ensure the reproducibility of the results.

RESULTS

Eight cationic, two neutral, and three anionic liposome compositions containing meropenem and gentamicin were prepared. The characteristics of the liposomal formulations are summarised in Tab. 1. For liposome preparation and drug

encapsulation, the thin lipid film method was chosen as the routine method. Due to the characteristics of the meropenem molecule, an active drug-loading method could not be applied. Other methods yielding high-efficiency encapsulation, such as reverse-phase evaporation, were not suitable because of the presence of residual toxic organic solvents in the liposome preparations. Another issue could be the use of the freeze-dried liposome rehydratation method, which often offers a higher encapsulation efficiency than methods such as the thin lipid film method; however, in the case of meropenem, we obtained similar results with the two methods (data not shown). Meropenem encapsulation efficiency using PBS was found to be in the range of 1.16 to 3.70%. The MEM-to-lipid ratios were low and varied from 1:23 to 1:72 w/w. Previous experiments of meropenem dissolved in 150 mM NaCl yielded a higher encapsulation capacity in the range of 3.7% to 7.2% (data not shown). The reason for this two-fold encapsulation decrease could be the slight amphipatic properties of meropenem.

Tab. 1. Properties of the liposomes containing the antibiotics

	Liposome composition	MEM encapsulation capacity [%]	MEM: lipid ratio [w:w]	GE encapsulation capacity [%]	GE: lipid ratio [w:w]
cationic	PC/Chol/SA 5:3:2 m/m	2.42 ± 0.40	1:35	4.77 ± 0.70	1:13
	PC/Chol/DDAB 4:4:2 m/m	2.17 ± 0.40	1:37	4.37 ± 0.70	1:14.3
	PC/DOPE/DOTAP 4:4:2 m/m	1.16 ± 0.40	1:72	6.61 ± 0.50	1:9.4
	PC/DOPE/DOTAP 3:4:3 m/m	1.43 ± 0.40	1:58	4.47 ± 0.40	1:14
	PC/DOPE/SA 4:4:2 m/m	2.40 ± 0.40	1:35	5.86 ± 0.70	1:10.6
	PC/Chol/DOTAP 5:3:2 m/m	3.58 ± 0.50	1:23	4.88 ± 0.50	1:12.8
	PC/Chol/DOTAP 3:4:3 m/m	2.10 ± 0.40	1:40	2.70 ± 0.30	1:23
	DPPC/Chol/DOTAP 5:3:2 m/m	3.70 ± 0.60	1:23	4.30 ± 0.40	1:14.5
neutral	PC/Chol 7:3 m/m	1.28 ± 0.40	1:65	2.90 ± 0.50	1:21.5
	DPPC/Chol 7:3 m/m	2.05 ± 0.40	1:39	1.79 ± 0.20	1:35
anionic	PC/Chol/PI 5.7:3:1.3 m/m	2.16 ± 0.40	1:39	4.80 ± 0.70	1:12.8
	PC/DMPG/DOPE 6:2:2 m/m	2.67 ± 0.30	1:31.5	2.60 ± 0.20	1:32
	DPPC/DMPG 18:1 w/w	2.15 ± 0.50	1:36	5.46 ± 0.70	1:11.4

However, in these experiments, meropenem was dissolved in PBS prior to encapsulation to give uniformity with the encapsulation of gentamicin. Gentamicin encapsulation efficiency in PBS was relatively higher than meropenem encapsulation (between 1.79 and 6.61%). For most of the charged formulations, the encapsulation efficiency was in the range of 4.30 to 6.61%, and the GE-to-lipid ratios were high, varying from 1:9.4 to 1:14.5 w/w (except for the PC/Chol/DOTAP 3:4:3 m/m formulation). Anionic liposomes containing the fusogenic lipid DOPE exhibited a lower encapsulation potency (2.6%). Lutwyche *et al.* [20] showed that 25 to 33% of the total gentamicin was associated with the outer surface of the anionic liposomes composed of DOPE

Tab. 2. *In vitro* activity of cationic liposome formulations containing meropenem against reference strains in MHB and NBS medium

Bacterial strains	MIC values in MHB (NBS) medium [μg/ml]								
	Free drug	PC/Chol/SA 5:3:2	PC/Chol/DDAB 4:4:2	PC/DOPE/DOTAP 4:4:2	PC/DOPE/DOTAP 3:4:3	PC/DOPE/SA 4:4:2	PC/Chol/DOTAP 5:3:2	PC/Chol/DOTAP 3:4:3	DPPC/Chol/DOTAP 5:3:2
P. aeruginosa ATCC 27853	0.5*[1]	0.25	0.5	0.25	0.125	0.125	0.125	0.125	0.5
	(1)[2]	(8)	(8)	(4)	(2)	(2)	(2)	(2)	(4)
K. pneumoniae ATCC 700601	0.125[1]	0.125	0.25	0.06	0.06	0.25	0.125	0.06	0.125
	(0.125)[2]	(0.25)	(0.5)	(0.25)	(0.06)	(0.5)	(0.25)	(0.06)	(0.125)
E.coli ATCC 25922	0.06*[1]	0.06	0.125	0.015	0.015	0.25	0.125	0.015	0.015
	(0.125)[2]	(0.25)	(0.25)	(0.125)	(0.015)	(0.125)	(0.25)	(0.06)	(0.06)
S.aureus ATCC 29213	0.25*[1]	0.5	0.5	0.25	0.06	0.5	0.25	0.125	0.5
	(0.5)[2]	(2)	(2)	(1)	(0.5)	(1)	(1)	(0.5)	(1)

*MIC range of MEM according to NCCLS: *P. aeruginosa* ATCC 27853 0.25-1 μg/ml; *E. coli* ATCC 25922 0.008-0.06 μg/ml; *S. aureus* ATCC 29213 0.03-0.12 μg/ml; [1]MHB medium; [2]NSB medium

lipid. They reported a gentamicin encapsulation capacity in the anionicformulation DOPE/DOPS/PEG at a level of 2.8%. The encapsulation efficiency of the drug was thus relatively low, but similar results were obtained by other investigators [20, 30, 31]. No noticeable differences in encapsulation efficiency were observed in relation to the composition, fluidity, or charge of the liposomes.

Liposome-encapsulated antibiotics are used both locally (wound treatment, intrabronchial/intratracheal administration) and intravenously [10]. To approximate *in vivo* conditions, in this study, we measured *in vitro* susceptibility to liposomal formulations of meropenem and gentamicin in both MHB medium and 50% normal bovine serum (NBS). The bactericidal activities of the cationic liposomes containing meropenem against the reference strains are presented in Tab. 2. The most efficient of the tested lipid formulations were fluid liposomes composed of PC/DOPE/DOTAP 3:4:3 and PC/Chol/DOTAP 3:4:3. The MICs for all the tested bacterial strains were 2 to 4 times lower than those of the free drug in MHB medium. In NBS medium, the values of MIC for these two formulations were almost the same for the *K. pneumoniae* and *E. coli* strains, but for the *P. aeruginosa* and *S. aureus* isolates, they were similar to or even higher than those determined for the free drug. The other cationic liposomes containing meropenem showed various antimicrobial activities towards the tested isolates, comparable to the MICs obtained for the free drug or for 1 dilution higher or lower. An interesting phenomenon was noticed in the case of *P. aeruginosa* susceptibility in the serum medium. The MICs of all the cationic formulations of meropenem were 4 to 32 times higher than those in the MHB medium conditions. This phenomenon was not observed for the other Gram-negative *K. pneumoniae* and *E. coli* strains, nor for the Gram-positive *S. aureus* strain. Meropenem loaded in neutral and anionic vesicles exhibited various *in vitro* antimicrobial activities against the tested isolates compared with the free drug in both kinds of medium (Tab. 3). The MICs of the liposomes for the reference strains were similar to or, most often, higher than those for free meropenem. In MHB medium, we found no significant differences in the bacterial susceptibility to the neutral liposomes (PC/Chol, DPPC/Chol) and the anionic formulations (PC/DMPG/DOPE, PC/Chol/PI, DPPC/DMPG) containing meropenem. Relatively rigid vesicles (PC/Chol, PC/Chol/PI, DPPC/Chol) exhibited significantly increased MICs compared with the efficacy of fluid liposomes in serum conditions. The lipid formulation DPPC/DMPG 18:1 (Fluidosomes®) had a bactericidal efficacy against *K. pneumoniae* and *E. coli* similar to that of the free drug in both media. In the serum medium, the *P. aeruginosa* strain was 4 to 32 times more resistant to the anionic and neutral liposomal forms of meropenem than under the MHB medium conditions. The same situation was observed in the case of the cationic formulations. Generally, cationic liposomes containing meropenem showed better antibacterial activity, because the MICs for meropenem encapsulated in the anionic and neutral formulations were equal to or higher than the MICs of the free drug.

Tab. 3. *In vitro* activity of neutral and anionic liposome formulations containing meropenem against reference strains in MHB and NBS medium

Bacterial strains	MIC values in MHB (NBS) medium [μg/ml]					
	Free drug	PC/Chol 7:3	PC/Chol/PI 5.7:3:1.3	PC/DMPG/ DOPE 6:2:2	DPPC/Chol 7:3	DPPC/DMPG 18:1
P. aeruginosa ATCC 27853	0.5*[1]	0.5	4	0.5	4	4
	(1)[2]	(16)	(16)	(4)	(32)	(4)
K. pneumoniae ATCC 700601	0.125[1]	0.5	8	1	1	0.25
	(0.125)[2]	(1)	(2)	(0.125)	(1)	(0.25)
E.coli ATCC 25922	0.06*[1]	0.25	8	1	0.5	0.125
	(0.125)[2]	(1)	(8)	(0.25)	(1)	(0.125)
S.aureus ATCC 29213	0.25*[1]	4	0.5	0.25	4	1
	(0.5)[2]	(8)	(8)	(0.5)	(16)	(2)

*MIC range of MEM according to NCCLS: *P. aeruginosa* ATCC 27853 0.25-1 μg/ml; *E. coli* ATCC 25922 0.008-0.06 μg/ml; *S. aureus* ATCC 29213 0.03-0.12μg/ml; [1]MHB medium; [2]NSB medium

The antibacterial efficacies of the same cationic, neutral, and anionic formulations were also tested with encapsulated gentamicin (data not shown). The most efficient liposomal formulation was composed of PC/DOPE/DOTAP 3:4:3, where the MICs for the *P. aeruginosa, E. coli* and *S. aureus* strains were similar to those of free gentamicin. The activity of the PC/Chol/DOTAP formulations also showed equal activity against the *E. coli* and *S. aureus* strains, but only in the serum medium. The remaining cationic, neutral and anionic liposomes containing gentamicin showed worse antimicrobial activity in MHB and NBS media compared with the free drug (4 to 64 times higher MIC values).
The PC/DOPE/DOTAP 3:4:3 and PC/Chol/DOTAP 3:4:3 formulations with encapsulated meropenem or gentamicin were revealed to be the most active against Gram-negative and Gram-positive bacteria. These compositions were stable at 37ºC. Retention of meropenem in the PC/DOPE/DOTAP 3:4:3

liposome in the culture medium was 91, 82, 80, 70 and 67% after 1, 3, 5, 20, and 24 h of incubation, respectively (Fig. 1). Retention of gentamicin in the same formulation was higher, reaching 78% after 24 h of incubation (Fig. 2). Drug leakage from the PC/DOTAP/Chol 3:4:3 formulation amounted to approximately 23 and 29% for meropenem and gentamicin, respectively (Figs 1 and 2).

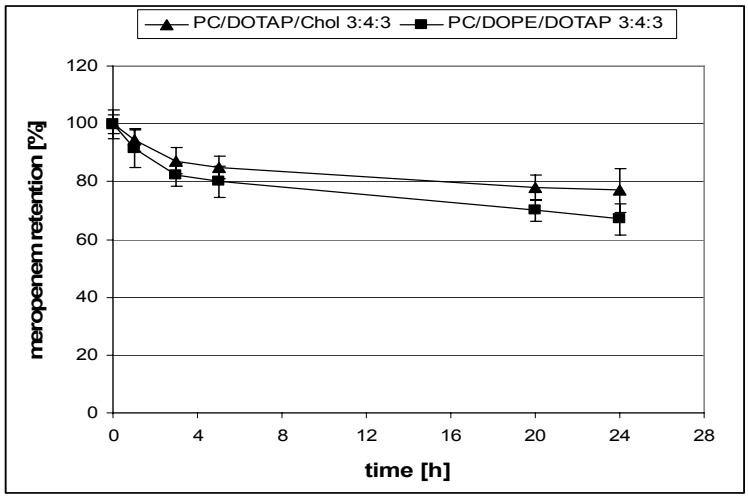

Fig. 1. Meropenem retention profile in PC/DOTAP/Chol 3:4:3 and PC/DOPE/DOTAP 3:4:3 liposome formulations in MHB medium at 37ºC.

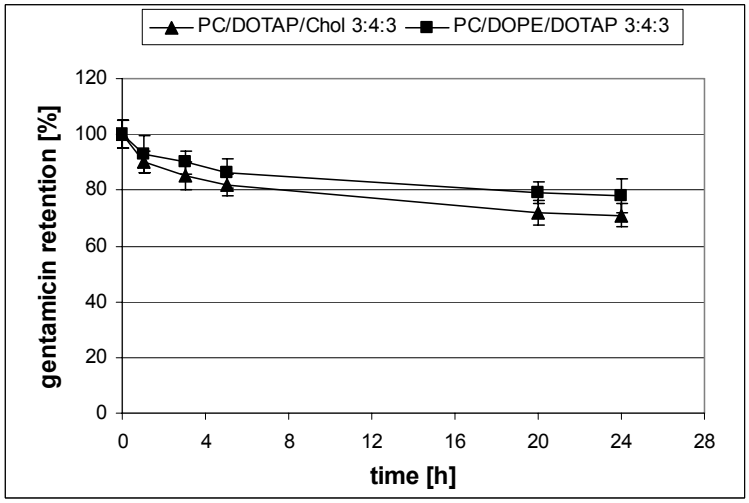

Fig. 2. Gentamicin retention profile in PC/DOTAP/Chol 3:4:3 and PC/DOPE/DOTAP 3:4:3 liposome formulations in MHB medium at 37ºC.

In the case of fluid formulations containing cationic DOTAP lipid, the liposomes showed better antimicrobial efficacy at 30 mol% than at 20 mol% regardless of the encapsulated antibiotic. The anionic and neutral formulations exhibited significantly lower antimicrobial activity because negatively charged bacterial cells interact more effectively with cationic vesicles.

DISCUSSION

Small unilamellar liposomes containing antibiotics exhibited bactericidal activity against extracellular pathogens in the treatment of various infections [13, 36, 37]. In our study, we constructed small (≈100 nm) unilamellar vesicles so that they could be applied as targeted carriers both in intravenous and local treatment. The charge of the liposomal carrier affects the blood circulation time because neutral vesicles are more slowly opsonised by plasma peptides and phagocytes than are cationic and anionic formulations, while charged liposomes accumulate in the spleen, liver and lungs more effectively than neutral vesicles [27]. It was shown that gentamicin encapsulated in cationic formulations exhibited better antibacterial efficacy against intracellular *Brucella* sp. compared with anionic and neutral formulations [38, 39]. Anionic vesicles containing aminoglycosides were bactericidal against *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* strains, extracellular pathogens causing pneumonia [12, 40]. In this study, both kinds of charged liposome were tested. It was demonstrated that cationic formulations exhibit much higher bactericidal efficacy against both Gram-positive and Gram-negative bacteria than anionic and neutral vesicles, regardless of the type of drug. The highest bactericidal activities was shown by the cationic PC/DOPE/DOTAP 3:4:3 and PC/Chol/DOTAP 3:4:3 formulations, as their MICs with meropenem were 2 to 4 times lower than those of the free drugs; with gentamicin, they were similar to the free drug activities. Similar results were obtained in previous studies using *Pseudomonas aeruginosa* strains and liposomal meropenem [41, 42]. Furthermore, formulations containing cationic DOTAP lipid at 30 mol% showed better antimicrobial efficacy than those containing this lipid at 20 mol%, despite the lower encapsulation capacity. We conclude that sub-MIC antimicrobial efficacy is not correlated to rapid drug release from liposomal formulations, but rather to electrostatic interaction/fusion between the liposomes and the bacterial cell. Cationic vesicles probably interact more readily with the negatively charged outer membrane of Gram-negative bacterial cells or, in the case of Gram-positive bacteria, with peptidoglycan, than do anionic and neutral liposomes. Besides the lipid charge, the enhancement of antibacterial activity against extracellular bacteria was also achieved by increasing liposome fluidity. Beaulac [12] noted that fluid and charged vesicles could be used to enhance liposome-bacteria interactions and, consequently, to increase antimicrobial activity.

Beaulac and Sachetelli [28, 32] demonstrated that liposomes consisting of tobramycin encapsulated in DPPC/DMPG 18:1 showed decreases in the bacterial counts in a sub-MIC concentration. In our experiments, the same formulation containing meropenem or gentamicin showed a bactericidal efficacy equal to or much lower than that of the free drug, depending on the bacterial strain.

As liposomes can be applied intravenously and locally, the efficacies of our liposome formulations were tested in two different media: serum and broth. An interesting phenomenon was observed for the *P. aeruginosa* strain. In the serum medium, the *P. aeruginosa* strain was 4 to 32 times more resistant to all the liposomal forms of meropenem than under the MHB medium conditions. In the serum medium, *P. aeruginosa* cells probably convert the properties or structure of their outer membrane by opsonisation of serum peptides or other plasma compounds, resulting in a disruption of the fusion between the liposomes and bacterial cells. This means that these liposome formulations could be used more effectively in local than intravenous treatment.

It is well known that cationic liposomes attach to the bacterial biofilm produced by staphylococci, *Pseudomonas aeruginosa* and oral bacteria [43], and liposomal vesicles are successfully used as the targeted carriers of antiseptic drugs in local applications even though most of the *in vivo* effective liposomes exhibited lower *in vitro* activity compared with the free drug. It seems promising to apply fluid cationic formulations to prevent bacterial biofilm formation, as the most active PC/DOPE/DOTAP 3:4:3 and PC/Chol/DOTAP 3:4:3 formulations exhibited *in vitro* bactericidal efficacy at concentrations 2 to 4 times lower than the free drug against biofilm-producing pathogens such as the *Pseudomonas aeruginosa* or *Staphylococcus aureus* strains. The *in vitro* experiments were performed to determine the role of antibiotic carriers mainly in the targeting of the bacterial cells of extracellular or intracellular pathogens. The *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* and *Staphylococcus aureus* strains used in this study can cause local and systemic infections. All these bacteria are known lung, intestinal, kidney, spleen, soft tissue and skin pathogens. They can cause acute and chronic community-acquired and nosocomial diseases. Our results show that liposomal antibiotics could be successfully used in local and intravenous applications in the eradication of these common pathogens. Further investigation verifying the antimicrobial activity of the tested cationic formulations will be carried out using an animal model.

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