International Journal of Pharmaceutics 367 (2009) 211–219

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

The interaction between *Pseudomonas aeruginosa* cells and cationic PC:Chol:DOTAP liposomal vesicles versus outer-membrane structure and envelope properties of bacterial cell

Zuzanna Drulis-Kawa[a,c,*], Agata Dorotkiewicz-Jach[a], Jerzy Gubernator[b,d], Grzegorz Gula[a], Tomasz Bocer[a], Wlodzimierz Doroszkiewicz[a]

[a] *Institute of Genetics and Microbiology, University of Wroclaw, Przybyszewskiego 63/77, 51-148 Wroclaw, Poland*
[b] *Institute of Biochemistry and Molecular Biology, University of Wroclaw, Przybyszewskiego 63/77, 51-148 Wroclaw, Poland*
[c] *Lower Silesian Centre of Paediatrics in Wroclaw, Kasprowicza 64/66, 51-147 Wroclaw, Poland*
[d] *Academic Centre for Biotechnology of Supramolecular Lipid Aggregates, Przybyszewskiego 63/77, 51-148 Wroclaw, Poland*

ARTICLE INFO

Article history:
Received 27 June 2008
Received in revised form
23 September 2008
Accepted 23 September 2008
Available online 5 October 2008

Keywords:
Pseudomonas aeruginosa
Outer membrane
Liposomes
Interactions

ABSTRACT

The interactions between cationic liposomal formulations (PC:Chol:DOTAP 3:4:3) and 23 *Pseudomonas aeruginosa* strains were tested. The study was undertaken because different antimicrobial results had been obtained by the authors for *Pseudomonas aeruginosa* strains and liposomal antibiotics (Drulis-Kawa, Z., Gubernator, J., Dorotkiewicz-Jach, A., Doroszkiewicz, W., Kozubek, A., 2006. The comparison of *in vitro* antimicrobial activity of liposomes containing meropenem and gentamicin. Cell. Mol. Biol. Lett., 11, 360–375; Drulis-Kawa, Z., Gubernator, J., Dorotkiewicz-Jach, A., Doroszkiewicz W., Kozubek, A., 2006. *In vitro* antimicrobial activity of liposomal meropenem against *Pseudomonas aeruginosa* strains. Int. J. Pharm., 315, 59–66). The experiments evaluate the roles of the bacterial outer-membrane structure, especially outer-membrane proteins and LPS, and envelope properties (hydrophobicity and electrostatic potential) in the interactions/fusion process between cells and lipid vesicles. The interactions were examined by fluorescent microscopy using PE-rhodamine-labelled liposomes. Some of the strains exhibited red-light emission (fusion with vesicles or vesicles surrounding the cell) and some showed negative reaction (no red-light emission). The main aim of the study was to determine what kinds of bacterial structure or envelope properties have a major influence on the fusion process. Negatively charged cells and hydrophobic properties promote interaction with cationic lipid vesicles, but no specific correlation was noted for the tested strains. A similar situation concerned LPS structure, where parent strains and their mutants possessing identical ladder-like band patterns in SDS-PAGE analysis exhibited totally different results with fluorescent microscopy. Outer-membrane protein analysis showed that an 18-kDA protein occurred in the isolates showing fusion with rhodamine-labelled vesicles and, conversely, strains lacking the 18-kDA protein exhibited no positive reaction (red emission). This suggests that even one protein may be responsible for favouring stronger interactions between *Pseudomonas aeruginosa* cells and cationic liposomal formulations (PC:Chol:DOTAP 3:4:3).

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Liposomes have a significant effect as antibiotic carriers on improving drug distribution and decreasing a drug's toxic properties (Omri et al., 2002; Sachetelli et al., 1999; Schiffelers et al., 2001a,b; Webb et al., 1998). 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). The most promising results regarding decreased toxicity, prolonged plasma half-life, and enhanced retention at infected sites were shown by liposomal formulations of aminoglycosides (Beaulac et al., 1996; Schiffelers et al., 2001b), fluoroquinolones (Schiffelers et al., 2001a), polymyxin B (Omri et al., 2002; Desai et al., 2003), and antitubercular drugs (Deol and Khuller, 1997; Labana et al., 2002; Mehta et al., 1993). Variations in the size, surface charge, fluidity, and lipid formulation of liposome vesicles make targeted delivery of encapsulated antibiotics possible. Pro-

* Corresponding author at: Institute of Genetics and Microbiology, University of Wroclaw, Przybyszewskiego 63/77, 51-148 Wroclaw, Poland. Tel.: +48 71 325 21 51; fax: +48 71 325 21 51.
E-mail address: kawa@microb.uni.wroc.pl (Z. Drulis-Kawa).

0378-5173/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2008.09.043

teoliposomes and cationic liposomes were investigated for their potential targeting ability to the bacterial biofilms produced by the skin and oral bacteria (Sanderson et al., 1996; Jones et al., 1993). Furthermore, it has been demonstrated that the same cationic lipids used in liposome preparations might act by themselves as anti-infective agents (Campanha et al., 1999; Carmona-Ribeiro, 2003; Vieira and Carmona-Ribeiro, 2006). Fluid liposomal drug formulations were developed to increase the bactericidal efficacy of antibiotics by promoting effective interaction between bacteria and liposomes. Various liposomes containing fluroquinolones and aminoglycosides demonstrated reductions in minimum inhibitory concentrations (MICs) compared with the free drug against Gram-positive and Gram-negative bacteria (Beaulac et al., 1998; Furneri et al., 2000; Puglisi et al., 1995). Sachetelli demonstrated that 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 (Sachetelli et al., 2000). Liposome–bacterium fusion has been reported by other investigators as well (Sekeri-Pataryas et al., 1985; Tomlinson et al., 1989).

Several hypotheses concerning the biochemical mechanism by which liposomes interact with cells were discussed in detail by Mugabe et al. (2006). In our previous studies (Drulis-Kawa et al., 2006a,b; Gubernator et al., 2007) a fluid cationic formulation (PC:Chol:DOTAP 3:4:3) containing meropenem and gentamicin showed better antimicrobial efficacy against *Pseudomonas aeruginosa* isolates compared with the free drugs, but not for all tested strains. We wondered what kind of bacterial features favour interaction (fusion) between liposomes and the cell. The present study was designed to examine the interactions between a cationic liposomal formulation (PC:Chol:DOTAP 3:4:3) and a wide group of control and clinical *Pseudomonas aeruginosa* strains. This time our intention was to evaluate the role of the bacterial outer-membrane structure, especially outer-membrane proteins and LPS, and envelope properties (hydrophobicity and electrostatic potential) in the fusion process between cells and lipid vesicles.

2. Materials and methods

2.1. Chemicals

1,2-Dioleoyloxy-3-trimethylammonium-propane (DOTAP) and phosphatidylcholine (PC) were purchased from Northern Lipids, Inc. (Vancouver, BC, Canada). Cholesterol (Chol) was obtained from E. Merck (Darmstadt, Germany). HPLC solvents were supplied by J.T. Baker (Deventer, the Netherlands). Rhodamine B was purchased from Acros Organics (Geel, Belgium) and PE-Rhodamine (fluorescent label-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl)) ammonium salt from Avanti Polar Lipids (Alabaster, USA). The *n*-hexadecane and Tris–HCl buffer were obtained from Sigma–Aldrich Chemie GmbH (Steinheim, Germany).

2.2. Bacterial strains

The strains used in this study are listed in Table 1. Bacteria were stored at −70 °C in Trypticase soy broth (Becton Dickinson and Company, Cockeysville, MD, USA) supplemented with 20% glycerol.

2.3. Preparation of PE-rhodamine containing liposomes

PE-rhodamine-labelled liposomes were prepared using a thin lipid film method. The appropriate lipids were mixed as chloroform solutions with a chloroform solution of 0.185 mg fluorescent label 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) ammonium salt in a 100-ml round-bottomed flask to obtain 1 mol% of fluorescent label in 10 mg of lipids in the final liposome preparation. Then the chloroform was evaporated under vacuum on a rotary evaporator and the resulting thin lipid film was hydrated by agitation with 1 ml of PBS at room temperature. The MLV liposomes were then extruded 10 times thru a 100-nm-pore filter using a 10-ml extruder. The size of the liposomes was determined with a ZetaSizer Nano-ZS (Malvern Instruments, UK) and was usually in the range of 105–110 nm.

Table 1
Bacterial strains.

P. aeruginosa	Relevant properties	Resistance mechanisms	Reference
ATCC 27853	MEM^S, IPM^S, GE^S, CIP^S	Wild type	ATCC collection
MEM	MEM^R, IPM^R, GE^S, CIP^S	OprM+; OprD−/OprN+	Selected on free meropenem
PAO1	MEM^S, IPM^S, GE^S, CIP^S	Wild type	Kohler et al. (1999)
PASE1	MEM^S, IPM^R, GE^S, CIP^S	OprM+; OprD−	Kohler et al. (1999)
PA 1425	MEM^S, IPM^R, GE^S, CIP^S	OprM−; OprD−	Kohler et al. (1999)
9/5	MEM^R, IPM^R, GE^S, CIP^S		Clinical strain
12/3	MEM^S, IPM^S, GE^S, CIP^S		Clinical strain
12/3 MEM	MEM^I, IPM^R, GE^S, CIP^S	OprM+; OprD−/OprN+	Selected on free meropenem
12/3 CHOL	MEM^I, IPM^R, GE^S, CIP^S	OprM+; OprD−/OprN+	Selected on liposomal meropenem
14/3	MEM^S, IPM^R, GE^R, CIP^R		Clinical strain
15/3	MEM^S, IPM^S, GE^S, CIP^S		Clinical strain
49/3	MEM^R, IPM^R, GE^S, CIP^S		Clinical strain
82/3	MEM^S, IPM^S, GE^S, CIP^S		Clinical strain
113	MEM^S, IPM^R, GE^R, CIP^S		Clinical strain
249/P	MEM^R, IPM^R, GE^S, CIP^S		Clinical strain
3	MEM^S, IPM^R, GE^S, CIP^S		Clinical strain
12	MEM^S, IPM^S, GE^S, CIP^S		Clinical strain
14	MEM^S, IPM^R, GE^R, CIP^S		Clinical strain
18	MEM^S, IPM^S, GE^S, CIP^S		Clinical strain
20	MEM^S, IPM^R, GE^S, CIP^S		Clinical strain
164	MEM^R, IPM^R, GE^R, CIP^S		Clinical strain
0013	MEM^S, IPM^R, GE^S, CIP^S		Clinical strain
0038	MEM^S, IPM^S, GE^S, CIP^S		Clinical strain

MEM: meropenem; IPM: imipenem; GE: gentamicin; CIP: ciprofloxacin; According to CLSI (NCCLS) breakpoints: R: resistant; S: sensitive; I: intermediate; OprM+: over expression of MexAB-OprM efflux system; OprD−: loss of OprD; OprN+: increased expression of MexEF-OprN efflux system.

2.4. Zeta potential measurements of Pseudomonas aeruginosa strains

Bacterial cells were washed twice in PBS by centrifugation and then diluted in PBS to attain a medium count rate as measured by the correlator of the ZetaSizer Nano-ZS. The bacterial suspension was introduced into the zeta potential measuring cell and the program macro FFR (fast field reversal) was run to measure the zeta potential of the living bacterial cells. The measurements were performed at 37 °C five times per strain.

2.5. Determination of hydrophobic properties

All bacterial cultures were grown in Trypticase soy broth (Difco, Sparks, MD, USA) with incubation at 37 °C for 18 h. Each culture was harvested in its stationary phase by centrifugation and washed twice with 100 mM phosphate buffer (pH 7). The harvested and washed cells were used to characterize the cell-surface properties. Bacterial adhesion to hydrocarbons (BATH method) was measured as described by Rosenberg et al. (1980). Cells were resuspended in phosphate buffer to an optical density at 600 nm (OD600) of about 0.6. An aliquot of cell suspension (1.2 ml) was mixed with 1 ml of *n*-hexadecane by vortexing for 120 s and allowed to separate into phase for 15 min. The OD600 of the aqueous phase was then measured spectrophotometrically. The difference between the optical densities of the aqueous phase before and after mixing with *n*-hexadecane was used to calculate the adhesion as a percentage: $100 \times [1 - (\text{OD600 after mixing/OD600 before mixing})]$.

2.6. Determination of outer-membrane protein patterns

The isolation of OMPs was carried out as follows. Bacteria were cultured on Mueller Hinton Agar, then scraped from the plates into 5 ml of PBS (0.01 M sodium phosphate). The outer membrane proteins of the *Pseudomonas aeruginosa* strains were obtained from Zwittergent Z 3-14 (Calbiochem-Behring) extracts according to Murphy's method (Murphy and Bartos, 1989). Polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). Discontinuous sodium dodecyl sulphate (SDS) gel electrophoresis was carried out on slabs with 12.5% acrylamide. Ten-microliters samples containing 10 μg of proteins were applied to the slabs after heating at 100 °C for 4 min. The same amount of protein sample was applied to the slab for each of the strains. The Sigma wide-range molecular-weight marker M4038 protein standard (6–205 kDa) was used for molecular-weight calibration. Electrophoresis was performed at 100 V for 25 min and then at 200 V for 45 min. After electrophoresis, the gel was stained for 1 h with a solution containing 25% methanol, 10% acetic acid, and 0.05% Coomassie Brilliant Blue and decolourised with 10% acetic acid for 3–5 h. Quantity One (BioRad) software was used for the molecular analysis of the OMPs according to the Sigma wide-range molecular-weight marker M4038 protein standard (6–205 kDa).

2.7. Determination of LPS patterns

Bacterial strains were cultivated in 5 ml of Trypticase soy broth (Difco, Sparks, MD, USA) at 37 °C. After 24 h the cells were harvested and the isolation of LPS was carried out using an LPS Extraction Kit according to the manufacturer's instructions (iNtRON Biotechnology, Korea). The LPS extracts were analysed by discontinuous sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) according to the Laemmli buffer system (Laemmli, 1970) using 5% stacking gel and 15% separating gel. Ten-microliter samples were applied to the slabs after heating at 100 °C for 10 min in sample buffer. Electrophoresis was conducted at 35 mA of constant current with Tris-glycine (pH 8.3) buffer containing 0.1% SDS until the dye reached the bottom of the gel. Silver staining was done according to the Tsai and Frash method (Tsai and Frash, 1982). LPS gave a dark staircase (ladder-like) band pattern due to the carbohydrate chain length variation of the O-antigen portion.

2.8. Fluorescent microscopy analysis

For the experiments the strains were inoculated on Mueller Hinton II Agar plates (MHA) (Becton Dickinson and Company, Cockeysville, MD, USA), incubated for 18 h at 35 °C, and then diluted

Table 2
Analysis of free rhodamine– and liposomal rhodamine–bacterium interactions by fluorescent microscopy; hydrophobic properties and zeta potential in *Pseudomonas aeruginosa* strains.

P. aeruginosa strains	Free rodamine uptake	Liposomal-rodamine uptake	Hydrophobic properties (%)	Zeta potential (mV)
ATCC 27853	+	+	17.7 ± 3.2	−12.8 ± 0.3
MEM	+	–	8.3 ± 1.5	−12.4 ± 0.9
PAO 1	+	+	3.8 ± 4.1	−12.7 ± 1.1
PASE 1	+	+	21.5 ± 4.7	−13.4 ± 0.9
PA 1425	+	+	23.0 ± 5.2	−14.4 ± 1.1
9/5	–	+++	32.0 ± 2.3	−5.8 ± 0.6
12/3	+++	–	0.5 ± 0.6	−11.0 ± 0.6
12/3 MEM	+++	+	1.5 ± 1.7	−11.5 ± 0.6
12/3 CHOL	+++	+	0.5 ± 1.0	−12.2 ± 0.9
14/3	+	–	11.3 ± 2.6	−5.1 ± 0.6
15/3	+	–	11.8 ± 3.5	−12.5 ± 0.9
49/3	+	+++	52.3 ± 3.5	−10.7 ± 1.0
82/3	+++	–	16.3 ± 1.5	−15.0 ± 0.6
113	+	–	17.5 ± 3.5	−7.8 ± 0.7
249/P	+	+	10.0 ± 3.6	−14.7 ± 0.8
3	+	+	30.3 ± 3.2	−14.4 ± 0.4
12	+	+	3.5 ± 0.7	−13.3 ± 1.5
14	+	–	10.3 ± 5.5	−3.9 ± 0.7
18	+	–	34.0 ± 2.9	−14.7 ± 1.1
20	+	+	14.7 ± 3.1	−5.8 ± 0.6
164	+	+	5.3 ± 3.2	−11.8 ± 0.5
0013	+	–	26.7 ± 9.3	−11.4 ± 1.0
0038	+	–	3.0 ± 3.5	−11.4 ± 0.7

(+) low level of red light emission; (+++) high level of red light emission; (–) no reaction.

in 1 ml of saline buffer to an optical density equal to McFarland No. 4 (10^9 cells/ml). Each culture was washed three times by centrifugation. Ten microliters of free rhodamine-B or 50 μl of rhodamine-labelled liposomes were added to a tube containing 0.5 ml of bacterial culture and incubated at 35 °C for 1 h. After incubation, the bacterial culture was washed three times in saline buffer by centrifugation (4000 rpm, 10 min). For microscopic analysis, 10 μl of the culture were embedded in 50 μl of 1% low-melting agarose. All observations were made with an Axio Imager M1 upright wide-field fluorescence microscope (Carl Zeiss, Germany) equipped with an illuminator (Zeiss HBO 100) and a 100× oil immersion objective (Zeiss Plan-Neofluar 100x/1.30). The Zeiss filter set 487915 (absorption 540 nm/emission 625 nm) was used for the observation of rhodamine-B. Exposure times were 500 ms. Images were collected using a Zeiss AxioCam MRc digital colour camera and processed with Zeiss AxioVision 4.5 software.

3. Results

The fluorescent microscopic analyses of liposome–bacterium interactions are presented in Table 2. First the strains were examined for their red self-fluorescence using the Zeiss filter set 487915 (absorption 540 nm/emission 625 nm). None exhibited a positive reaction. The second step was to determine the penetration of free rhodamine B into the bacterial cells. The positive reactions are presented in Fig. 1a. Most of the isolates took up the rhodamine at a low level and four of the *P. aeruginosa* strains (12/3, 12/3 CHOL, 12/3 MEM, and 82/3) exhibited a strong red-light emission effect.

In the case of interactions between liposomes and bacterial cells, three kinds of results were obtained: (i) no visible reaction (no emission); (ii) a positive reaction as liposomes surrounding the bacterial cells (+); (iii) a positive reaction as visible fusion, with red emission of the bacterial membranes (+++). The positive reactions for rhodamine-labelled liposomes are presented in Fig. 1c.

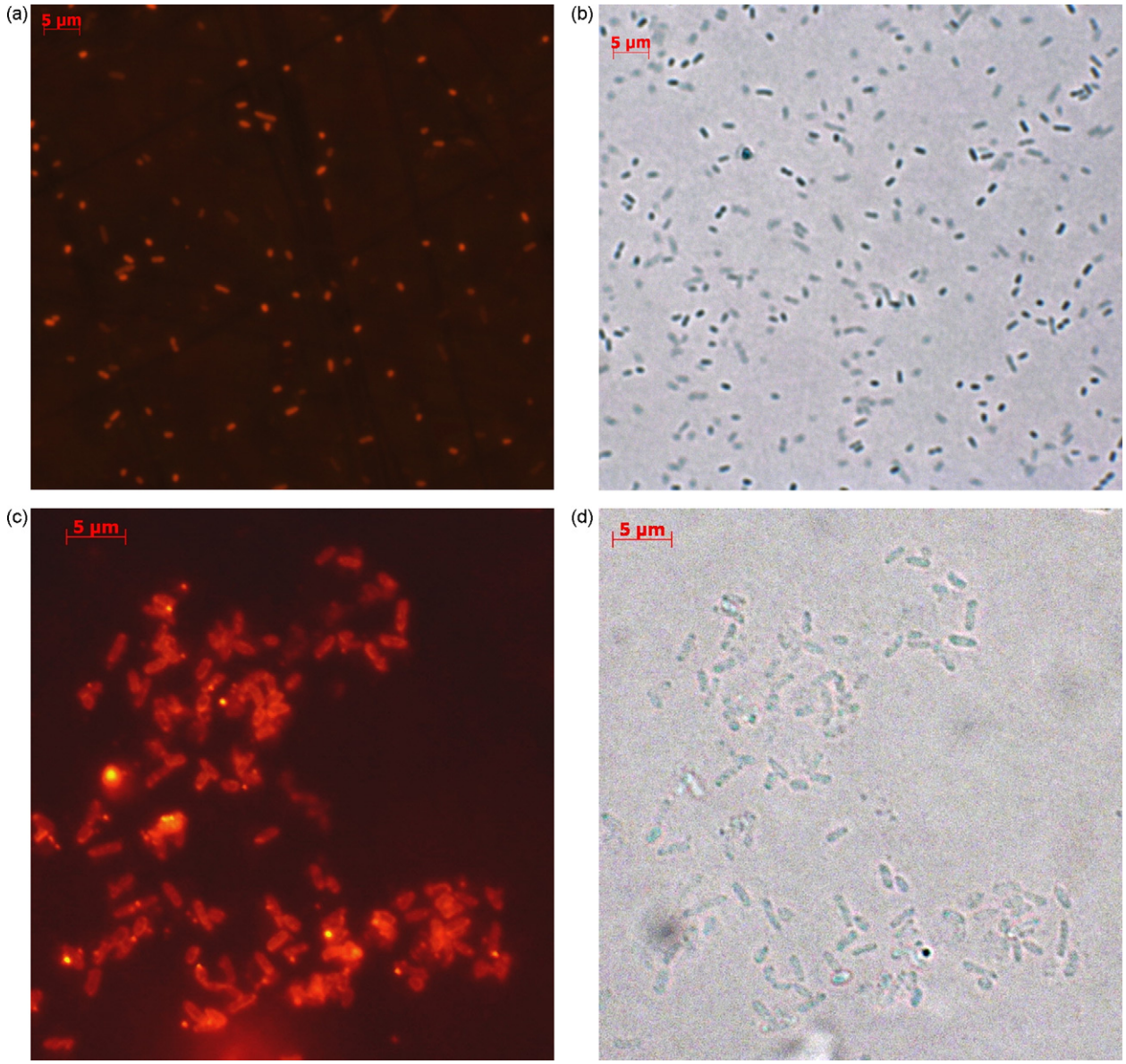

Fig. 1. The fluorescent microscopic analyses of liposome–bacterium interactions. Penetration of free rhodamine B into the bacterial cells: fluorescent microscopy image (a), transparent microscopy image (b). Interactions between rhodamine-liposomes and bacterial cells as visible fusion: red light emission of bacterial membranes: fluorescent microscopy image (c), transparent microscopy image (d). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Of the 23 isolates, only two (9/5 and 49/3) showed visible fusion after 1 h of incubation with rhodamine-liposomes and eleven of them exhibited a positive reaction as liposomes surrounding the cell. An interesting behaviour of bacteria was noticed for the uptake of free and liposomal rhodamine. For example, isolate 12/3 and its mutants took up the free rhodamine very effectively, but there was no positive reaction with liposomal rhodamine in the case of the 12/3 parent strain. In contrast, strain 9/5 exhibited high red emission for liposomal rhodamine but a negative reaction with the free dye.

To verify the influence of bacterial surface properties on the fusion process, zeta potentials and hydrophobic properties were determined (Table 2). All strains were negatively charged, which might favour interactions with the cationic lipid vesicles. The zeta potential varied from −3.9 mV to −15.0 mV. The 9/5 and 49/3 isolates exhibited relatively small negative charges (−5.8 mV and −10.7 mV, respectively). Remaining strains showing positive interactions in fluorescent microscopy belonged as well to low negative and high negative charged bacteria. We therefore conclude that the negative potential of the cell favour the electrostatic interactions between liposomal vesicles and bacterial cell, but probably there are some other factors responsible for observed fusion with liposomes.

The BATH method was chosen to determine hydrophobicity and only one isolate, 49/3, seemed to be hydrophobic (52.3%). The other strains were found to have no hydrophobic properties. The bacterial adhesion of the remaining strains to hydrocarbons was in the range of 0.5–34.0%. Although the adhesion of liposomes to the bacterial surface depends on the balance of electrostatic and hydrophobic interactions, no correlations between hydrophobic properties and fusion results were noted. Some of the strains (9/5 or 49/3) with a higher percentage of adhesion to hydrocarbons showed fusion with liposomal vesicles, but at the same time others (18 and 0013) with similar hydrophobic properties exhibited negative interactions with liposomes.

The next aspect taken into account was LPS structure as an element located on the surface of Gram-negative bacteria. Silver staining of LPS separated by gel chromatography revealed a progressive ladder-like band pattern up the gel (Fig. 2a and b). Three or four (1, 2, 3, 4) band regions can be distinguished presenting populations of molecules differing in O-antigen length. PAO1, PASE1, and PA1425 showed the same band patterns. The same situation

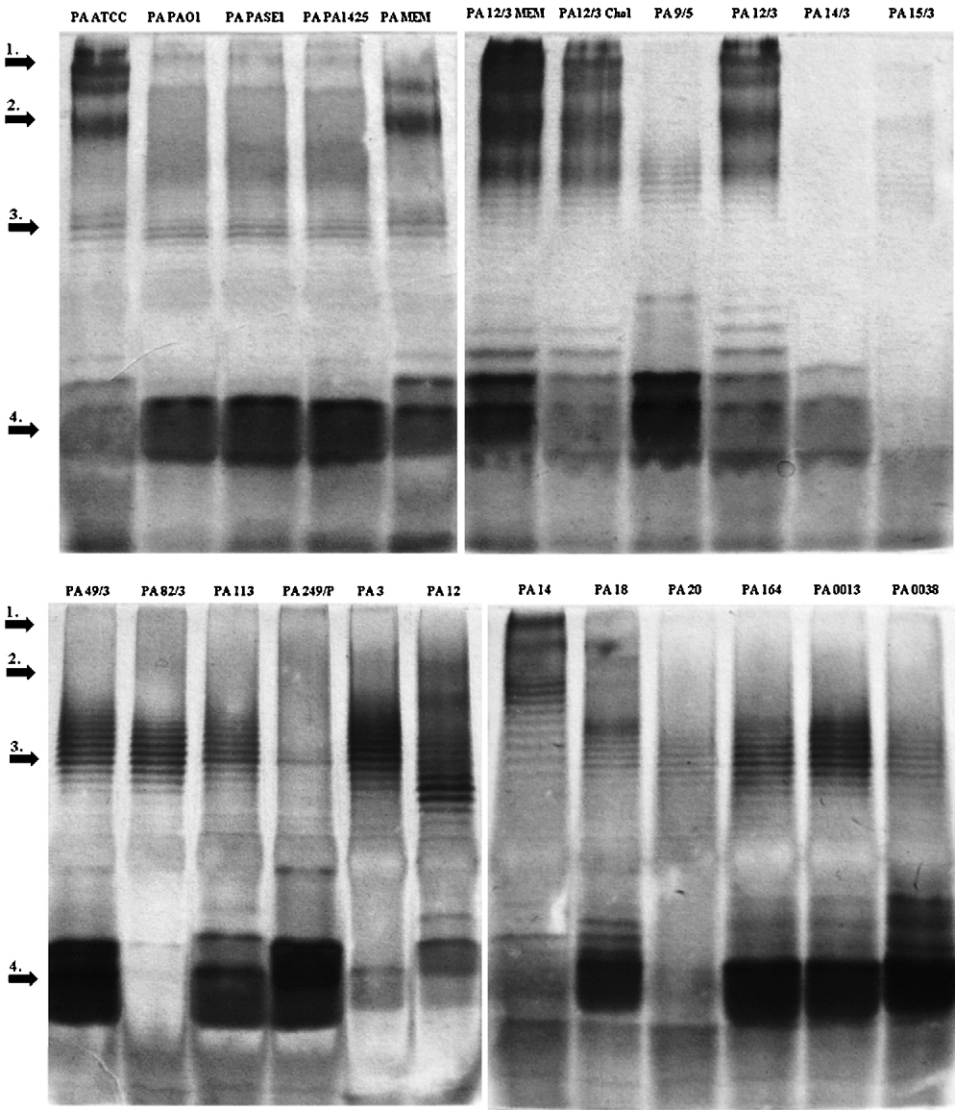

Fig. 2. (a and b) Silver-stained SDS-polyacrylamide gel of LPS from *P. aeruginosa*. Arrows indicate the four intensively stained regions of the *P. aeruginosa* samples: band sets 1–4.

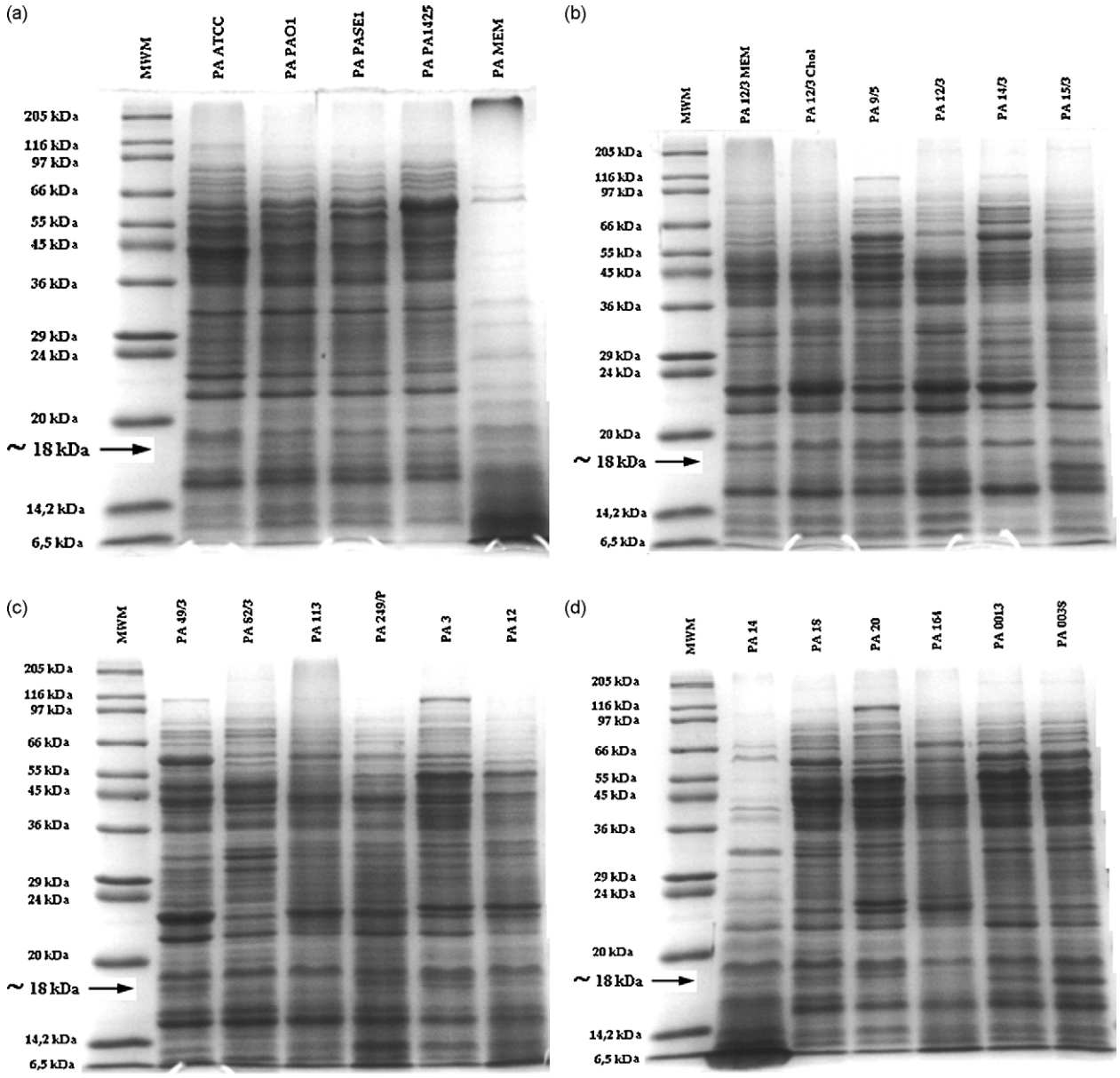

Fig. 3. (a–d) The outer membrane protein patterns of the *P. aeruginosa* isolates.

was noted for the parent strains ATCC and 12/3 and their mutants. This means that meropenem resistance has no effect on changing LPS. For the remaining clinical isolates, various ladder-like patterns were observed. Most of them possessed at least two populations of different O-antigen length, including short, fast-migrating LPS chains, and the LPSs of all the isolates were classified as smooth. LPS, an outer-membrane structure covering the whole surface of the cell, may play a significant role in the interaction between the bacterial cell and liposomal vesicles. When looking for these dependences it was found that LPS is probably of minor importance, as previously thought. It was demonstrated that in the case of the parent strains ATCC and 12/3 and their mutants, the same ladder-like pattern was observed, but quite different interactions with liposomal rhodamine. This means that other bacterial cell-surface molecules should be responsible for favouring the fusion process.

Outer-membrane proteins might also have a role in interactions with lipid vesicles, which is why the outer-membrane protein patterns of the tested isolates were determined. The procedure were carried out according to Murphy's method using Zwittergent detergent. The gel analysis is shown in Fig. 3a–d. In the electrophoregrams we acquired many protein patterns that represented OMPs. It was decided to compare first the differences in OMP profiles between parent and mutant strains that exhibited different reactions with liposomal vesicles. We decided to analyse protein patterns in those cases using QuantityOne software (Table 3). We analysed the correlation of particular proteins acquired or lost in the parents and their mutant strains. Additionally we analysed the protein patterns of the remaining reference strains and two clinical ones (9/5 and 49/3) which gave the most intensive reaction in the visualisation test. Visible reactions within one hour of incubation with liposomal rhodamine were observed in the strains possessing the 18-kDa protein bands (Fig. 3a–d arrow and Table 3 frame). The most intensive red emission in fluorescent microscopy after interaction with liposomal vesicles containing rhodamine was noted for the 9/5 and 49/3 isolates, which exhibited the highest amount of 18-kDa protein among the tested strains. We decided to analyse the protein patterns of the rest of strains and we confirmed the same

Table 3
Analysis of outer membrane protein patterns of chosen *P. aeruginosa* isolates.

Molecular weight [kDa]	ATCC 27853	MEM	PAO1	PASE1	PA1425	12/3	12/3 MEM	12/3 CHOL	9/5	49/3
106							+	+		
84	+							+	+	
77	+		+		+					
72	+		+	+			+			
66	+									
60	+		+	+						
58							+	+		
56	+			+		+			+	+
50	+									
43							+	+		+
37							+	+		
36	+		+	+	+	+			+	+
32		+			+		+	+		+
27	+					+		+		
24	+					+	+			+
18	+		+	+	+		+	+	+	+
17	+	+	+	+	+		+	+	+	+
16	+	+			+		+	+	+	+
15	+		+	+	+	+	+	+	+	+
8	+					+	+	+	+	

+ presence of protein band

correlation. We concluded that one protein (18 kDa) might promote interaction with the liposomal vesicles.

4. Discussion

Fusion interactions between bacterial membrane phospholipids and liposome-containing antibiotic could hold promise in overcoming non-enzymatic drug resistance (Beaulac et al., 1998; Omri and Ravaoarinoro, 1996; Puglisi et al., 1995). Omri et al. (Omri and Ravaoarinoro, 1996) demonstrated an increase in bactericidal activity of netilmicin entrapped in liposomes (PC/Chol 7:1) against Gram-negative *E. coli*, *Pseudomonas aeruginosa*, and *Stenotrophomonas* (*Xanthomonas*) *maltophilia*. Higher antibacterial efficacy of the liposomal form of polymyxin B and aminoglycosides (DPPC:Chol 2:1) against *Pseudomonas aeruginosa* strains compared with the free drug was also described (Omri et al., 2002; Mugabe et al., 2006). Mugabe demonstrated fusion between DPPC:chol liposomal vesicles and bacterial cells using three different methods: transmission electron microscopy, flow cytometric assay, and lipid mixing assay (Mugabe et al., 2006). It was shown that in the case of a clinically resistant *P. aeruginosa* strain, liposomal fusion was significantly delayed in comparison with the susceptible ATCC 10145 strain, but the overall level of fusion was not affected.

In our experiment, the interactions between liposomes and bacterial cells were tested by fluorescent microscopy using rhodamine vesicles. We wanted to examine a higher number of strains to discover the dependencies between antibiotic resistance, outer-membrane properties of the bacteria, and the fusion effect with lipid vesicles. In our study, 23 *P. aeruginosa* strains exhibiting different antibiotic susceptibilities were tested. The ATCC 27853, PAO 1, PASE 1, and PA 1425 isolates were used as controls with strictly defined resistance mechanisms (Kohler and Pechere, 2001). Three mutants with strictly defined resistance mechanisms obtained by free or liposomal meropenem were also included to verify the influence of outer-membrane proteins on the fusion process. The remaining 16 clinical isolates were used to determine the variety of interactions with liposomal vesicles. The penetration of free rhodamine B into bacterial cells was previously tested. Most of the strains, except for 82/3, 12/3, and its mutants, exhibited a low level of uptake of free rhodamine. The results were convergent with a previous finding that xenobiotic efflux pumps, especially MexAB-OprD, interact with the outer-membrane permeability barrier in fluorescent dye extrusion in *Pseudomonas aeruginosa* rods (Germ et al., 1999). Different results were obtained for liposomal rhodamine. Unexpectedly, the antibiotic resistant 9/5 and 49/3 strains showed the most intensive red emission (visible fusion). An interesting behaviour of mutants and their parent strains in rhodamine vesicle fusion was noted. ATCC 27853 exhibited positive results (red emission) under fluorescent microscopy, in contrast to its MEM mutant. The opposite situation was found in the case of the 12/3 isolate and its mutants. PAO 1, possessing wild-type efflux pump patterns, exhibited the same positive reaction as the resistant strains PASE 1 and PA 1425. We concluded that resistance mechanisms, especially outer-membrane proteins involved in the efflux pump system (OprD, OprN, OprM), probably play an imperceptible role in the interaction between liposomal vesicles and bacterial cells.

Another aspect studied was the influence of bacterial cell charge and fusion with liposomes. All the cells were negatively charged in the range of −3.9 mV to −15.0 mV, which forced the interplay with the cationic vesicles. Liposome-cell contact may also be favoured by hydrophobic features of the bacterial cell surface. It is well known that some microorganisms can adhere to hydrocarbons, which is associated with the surface properties of the cells, as a result of attachment to the oil-water interface by general hydrophobic interactions rather than specific recognition of the substrate (Dorobantu et al., 2004). The hydrophobic interactive forces are strongly attractive and are determined by the ratio of the hydrophilic and hydrophobic surface components (Matz and Jurgens, 2001). In this study the hydrophobicity of the tested strains was determined, but no direct influence on fusion with liposome vesicles was shown. Both low-level (0.5–20% adhesion to hydrocarbons) and higher-level (20–50% adhesion to hydrocarbons) hydrophobic cells interacted with liposomes. We conclude that zeta potential and hydrophobic features probably play a role in the general interaction between liposomes and bacterial cells, but there are other specific elements/structures which tend to strengthen this

process because of the different behaviours of the parent strains and their mutants with similar negative charges and hydrophobic properties.

We therefore considered the changes in liposomal fusion in the cases of ATCC 27853 and 12/3 and their mutants (MEM, 12/3 MEM, and 12/3 CHOL, respectively). We analysed the changes in such cell-surface structures as LPS and outer-membrane proteins. LPS is a major membrane component of both structural and functional importance (Kropinski et al., 1985), so it seems that the molecules of the LPS chains may interact with liposomal vesicles. However, LPS molecules had no significant impact on the interaction (fusion) between *Pseudomonas aeruginosa* cells and the cationic liposomal formulation (PC:Chol:DOTAP 3:4:3). It was shown that isolates possessing the same kind of LPS (parents and mutants) exhibited different results in the test when liposomal rhodamine was used. Analysing the outer-membrane protein patterns we found differences in the appearances of the bands. In both 12/3 MEM and 12/3 CHOL the 18-kDa protein bands were observed, in contrast to the 12/3 parent isolate. In the MEM mutant the protein patterns were totally different from those of the parent strain, and the 18-kDa protein was not detected, contrary to the parent strain. To verify the hypothesis of a role of the 18-kDa protein in the fusion process with cationic vesicles, the outer-membrane patterns of the remaining 18 strains were analysed. It turned out that all strains possessing the 18-kDa protein exhibited a visible interaction with rhodamine vesicles under fluorescent microscopy. In addition, no red emission was noted for the isolates which lacked the 18-kDa band.

In vitro experiments were performed to determine the role of antibiotic carriers mainly in the targeting of bacterial cells. Direct interactions between lipid vesicles and bacterial cells strongly depend on the liposomal formulation, but also on bacterial surface patterns. It seemed that not only global surface charge, hydrophobic properties, and LPS structure promote fusion, but that one protein might even influence this process. In the case of the cationic PC:Chol:DOTAP formulation, the 18-kDa outer-membrane protein probably increases the liposomes' affinity to the bacterial cell. Because of these aspects, further investigations verifying the influence of bacterial surface features on the antimicrobial activity of liposomal drug formulations should be carried out.

Acknowledgements

We are grateful to Thilo Kőhler (Department of Genetics and Microbiology, Centre Medical Universitaire, Geneva, Switzerland) for providing the *P. aeruginosa* PAO1, *P. aeruginosa* PASE1, and *P. aeruginosa* PA 1425 strains. This article was co-financed by the European Social Fund within the "Project of Lower Silesia Research Grants for increasing innovations for PhD students" number Z/2.02/II/2.6/04/04U/03/05. This study was supported by Polish Scientific Research Committee research grant No. NN401230533.

References

Beaulac, C., Clement-Major, S., Hawari, J., Lagace, J., 1996. Eradication of mucoid *Pseudomonas aeruginosa* with fluid liposome-encapsulated tobramycin in an animal model of chronic pulmonary infection. Antimicrob. Agents Chemother. 40, 665–669.

Beaulac, C., Sachetelli, S., Lagace, J., 1998. In-vitro bactericidal efficacy of sub-MIC concentrations of liposome-encapsulated antibiotic against Gram-negative and Gram-positive bacteria. J. Antimicrob. Chemother. 41, 35–41.

Campanha, M.T., Mamizuka, E.M., Carmona-Ribeiro, A.M., 1999. Interactions between cationic liposomes and bacteria: the physical-chemistry of the bactericidal action. J. Lipid Res. 40, 1495–1500.

Carmona-Ribeiro, A.M., 2003. Bilayer-forming synthetic lipids: drugs or carriers? Curr. Med. Chem. 10, 2425–2446.

Deol, P., Khuller, G.K., 1997. Lung specific stealth liposomes: stability, biodistribution and toxicity of liposomal antitubercular drugs in mice. Biochim. Biophys. Acta 1334, 161–172.

Desai, T.R., Tyrrell, G.J., Ng, T., Finlay, W.H., 2003. *In vitro* evaluation of nebulization properties, antimicrobial activity, and regional airway surface liquid concentration of liposomal polymyxin B sulfate. Pharm. Res. 20, 442–447.

Dorobantu, L.S., Yeung, A.K., Foght, J.M., Gray, M.R., 2004. Stabilization of oil–water emulsions by hydrophobic bacteria. Appl. Environ. Microbiol. 70,6333–6336.

Drulis-Kawa, Z., Gubernator, J., Dorotkiewicz-Jach, A., Doroszkiewicz, W., Kozubek, A., 2006a. The comparison of *in vitro* antimicrobial activity of liposomes containing meropenem and gentamicin. Cell. Mol. Biol. Lett. 11, 360–375.

Drulis-Kawa, Z., Gubernator, J., Dorotkiewicz-Jach, A., Doroszkiewicz, W., Kozubek, A., 2006b. *In vitro* antimicrobial activity of liposomal meropenem against *Pseudomonas aeruginosa* strains. Int. J. Pharm. 315, 59–66.

Furneri, P.M., Fresta, M., Puglisi, G., Tempera, G., 2000. Ofloxacin-loaded liposomes: *in vitro* activity and drug accumulation in bacteria. Antimicrob. Agents Chemother. 44, 2458–2464.

Germ, M., Yoshikara, E., Yoneyama, H., Nakae, T., 1999. Interplay between the efflux pump and outer membrane permeability barrier in fluorescent dye accumulation in *Pseudomonas aeruginosa*. Biochem. Biophys. Res. Commun. 261,452–455.

Gubernator, J., Drulis-Kawa, Z., Dorotkiewicz-Jach, A., Doroszkiewicz, W., Kozubek, A., 2007. *In vitro* antimicrobial activity of liposomes containing ciprofloxacin, meropenem and gentamicin against Gram-negative clinical bacterial strains. Lett. Drug Des. Disc. 4, 297–304.

Jones, M.N., Francis, S.E., Hutchinson, F.J., Lyle, I.G., 1993. Targeting and delivery of bactericide to adsorbed oral bacteria by use of proteoliposomes. Biochim. Biophys. Acta 1147, 251–261.

Kohler, T., Pechere, J.C., 2001. *In vitro* selection of antibiotic resistance in *Pseudomonas aeruginosa*. Clin. Microb. Infect. 7, 7–10.

Kohler, T., Michea-Hamzehpour, M., Epp, S.F., Pechere, J.C., 1999. Carbapenem activities against *Pseudomonas aeruginosa*: respective contributions of OprD and efflux systems. Antimicrob. Agents Chemother. 43, 424–427.

Kropinski, A.M., Jewell, B., Kuzio, J., Milazzo, F., Berry, D., 1985. Structure and functions of *Pseudomonas aeruginosa* lipopolysaccharide. Antibiot. Chemother. 36, 58–73.

Labana, S., Pandey, R., Sharma, S., Khuller, G.K., 2002. Chemotherapeutic activity against murine tuberculosis of once weekly administered drugs (isoniazid and rifampicin) encapsulated in liposomes. Int. J. Antimicrob. Agents 20, 301–304.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–686.

Matz, C., Jurgens, K., 2001. Effects of hydrophobic and electrostatic cell surface properties of bacteria on feeding rates of heterotrophic nanoflagellates. Appl. Environ. Microbiol. 67, 814–820.

Mehta, R.T., Keyhani, A., McQueen, T.J., Rosenbaum, B., Rolston, K.V., Tarrand, J.J., 1993. *In-vitro* activities of free and liposomal drugs against *Mycobacterium avium–M. intracellulare* complex and *M. tuberculosis*. Antimicrob. Agents Chemother. 37, 2584–2587.

Mugabe, C., Halwani, M., Azghani, A.O., Lafrenie, R.M., Omri, A., 2006. Mechanism of enhanced activity of liposome-entrapped aminoglycosides against resistant strains of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 50, 2016–2022.

Murphy, T.F., Bartos, L.C., 1989. Surface-exposed and antigenically conserved determinants of outer membrane proteins of *Branhamella catarrhalis*. Infect. Immun. 57, 2938–2941.

Omri, A., Ravaoarinoro, M., 1996. Preparation, properties and the effects of amikacin, netilmicin and tobramycin in free and liposomal formulations on Gram-negative and Gram-positive bacteria. Int. J. Antimicrob. Agents 7, 9–14.

Omri, A., Suntres, Z.E., Shek, P.N., 2002. Enhanced activity of liposomal polymyxin B against *Pseudomonas aeruginosa* in a rat model of lung infection. Biochem. Pharmacol. 64, 1407–1413.

Puglisi, G., Fresta, M., Mazzone, G., Furneri, P.M., Tempera, G., 1995. Formulation parameters of fluoroquinolone-loaded liposomes and *in vitro* antimicrobial activity. Int. J. Pharm. 118, 65–76.

Rosenberg, M., Gutnick, D., Rosenberg, E., 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell–surface hydrophobicity. FEMS Microbiol. Lett. 9, 29–33.

Sachetelli, S., Beaulac, C., Riffon, R., Lagace, J., 1999. Evaluation of the pulmonary and systemic immunogenicity of Fluidosomes, a fluid liposomal-tobramycin formulation for the treatment of chronic infections in lungs. Biochim. Biophys. Acta 1428, 334–340.

Sachetelli, S., Khalil, H., Chen, T., Beaulac, C., Senechal, S., Lagace, J., 2000. Demonstration of a fusion mechanism between a fluid bactericidal liposomal formulation and bacterial cells. Biochim. Biophys. Acta 1463, 254–266.

Sanderson, N.M., Guo, B., Jacob, A.E., Handley, P.S., Cunniffe, J.G., Jones, M.N., 1996. The interaction of cationic liposomes with the skin-associated bacterium *Staphylococcus epidermidis*: effects of ionic strength and temperature. Biochim. Biophys. Acta 1283, 207–214.

Schiffelers, R., Storm, G., Bakker-Woudenberg, I.A.J.M., 2001a. Host factors influencing the preferential localization of sterically stabilized liposomes in *Klebsiella pneumoniae*-infected rat lung tissue. Pharm. Res. 18, 780–787.

Schiffelers, R., Storm, G., Bakker-Woudenberg, I.A.J.M., 2001b. Liposome-encapsulated aminoglycosides in pre-clinical and clinical studies. J. Antimicrob. Chemother. 48, 333–344.

Sekeri-Pataryas, K.H., Vakirtzi-Lemonias, C., Pataryas, H.A., Legakis, J.N., 1985. Liposomes as carriers of ^{14}C-labelled penicillin and ^{125}I-labelled albumin

through the cell wall of *Pseudomonas aeruginosa*. Int. J. Biol. Macromol. 7, 379–381.
Tomlinson, S., Taylor, P.W., Luzio, J.P., 1989. Transfer of phospholipids and protein into the envelope of Gram-negative bacteria by liposome fusion. Biochemistry 28, 8303–8311.
Tsai, C.M., Frash, C.E., 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. 119, 115–119.
Vieira, D.B., Carmona-Ribeiro, A.M., 2006. Cationic lipids and surfactants as antifungal agents: mode of action. J. Antimicrob. Chemother. 58, 760–767.
Webb, M.S., Boman, N.L., Wiseman, D.J., Saxon, D., Sutton, K., Wong, K.F., Logan, P., Hope, M.J., 1998. Antibacterial efficacy against an in vivo *Salmonella typhimurium* infection model and pharmacokinetics of a liposomal ciprofloxacin formulation. Antimicrob. Agents Chemother. 42, 45–52.