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INTERACTION OF CIPROFLOXACIN LOADED LIPOSOMES WITH *PSEUDOMONAS AERUGINOSA* CELLS

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Abstract

An antibiotic delivery system has been developed using ciprofloxacin (CIPRO) encapsulated within sonicated unilamellar vesicles (SUV) of different lipid compositions composed of dipalmitoylphospatidylcholine (DPPC), L-α-phosphatidylcholine (PC), phosphatidylethanolamine (PEA) and cholesterol (CHOL). The interaction of SUV (i.e. liposomes) with *Pseudomonas aeruginosa* (T12977) cells was studied and the effect of the liposome encapsulated antibiotic was tested. Encapsulation of CIPRO apparently increased the amount of antibiotic resident in the vicinity of bacteria in aqueous solution at neutral pH.

Electrophoretic mobility measurements showed that *P. aeruginosa* cells were negatively charged. The zeta potential of *P. aeruginosa* was -11.4 ± 2.9 mV in phosphate buffered saline at pH 7.0, and the corresponding surface charge density was -14.7 ± 3.9 mC/m^2. DPPC liposomes and PC liposomes had mean diameters of 103 ± 35 nm and 80 ± 25nm, respectively. They were electrically neutral or had a small positive charge of +5.0 ± 3.0 mC/m^2 when prepared with PC:CHOL:PEA=5:1:1. The interaction and attachment of neutral or positively charged SUV to negatively charged bacterial cells was studied in an effort to increase the residency of the liposomes and CIPRO, in the vicinity of the bacterial cells.

Key Words: Ciprofloxacin, liposomes, bacteria, *Pseudomonas aeruginosa*.

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Introduction

Encapsulation of antibiotics into phospholipid vesicles has several therapeutic advantages [2-8], which include an enhanced drug action and increased drug stability. It has been reported frequently [6,16-18] that liposome encapsulated antibiotics are more effective than antibiotics in solution in the elimination of infection. The encapsulation of antibiotics in liposomes also protects the drug from hydrolysis and reduces drug toxicity [7]. Some liposome encapsulated drugs are currently being evaluated for use in human bacterial infections [2].

In this communication, as a first objective, we studied the encapsulation of CIPRO in sonicated unilamellar vesicles (SUV). CIPRO is a fluoroquinolone with substantial antimicrobial activity against gram-negative and gram-positive bacteria. Among five new quinolones, CIPRO was reported [4] as most active against 51 *P. aeruginosa* strains. Minimum inhibitory concentration (MIC) was determined to be in the range of 0.125-1.0 mg/l against planktonic *P. aeruginosa* cells [4]. Much higher concentrations are required for treatment of *P. aeruginosa* biofilms [1]. The use of increased doses of CIPRO is limited by toxicity considerations and by the poor solubility of the antibiotic in solutions at neutral pH. By encapsulating CIPRO into liposomes, it was possible to expose bacteria to a higher absolute antibiotic concentration and use the vesicles as a depot for controlled drug release.

The second objective of liposomal encapsulation of CIPRO was to prolong the residency of the drug in the vicinity of the bacterial cell by first – increasing unspecific interactions between SUV and *P. aeuginosa*, and second – by decreasing the rate of drug efflux. The unspecific, electrostatic interactions of liposomes with bacterial cells were manipulated by modifications of the lipid composition of the SUV. It was expected that by formulating a neutral, or positively charged liposome membrane, we could increase the electrical attraction between the negatively charged bacterial cells and the liposomes. It is also possible that specific head groups of the lipid molecules would be recognized by *P. aeruginosa* to different extents leading to specific interactions [15].

Abbreviations:
DPPC - Dipalmitoylphosphatidylcholine
PC - L-α-phosphatidylcholine Type III
PEA - Phosphatidylethanolamine
CHOL - Cholesterol

Materials and Methods

Chemicals. L-α-phosphatidylcholine Type III (PC), cholesterol (CHOL), dipalmitoylphosphatidylcholine (DPPC) and phosphatidylethanolamine (PEA) were purchased from Sigma Chemical Company (St. Louis, MO). [^{14}C]-cholesterol was purchased from Amersham (Ontario, Canada). CIPRO was from Miles Pharmaceutical (West Haven, Connecticut, USA). All other chemicals were of reagent grade.

Preparation of liposomes. Liposomes were prepared using a modified procedure found in reference [14] from total of 120-125 μmoles phospholipid and cholesterol in methanol:chloroform (2:1) in the compositions as shown in Table I. Radiolabelled cholesterol [^{14}C]-CHOL in a concentration of 8 x 10^4 dpm (spec. act. 95.0 mCi/mmol) was added as a tracer. Solvents were removed by evaporation at room temperature under a stream of nitrogen followed by vacuum desiccation. Dried lipid films were hydrated overnight with 25-100 nmole/ml (8-33 μg/ml) CIPRO in 100 mM PBS, pH 7.0. A control sample of liposomes was hydrated overnight in 1 ml 100 mM PBS, pH 4.0. The hydrated mixtures were subjected to sonication for 5 min with a Biosonic, (Bronwill Scientific, USA) equipped with a titanium tip. The liposomal suspension was centrifuged at 1,500 xg for 10 min to eliminate the larger membranes and titanium particles from the sonifier probe followed by centrifugation at 120,000 xg for 30 min.

Determination of radioactivity. For determination of radioactivity the samples were mixed with a scintillation mixture and counted with a Beckman LS 6000IC scintillation counter. The reported results were the mean of two counting cycles and three independent experiments. Liposomal concentration in the samples was determined by relative [^{14}C]-CHOL radioactivity.

Electrophoretic mobility measurements. The mobility of liposomes and bacterial cell suspensions was measured using an automated electrokinetics analyzer, Pen Kem 3000. Zeta potential was determined using the Smolukowski equation [12] and the surface charge density (σ) of bacterial cells and liposomes was calculated from the Gouy-Chapman equation:

$$\sigma^2 = 2\varepsilon\varepsilon_0 kT\ ((\Sigma c_i \exp Fz_i\phi/RT)-1))$$

where c_i is the bulk molar concentration of i ion, z_i is the valency of the i ion, R is the gas constant, T is the absolute temperature and ϕ is an approximation of the zeta potential [12].

Size determination. The size of liposomes was determined by a submicron particle size analyzer (Cauter, Model N4SD) and by size analysis mode of the Pen Kem System 3000. The mean size was determined from three separate liposome preparations. The mean dimension of *P. aeruginosa* cells was measured by the Pen Kem 3000.

Drug encapsulation efficiency. The amount of ciprofloxacin encapsulated into liposomes was determined by mass balance from the total concentration, and the concentration in the supernatant after centrifugation of SUV at 130,000 xg in a Sorval ultracentrifuge OTD 65B. CIPRO concentration in the supernatant was determined from UV absorbance at 272 nm (Fig. 1). The CIPRO concentration in liposomal suspensions was determined from the aqueous phase after phase separation.

Susceptibility testing. The antibiotic activity was determined using a clinical isolate of *P. aeruginosa* (T12977) by the agar incorporation technique [19] containing dilutions of antibiotic and liposomal suspension. The inocula were prepared from 24 h broth cultures of the organisms adjusted to 10^6-10^7 colony forming units (cfu)/ml. One (or more) μl of the final inoculum was applied to unsupplemented Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Maryland). The colonies were counted following 24 h incubation at 37°C.

Interaction of bacteria with liposomes. To test the cell association of the liposomes a bacterial suspension (10^6 cells/ml) was incubated with liposomes, which contained a trace amount of ^{14}C-CHOL(1-8 x 10^4 dpm). Bacterial cells were separated from liposomes by differential centrifugation. Radioactivity was determined in the cell pellet and the supernatant. ^{14}C-CHOL radioactivity is indicative of the amount of liposomes attached to the bacterial surface.

Results and Discussion

Lipid composition of SUV and the molar ratio of the lipids used for each preparation are shown in Table 1. The lipid compositions of SUV were selected considering the physicochemical characteristics of the bilayer of liposomes [5,10]. DPPC, PC and PEA are zwitterionic phospholipids at neutral pH and form noncharged liposomes; PEA presents the most freely exposed positively charged amino group on the surface of the bilayer, and the SUV with a lipid composition including 18% PEA showed a low positive charge. DPPC was selected because it has a relatively high phase transition temperature (41.5°C) which is considered as an advantage for medical applications of SUV. CHOL was included in the lipid composition because of its influence on the rate of drug release [6]. The presence of cholesterol into the bilayer also increases the stability of liposomes [9].

The efficiencies of CIPRO encapsulation by our liposomal preparations (Table 1) were 40.2-54.3%; large lipid aggregates and micelles were pelleted at 1,600 rpm.

The concentration of CIPRO in a lipid dispersion varied in the range of 0.2-33.0 mg/ml. Data in Table 1 represent SUV of lipid dispersion of 20 μmole/ml lipid and 0.2 mg/ml CIPRO in PBS, pH 7.0. The amount of encapsulated CIPRO in liposomes (Table 1) with different lipid compositions was determined by the CIPRO adsorption maximum at 272 nm (Fig. 1a). However, it must be pointed out that after encapsulation into SUV, CIPRO had a broad absorption maximum around 272 nm, which could not be used for quantitative analyses (Fig. 1b). Therefore the amount of encapsulated antibiotic was determined from the aqueous phase after phase separation of the liposomal suspension (Fig. 1c). The partitioning of CIPRO in n-octanol phosphate buffer phases was reported in reference [11].

As is seen in Table 1, the amount of CIPRO encapsulated into SUV remained relatively constant as a function of variation of the lipid composition. The maximum amount of CIPRO (11.76 ± 0.72 μg/μmole lipid) was found in DPPC liposomes. Addition of PEA into the bilayer decreased slightly the amount of the encapsulated antibiotic

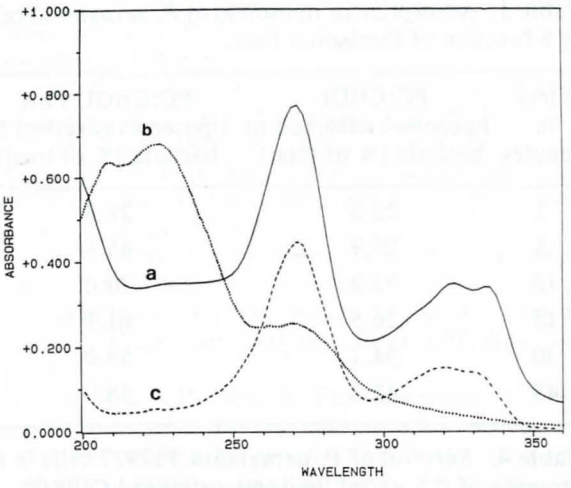

Figure 1. Ultraviolet adsorption spectra of free ciprofloxacin (**a**), encapsulated ciprofloxacin (**b**), and ciprofloxacin extracted from liposomes (**c**).

(10.16 ± 1.02 μg/μmole of lipid). The lowest amount of CIPRO was recorded in DPPC: CHOL liposomes (8.02 ± 1.2 μg/μmole lipid). The differences were insignificant. However, the phospholipid concentration was varied in the range of 0.2-20 μmole/ml; at the highest concentration, the amount of CIPRO encapsulated reached >30 μmole/ml (data not shown). The value of 30 μmole/ml for encapsulated CIPRO is significantly higher than the concentration achieved in aqueous solutions at neutral pH. Thus, relatively high doses may be delivered via SUV.

Liposomes having membranes with a lipid concentration of 6 μmole/ml were mixed with *P. aeruginosa* cells (10^6-10^7 cells/ml) and incubated for different periods of time at room temperature. Bacterial cells were later separated from liposomes by differential centrifugation. As mentioned in the "Materials and Methods" section, all liposomal preparations, independent of their lipid composition, contained ^{14}C-CHOL as a tracer. Therefore, the ^{14}C-CHOL radioactivity in liposomal and bacterial fractions was indicative of liposomal association. The liposomes that pelleted together with bacterial cells are considered to be attached to the cells. Approximately 30 per cent of the total SUV were pelleted together with bacterial cells after 5 min (Table 2). The quantity varied little for DPPC, DPPC:CHOL and PC:CHOL liposomes, but increased significantly for PC:CHOL:PEA liposomes.

One of the reasons for the increased PC:CHOL:PEA liposome-bacterial association could be due to the electrostatic attractive force designed into the liposomal membrane by inclusion of PEA. Indeed, a positive ξ-potential of +5.0 ± 0.3 mV was recorded for PC:CHOL:PEA liposomes. *P. aeruginosa* bacterial cells were negatively charged with an electrophoretic mobility of -1.304 ± 0.622 x 10^{-8} $m^2s^{-1}V^{-1}$, and had a ξ-potential of -11.4 ± 2.9 mV in PBS at pH 7.0, with a corresponding surface charge density of -14.7 ± 3.9 mC/m^2. These results are consistent with published reports of surface charge properties of microbial cells [13].

Table 1. Encapsulation of ciprofloxacin into small unilamellar liposomes with different lipid composition.

Lipid composition molar ratio[a]	μg cipro per μmoles lipid[a]	% recovery ^{14}C-CHOL[a]
DPPC	11.76 ± 0.72	40.2 ± 5.3
DPPC:CHOL 9:1	8.02 ± 1.20	52.7 ± 4.8
PC:CHOL 9:1	9.07 ± 0.91	53.2 ± 4.5
PC:CHOL:PEA 10:2:2	10.16 ± 1.02	54.3 ± 5.0

[a]Results of three independent measurements; SUV were sonicated in 0.2 mg/ml CIPRO with 20 μmole lipid dispersion.

Table 2. Distribution of ^{14}C-CHOL between liposomes and *P. aeruginosa* cells after differential centrifugation.

composition	^{14}C-CHOL in SUV supernatant (%)	bacteria pellets[a](%)
DPPC	72.0 ± 4.3	26.4 ± 5.0
DPPC:CHOL	73.6 ± 4.0	27.7 ± 7.5
PC:CHOL	76.1 ± 4.8	23.9 ± 4.6
PC:CHOL:PEA	54.2 ± 5.5	45.8 ± 4.4

[a]Bacteria were incubated with ^{14}C-CHOL liposomal suspension for 5 minutes; free liposomes were separated from *P. aeruginosa* cells and the radioactivity of the fractions was measured.

Bacterial cells incubated with suspensions of liposomes and separated from the free liposomes by differential centrifugation had a significantly suppressed electrophoretic mobility and decreased negative ξ-potential (Fig. 2).

This demonstrates that liposomal attachment to bacterial cells due to electrostatic attraction screened part of the bacterial negative surface charges. The electrostatic attraction phenomenon was observed with all types of SUV, but was more pronounced for PC:CHOL:PEA liposomes as expected. However, it is not excluded that PEA may serve as a specific adsorption site for *P. aeruginosa*. Prolongation of the incubation time of SUV with bacteria resulted in an increase in the amount of liposomes attached to bacterial cells (Table 3) for the first 10 min. Subsequently, an average plateau value of 60% of the total liposome population was adherent to the bacteria.

The clinical isolate of *P. aeruginosa* T12977 was tested for liposome encapsulated CIPRO susceptibility. The results in Table 4 show that DPPC and PC:CHOL liposomal encapsulated samples were equal in effectiveness as free CIPRO; PC:CHOL:PEA liposomes showed significantly increased suppression of bacterial growth. After 24 hours, the number of the colonies was reduced in all samples containing SUV especially for the PC:CHOL:PEA-containing samples (Table 4).

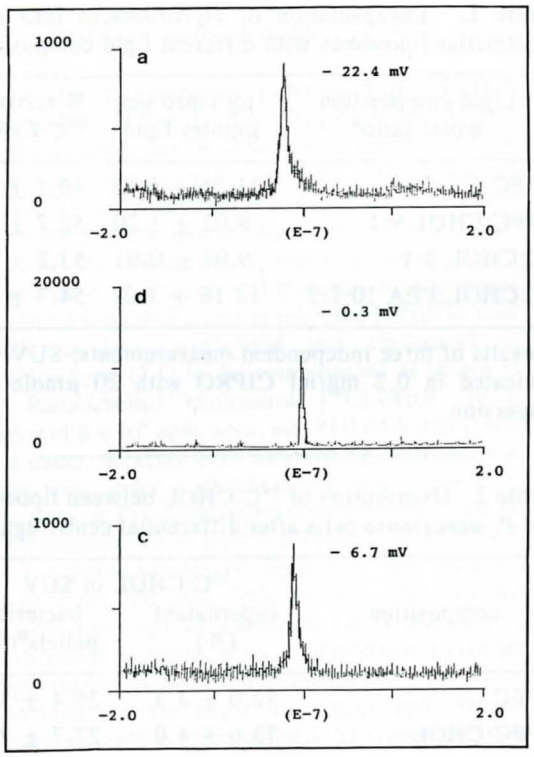

Figure 2. Electrophoretic mobility of bacteria (**a**), liposomes (**b**), and bacteria with liposomes (**c**).

We have demonstrated that CIPRO can be readily encapsulated into liposomes of varying lipid composition, and surface electrical charge. The positively charged liposomes were shown to adhere to bacterial cells and improve the antibiotic efficacy of CIPRO.

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Table 3. Adsorption of liposomes to *P. aeruginosa* cells as a function of incubation time.

Time in minutes	PC:CHOL liposomes adsorbed to bacteria (% of total)	PC:CHOL:PEA liposomes adsorbed to bacteria (% of total)
1	20.3	28.3
5	27.7	45.8
10	32.2	70.0
15	26.5	61.8
30	34.1	58.0
45	47.3	56.9

Table 4. Survival of *P. aeruginosa* T12977 cells in the presence of 0.5 μg/ml liposome-entrapped CIPRO[a].

Liposome sample	viable cells (% of control) 4 hours	24 hours
DPPC	110.0	32.0
PC:CHOL	105.0	10.8
PC:CHOL:PEA	10.5	2.7

[a]The control consisted of 0.5 μg/ml CIPRO in solution applied to a bacterial suspension of 10^6 CFU/ml, at 37°C.

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Discussion with Reviewers

R.J. Doyle: In studying the binding of the liposomes to the bacteria, only one concentration of liposome was used with only one bacterial concentration (or density). In equilibria, the amount of ligand bound by the receptor may be dependent on the concentrations of the ligands and receptors. If cooperative effects (positive or negative cooperativity) occur the authors would never have observed them by using only single concentrations of reactants.
Authors: Correct. We have considered these problems previously and our results will be submitted for publication elsewhere.

R.J. Doyle: The authors suggest that liposome(s) delivery methods may be useful in delivering antibiotic therapy. Yet, liposomes may have greater affinities for blood cells and proteins than for *P. aeruginosa*.
Authors: This topic has been under intensive investigation by others. For example see Ref. 10 and 15 of the manuscript.

R.J. Doyle: There is a growing body of literature suggesting that during metabolism, many bacteria are positively charged. This is because the protonmotive force is extruding protons which can bind to cell wall (peptidoglycan especially). Dissipation of the PMF would cause the wall pH to assume that of the surrounding medium. Do the authors have any results of liposome binding to metabolically active and dead cells ($\Delta p = 0$)?
Authors: We have not investigated this aspect.

H.J. Busscher: The authors mention higher antibiotic concentrations are required for treatment of *P. aeruginosa* biofilms. Could the authors go into this a little deeper as this is also the rationale for this study?
Authors: Biofilm eliminatory concentration can be as high as 500 times when compared with minimum inhibitory concentration; see: Khoury et al. (1992) Inter. Biodeter. & Biodegr. 30: 187-199; Anwar et al. (1989) J. Antimicrob. Chemother. 1,24: 647-655; Nichols, W.W. et al. (1989) J. Gen. Microbiol. 135: 1291-1303.

H.J. Busscher: Would hydrophobicity and/or Van der Walls forces not play a role in the interaction of the liposomes with *P. aeruginosa* cells?
Authors: Definitely yes, but this was not the focus for our study.

J.W. Costerton: I urge the authors to examine the efficacy of liposome-enclosed CIPRO on biofilms of this same organism.
Authors: Thank you for the suggestion.

R. Proctor: Table 4. These data should be more meaningful by presenting numbers of bacteria rather than % of control. If the control bacteria fell 2 orders of magnitude while the treated cells fell 3 orders of magnitude, then this would be 10% of control. However, if the control cells grew 3 orders of magnitude while the experimental rose 2 orders of magnitude, then this too would be 10%, but it would be viewed differently.
Authors: The concentration of bacterial cells is known as a number per volume, see p.2 line 15. For the liposomal suspension, the optical density OD and ^{14}C-CHOL radioactivity were known, but not the number of SUV per volume. These were the reasons why we expressed the data as % of control.

G. Reid: The introductory paragraph quotes references of no specific correlation with the system (urology) or agent (ciprofloxacin) in question. Is this information still relevant?
Authors: It provides useful background information.

G. Reid: Authors suggest that ^{14}C-CHOL is taken up by bacteria. Is this true or it is just an indication of liposomal binding to bacteria?
Authors: We suppose that it is indication of liposome binding or adherence to the bacterial surface. Additional experiments are needed to show that significant disintegration of liposomes did not occur.

G. Reid: What viability drop occurred, if any, in the controls over time?
Authors: We have expressed survival of *P. aeruginosa* cells as a % of control at 4 h" in Table 4.

G. Reid: The data for 1-5 hours on Table 3 shows minor difference, yet that on Table 4 is vastly different. Can this be explained more clearly? If adsorption if similar, why is killing so different? Is the killing explained by the 30 μmol/ml levels?
Authors: The data on Table 3 measured time in minutes, however the time in Table 4 is in hours.

G. Reid: Is there any evidence for specificity in binding of the liposomes or did it occur by chance by being in the tubes?
Authors: The interaction between cells and liposomes depends on specific design of the liposomal bilayer; adsorption of SUV onto bacteria cell wall is not precluded.