IN VITRO ANTIBACTERIAL ACTIVITY OF LIPOSOMAL CEPHALEXIN AGAINST *STAPHYLOCOCCUS AUREUS*

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Abstract. Liposomes encapsulating antibiotics have always shown an improved therapeutic index and reduced toxicity. Among the factors which affect the efficacy of liposomes loaded with antibacterial agents are the surface charge on liposomes and the interaction of the antibiotic with phospholipid vesicles. In this work, the incorporation of cephalexin (antibiotic) with neutral, negative and positive liposomes was studied using phase transition measurements. The main transition temperature of neutral liposomes was shifted to 42.5 ºC when liposomes were loaded with cephalexin while the main transition temperature of empty liposomes was found to occur at 40.5 ºC. Negative liposomes encapsulating cephalexin showed a pre-transition at 32.5 ºC and a main transition at 42.5 ºC. Positive liposomes loaded with cephalexin showed a phase transition temperature, equal to 38 ºC. The characteristics of growth of *Staphylococcus aureus* were also studied after treatment with the given liposome formulations. These characteristics of bacterial growth were found to be highly correlated to the physical properties of the liposome complexes applied. The *in vitro* antibacterial effect of negative liposomes encapsulating cephalexin was found to be superior as compared to neutral and positive liposome formulations.

Key words: liposomes, antibiotics, cephalexin, *Staphylococcus aureus*, phase transition.

INTRODUCTION

Cephalexin is in a group of drugs called cephalosporin antibiotics and is used to fight bacteria in the body. It works by interfering with the bacteria's cell wall formation, causing it to rupture, and killing the bacteria. Cephalexin is used to treat infections caused by bacteria including upper respiratory infections, eye and ear infections, skin infections, and urinary tract infections. Cephalexin may also be used for other purposes than those listed [11].

Many antimicrobial agents have been developed with significant success. They do not, however, provide complete solutions, since the development of drug resistance, the inability of some drugs to penetrate target cells or reach the infection

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site with sufficient concentrations, and the appearance of toxicity of some drugs, in spite of their potent antimicrobial activities [5].

In recent years, encapsulation of antimicrobial agents in lipid formulations as a drug delivery system has been a popular practice in research work aimed at improved therapeutic indices. Phospholipid, a component of the cell membrane, is amphipathic, consisting of a hydrophilic head and a hydrophobic tail. On mixing with water, bilayered lipid membranes enclosing water are formed, called liposomes. Liposomes can incorporate hydrophilic antibiotics in aqueous spaces and hydrophobic antibiotics in lipid membranes.

Liposome-incorporating antimicrobial agents might change the antimicrobial activity and pharmacokinetics and increase the clinical efficacy with decreased toxicity. Liposomes encapsulating antimicrobial agents are selected by many authors for the treatment of bacterial infections [1, 4, 7, 8]. The success of using liposomes as carriers of antimicrobial drugs depends on many factors. Among these factors are liposomes size, charge, membrane fluidity and biodegradability [3, 6, 9, 10]. Although encapsulated liposomes have been widely investigated for different drugs for the treatment of different bacterial infections little attention has been given to the study of both encapsulation efficiency of liposomes and liposomes phase transition behavior which are the most crucial factors of its commercial use.

In this work, the incorporation of cephalexin with phospholipid vesicles was studied using phase transition measurements. Different liposome formulations were used and classified according to surface charge into neutral, negative and positive liposomes. These complexes were applied, *in vitro*, to *Staphylococcus aureus* (isolated from human eyes). The possibility to correlate the observed bacterial growth characteristics to the physical properties of the applied liposomes (encapsulating cephalexin) is discussed.

MATERIALS AND METHODS

CHEMICALS

L-α-dipalmitoyl phosphatidyl choline (DPPC), with a molecular weight of 734 (99% pure), Trizma buffer with a molecular weight of 121.1), Dicetyl Phosphate (DCP), with a molecular weight of 546.9 (99% pure) and, Stearyl Amine (SA), with a molecular weight of 269.5 (99% pure) were purchased from Sigma chemicals. Cephalexin drug with a molecular weight of 365.41 was purchased from Glaxosmithkline. Chloroform was purchased from BDH chemical Ltd. (ARISTAR grade). All chemicals were used without further purification.

PREPARATION OF LIPOSOMES

L-α-dipalmitoyl phosphatidyl choline (DPPC): cephalexin molar ratio 7:2 was used to prepare neutral MLV's using the method of Bangham *et al.* [2]. Briefly, 10 mg of DPPC and 1.4 mg of the drug powder were transferred to a 50 mL round bottom flask. Then 15 mL of chloroform was added, and the flask was shaken until all lipid dissolved in the chloroform. The solvent was evaporated under vacuum using a rotary evaporator until a thin dry film of lipid was formed. The flask was then left under vacuum for 12 hrs to insure the evaporation of all traces of chloroform. Ten mL of buffer (10 mM Trizma adjusted to pH 7) was then added to the flask which was flashed through with nitrogen stream and immediately stoppered. The flask was mechanically shaken for 1 hr at temperature of 45 ºC. The suspension was then centrifuged at 8000 rpm for 20 min. The supernatant was collected for calculation of encapsulation efficiency. Liposomes were then re-suspended in 10 mL buffer solution. DPPC: cephalexin: DCP molar ratio 7:2:1 was used to prepare negatively charged liposomes. Liposomes with a positive charge were composed of DPPC: cephalexin: SA in the molar ratio 7:2:1.

ENCAPSULATION EFFICIENCY MEASUREMENTS

The encapsulation efficiency of the samples was measured using spectrophotometer (Uvikon 930, Italy). The wavelength was adjusted at 261 nm (the resonance absorption of cephalexin). The absorption of the supernatant of each sample (centrifuged at 8000 rpm for 20 minutes) was compared to the standard curve relating absorption to cephalexin concentration (Fig.1). Mixing cephalexin with the lipid powder before dissolving in chloroform was found to increase the encapsulation efficiency up to 96%. If cephalexin is dissolved in buffer and added to the dry film of lipid upon hydration, the encapsulation efficiency is significantly reduced.

PHASE TRANSITION MEASUREMENTS

The phase transitions of the investigated liposome complexes were determined by using spectrophotometer (Uvikon 930, Italy) which is attached to a temperature control unit. Absorbance changes associated with phase transitions of MLV suspensions were continuously recorded by measuring optical density changes at 600 nm (where absorption measurements are only sensitive to the number of bacterial cells in the medium) during temperature scanning with spectrophotometer.Temperature was monitored in the cuvette by an internally attached thermocouple. The samples were temperature scanned from 25 ºC up to 45 ºC at a scan rate of 1 ºC/min.

BACTERIAL STRAIN

Clinical isolates of *Staphylococcus aureus* were isolated from the conjunctiva of patients with mucopurulent conjunctivitis and identified by coagulase test and API system. The isolated strain was tested for antibiotic susceptibility using antibiotic discs on agar plates and an agar dilution method. Pre-culture of test bacteria for 17 h with a 250 mL peptone broth (Difco Laboratories, Detroit, MI, USA) produced a stock preparation containing a log-phase cell density of approximately 10^7 colony forming units (CFU)/mL as evaluated initially by measurements of the optical density at 600 nm. Culture dilution was plated in triplicate and the CFU was counted for final evaluation of the results. All inhibitory tests were performed in triplicate.

RESULTS AND DISCUSSION

Fig. 1 presents the absorption spectra of different concentrations of cephalexin. Two distinct peaks are observed. The first one is at 200 nm and the second is at 261 nm. The first peak is less sensitive to changes in cephalexin concentration, besides it shows a gradual shift to higher values of wave length with increase in cephalexin concentration.

A standard curve is plotted in the upper right side of Fig. 1, showing a linear relationship between the concentration of cephalexin and the absorbance at the 2nd peak (261nm). Using this relation, the concentration of the non-encapsulated drug in the supernatant was determined. Consequently, the encapsulation efficiency of the different types of liposomes entrapping cephalexin is easily calculated and found to be around 96%.

Fig. 2 illustrates the measured phase transition diagrams for empty liposomes and liposomes encapsulating cephalexin. Empty liposomes have a phase transition temperature nearly equal to 40.5 ºC (Fig. 2a). After the incorporation with cephalexin, the phase transition temperature of neutral liposome is shifted towards a higher temperature of about 42.5 ºC (Fig. 2b).

Negative liposomes encapsulating cephalexin show a pre-transition at 32.5 ºC and a main transition at 42.5 $^{\circ C}$ (Fig. 2c). Positive liposomes have a phase transition temperature of about 38 ºC (Fig. 2d). One can say that the incorporation of cephalexin with neutral liposomes causes a shift in the phase transition temperature from 40.5 ºC up to 42 ºC. The other two complexes (negative liposomes and positive liposomes) also show their own characteristic transitions which were significantly different from the empty liposomes. It is expected that liposomes having lower transition temperatures provide a higher rate of drug release unless influenced by other possible mechanism of interaction with bacteria (e.g. fusion to bacterial membrane).

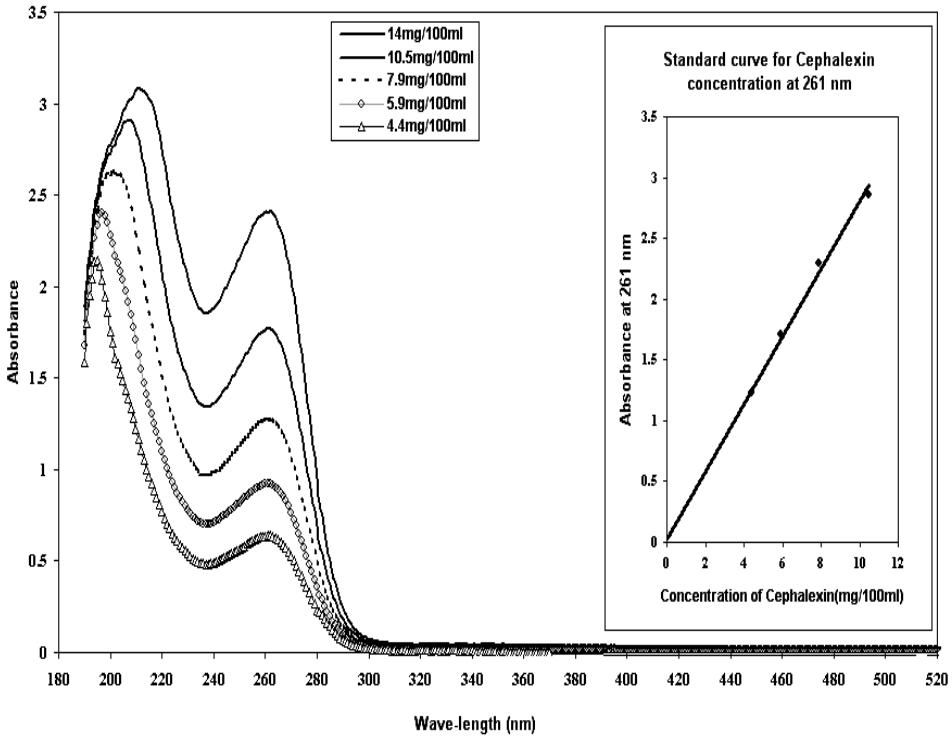

Fig. 1. Absorption spectra of different cephalexin concentrations.

Fig. 3 presents the growth curves of *Staphylococcus aureus* after treatment with different formulations of liposomes encapsulating cephalexin. The first remark is that the inhibition of growth lasted for 150 min for negative liposomes, 110 min for positive liposomes and 20 min for neutral liposomes (Fig. 4).

This behavior is consistent with the phase transition properties presented in Fig. 2, knowing that the growth curves were measured at 37 ºC. The lower the phase transition temperature, the higher the rate of drug release and, consequently, the higher the period of inhibition of growth.

When bacteria started to grow again, after inhibition, the minimum period of growth taken to reach the stationary phase is reported for bacteria treated using a negative liposome complex (305 min).

Bacteria treated with positive and neutral liposome complexes reported periods of growth equal to 400 min and 435 min respectively (Fig. 4). Empty liposomes containing no cephalexin did not affect *Staphylococcus aureus* growth (data not shown).

These results are also consistent with the discussed characteristics of drug release from the investigated types of liposomes encapsulating cephalexin. Thus,

the biophysical characteristics of liposomal membranes are very important in determining drug release, persistence in the targeted organs and interaction with cells, including bacterial cells.

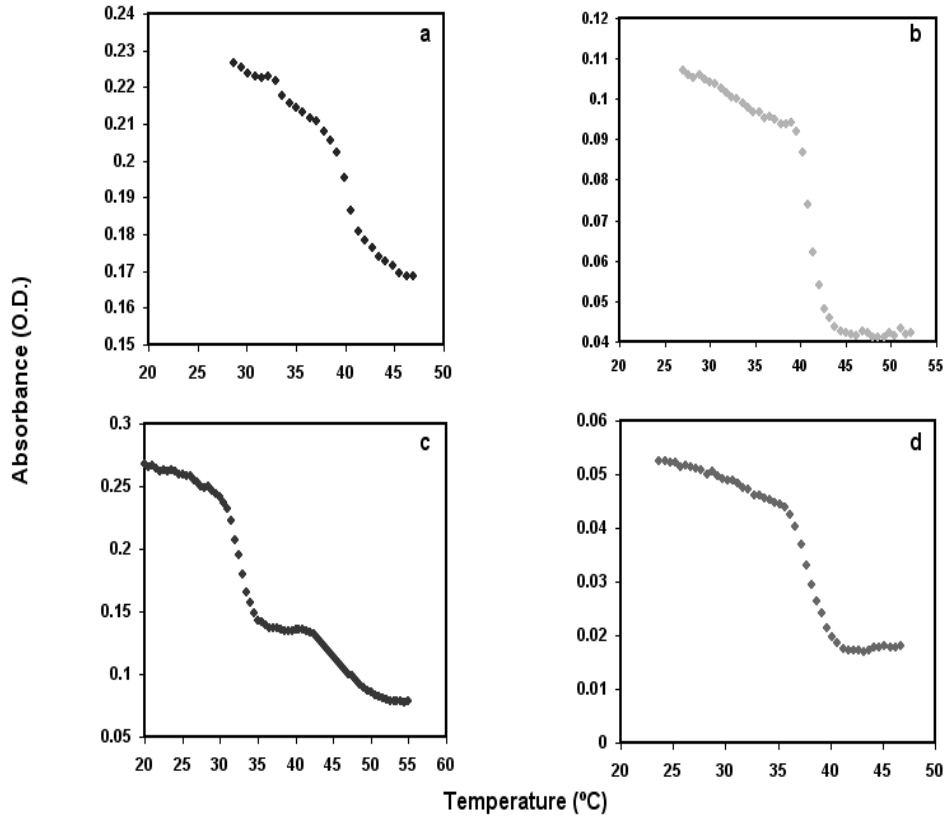

Fig. 2. Measured phase transition diagrams as temperature dependence of absorbance at 600 nm for DPPC liposomes: (a) empty liposomes; (b) neutral charged liposomal cephalexin; (c) negatively charged liposomal cephalexin and (d) positively charged liposomal cephalexin.

It has been reported that the ciprofloxacin or azithromycin encapsulated in stable liposomes having substantial negative surface charge is superior to nonencapsulated drug in inhibition of *Mycobacterium avium* growth within cultured macrophages and may provide more effective therapy of *Mycobacterium avium* infections [7].

They observed that negatively charged liposomes containing ciprofloxacin were superior to neutral liposomes with respect to both cellular uptake and antimycobacterial potency of the antibiotics.

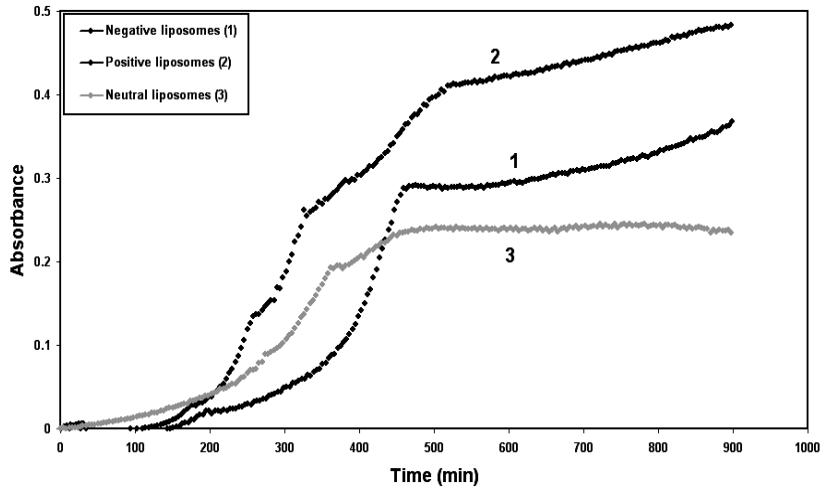

Fig. 3. Growth curves for *Staphylococcus aureus* exposed to different formulation of liposomes encapsulated cephalexin.

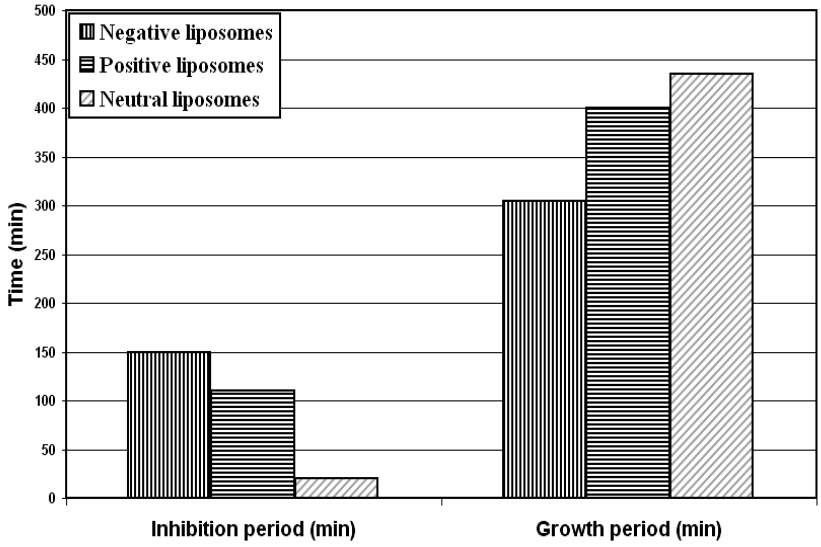

Fig. 4. Characteristics of growth of *Staphylococcus aureus* treated with different formulations of liposomes encapsulated cephalexin.

CONCLUSION

The phase transition characteristics of neutral, negative and positive liposomes encapsulating cephalexin were considerably different. They were also different from empty liposomes. The interaction of cephalexin with neutral

liposomes is expressed in the form of a shift to higher values of the phase transition temperature. The characteristics of growth of *Staphylococcus aureus* treated with the investigated liposome formulations were highly correlated to the phase transition temperature of the applied liposomes. In general, negative liposomes encapsulating cephalexin were superior in their *in vitro* antibacterial behavior compared to neutral and positive liposomes.

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