



## Investigating the Antimicrobial Efficacy of Liposomal Vancomycin in Gram-positive and Gram-negative bacteria- A Preliminary Mechanistic Study

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### Abstract

Outer membrane of Gram-negative bacteria is a permeability barrier to many antibacterial agents, including the glycopeptide antibiotics such as vancomycin hydrochloride and as a result these antibiotics are ineffective against Gram negative bacteria. Different strategies have been described to overcome such limitation, including application of nanoparticles, as was shown in our previous studies for polymeric nanoparticles. On the other hand, some nanoparticles have the ability to reduce the permeation of drugs through biological barriers. Therefore, in this investigation, the effects of fusogenic liposomes, which are expected to interact well with biological barriers, toward antimicrobial effects of vancomycin in different bacteria, are investigated. Vancomycin-loaded liposomes were prepared by lipid film hydration method from a phospholipid mixture composed of either DPPC: DOPE: Chol or DPPC: DOPE: CHEMS, both in 1: 0.5: 1 molar ratios. Obtained liposomes were then assessed in regard to their antibacterial properties using broth microdilution method. Liposomes were prepared by lipid-film hydration followed by extrusion and probe sonication for size reduction. Encapsulation efficiency for large hydrophilic vancomycin in liposomes was found to be in the range of 0.1 to 9 % for different formulations. Probe sonicated liposomes showed smaller size and were more stable than those prepared by extrusion. Antimicrobial results showed that encapsulation of vancomycin in liposomes decreased antibacterial efficacy of vancomycin and caused MIC increments, compared to those of free vancomycin. This might indicate negligible release of this large and charged molecule from liposomes into the bacterial preplasmic space (retention of vancomycin inside liposomal cavity or lipid-drug complexation) accompanied by inability of liposomes to permeate the bacterial barrier. Further investigations are needed to explain the interaction of liposomes with bacterial membranes.

*Keywords:* Vancomycin; Fusogenic liposomes; Antibacterial efficacy; Bacterial resistance; Outer membrane; Retardation

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## 1. Introduction

Antimicrobial resistance is widely regarded as one of the main health issues of the current century. Several studies have provided estimates of the burden of bacterial resistance on health care system. Such data are provided for Europe [1], the USA [2], and the world [3]. According to one WHO survey, annual cost to the US health system alone has been estimated at US \$21 to \$34 billion [4]. Pharmaceutical companies are reluctant to develop new antibiotics because of reason of time consuming and costly process of antibiotic production [5], which requires an average investment of at least \$800 million and 10 years.

Vancomycin, a large glycopeptide antibiotic, forms high affinity complexes with terminal D-Alanine-D-Alanine dipeptides in peptidoglycan wall of bacteria, sterically inhibits bacterial cell wall production, which leads in osmotic lysis and eventually cell death [6-8]. Vancomycin is the first line antibiotic in the treatment of resistant Gram-positive bacteria like *S. aureus* and *methicillin-resistant S. aureus (MRSA)*. Since Gram-negative bacteria have an outer membrane

which acts as a permeation barrier for vancomycin, it cannot diffuse through this membrane to reach its site of action, as a result, vancomycin is ineffective against Gram-negative bacteria [9-12].

Antibiotic encapsulation into nanocarriers can effectively promote their interaction with different microorganisms. Among these carriers, liposomes are bilayer carriers which are formed by amphipathic phospholipids, with aqueous interior that can entrap hydrophilic molecules. Their structure resembles the cell membrane, thus provide a possibility to interact between liposomes and various cell membranes. This interaction has been suggested to have four mechanisms: adsorption, endocytosis, lipid exchange, and fusion [13]. The reports on how liposome encapsulation affect drug efficacy are really controversial. For example, liposome-entrapped ceftazidime and gentamicin have low bactericidal activity in vitro, compared with antibiotic alone [14], while when used in vivo, liposome-entrapped gentamicin or ceftazidime increased the therapeutic effect of the drugs, survival of rats, and bacterial killing in lungs. This enhanced in vivo activity of liposomes was attributed to the localization and subsequent degradation of liposomes and the resulting release of entrapped antibiotic at the infection site [14]. Conversely, in another study aminoglycoside loaded liposome showed to have better efficacy compared to free drug against *P. aeruginosa*, owing to interaction with bacterial membrane and

enhanced permeation through the bacterial membrane [15].

In the present work, we have investigated the possibility of increasing the antimicrobial effects of vancomycin by liposomes and, to perform this, have focused on fusion mechanisms and tried to verify the hypothesis that liposomes containing DOPE as a fusogenic lipid can interact with bacterial outer membrane and hence, increase the efficacy of loaded vancomycin against Gram-negative and Gram-positive bacteria.

## 2. Materials and Methods

### 2.1. Materials

1, 2-Dioleoyl-sn-glycero-3 Phosphoethanolamine (DOPE) and 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were purchased from Avanti Polar Lipids (Alabaster, USA) and lipid (GmbH, Germany). Cholesterol (Chol), cholesterol hemisuccinate (CHEMS), Triton X-100 and polycarbonate filters were obtained from Sigma-Aldrich chemical company (USA). Vancomycin hydrochloride was purchased from Gold biotechnology INC. (USA). Polyethersulfone (PES) membrane filters were acquired from JET Bio-Filtration Co., Ltd

(China). Muller-Hinton agar (MHA) and Muller-Hinton broth (MHB) were purchased from Merck Company (Germany).

### 2.2. Microorganisms

Gram-positive bacteria including *Staphylococcus aureus* ATCC 6538, Methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 33591 and Gram-negative bacteria including *Escherichia coli* ATCC 8739, *Klebsiella pneumonia* ATCC 10031, *Salmonella typhimurium* ATCC 14028 and *Pseudomonas aeruginosa* ATCC 9027 were obtained from Iranian Research Organization for Science and Technology, Persian type Culture Collection (PTCC), Tehran, Iran.

### 2.3. Methods

#### 2.3.1. Preparation of Vancomycin Liposomes by Thin Film Hydration

Vancomycin-containing liposomes were prepared by thin film hydration method [16]. Briefly DPPC/DOPE/Chol (A formulation) and DPPC/DOPE/ CHEMS (B formulation) (1:0.1 molar %) were dissolved in 4 mL chloroform: methanol (3:1) and the solvent was evaporated in a rotary evaporator balloon (Heidolph WB eco, Germany and shaken for

**Table 1.** Type and amount of lipids used in liposome preparation.

Lipid type	Amount (mg/mL)
DPPC	5.87
DOPE	2.98
Cholesterol	3.09
CHEMS	3.89

2 h at 45 °C (A formulation) or at 35 °C (B formulation), (above the transition temperature of lipid mixture). The obtained lipid film was then hydrated with either PBS (pH 7.4) for A formulation or PBS (pH: 7.4) for B formulation, to form initial liposome suspension. Table 1 shows type and amount of lipids used in this study.

### 2.3.2. Size Reduction Methods

Liposomes were then extruded (10 times through 1000 nm, 400 nm, 200 nm and 100 nm) through polycarbonate filter membranes (Mini Extruder, Lipex, Canada). Probe sonication) Topsonic, Iran) (400W, 20 KHz), was used to compare the size reduction efficacy of these two techniques.

### 2.3.3. Particle Size and Zeta-Potential Determination

Zeta potential and particle size of liposomes were determined by Malvern Zetasizer (UK). Liposomes were diluted with PBS. Measurements were carried out at 25 °C, under conditions of: viscosity, 0.88 cP; reflex index, 1.33.

### 2.3.4. Phospholipid Assay

The amounts of phospholipids present in the liposomal formulations were determined using colorimetric method [17]. Five-point calibration curves were prepared for DPPC and DOPE for estimation of unknown concentration of lipids in vesicles obtained after each preparation methods. Briefly, 0.1 mL of the liposome suspension (approximately

at a concentration of 0.1 mg mL<sup>-1</sup>) was added to 1.9 mL of an aqueous 0.1 M ammonium ferrothiocyanate solution in a test tube. The resulting suspension was mixed with 2 mL of chloroform using a vortex, and then centrifuged at 1500× g for 5 min. The upper layer was recovered and analyzed at 488 nm by a UV-vis spectrophotometer (Shimadzu, Japan).

### 2.3.5. Determination of Vancomycin Encapsulation Efficiency

Vancomycin liposomes were separated from untrapped drug by sephadex column. vancomycin encapsulated in the internal aqueous void of liposomes were separated from free vancomycin on sephdex-G50 column. The gel column was prepared first by boiling about 1 g of sephdex-G50 fine powder in 20 mL of DI-water for an hour in a covered beaker. The gel was cooled and packed into column. The pooled liposomes were then disrupted by triton X-100 (0.1% w/v) solution and contained vancomycin was assayed by an HPLC method previously developed by this team [18]. Encapsulation efficiency (EE) was calculated using the following formula [19]:

$$EE\% = \frac{D2}{D1} \times \frac{P1}{P2}$$

Where D1 is the concentrations of initial drug in the liposomes, D2 is the concentration of entrapped drug, P1 is phospholipids concentration in initial liposomes, and P2 is phospholipid concentration in purified liposomes.

### 2.3.6. MIC and MBC Determination of Liposomes

Minimum inhibitory concentrations (MICs) were determined by the standard broth microdilution method. Each microplate well was filled with 100  $\mu$ L of Müller–Hinton broth and then 100 Minimum inhibitory concentrations (MICs) were determined by the standard broth microdilution method. Each microplate well was filled with 100  $\mu$ L of Müller–Hinton broth and then 100  $\mu$ L of VAN-loaded liposomal suspension or a corresponding volume of unloaded liposomes as negative control was added. To test the free drug, 100  $\mu$ L of an aqueous solution of vancomycin was used. By following scalar dilutions with the same broth, the different drug concentrations were thus obtained. The control well consisted of 100  $\mu$ L of Müller–Hinton broth.

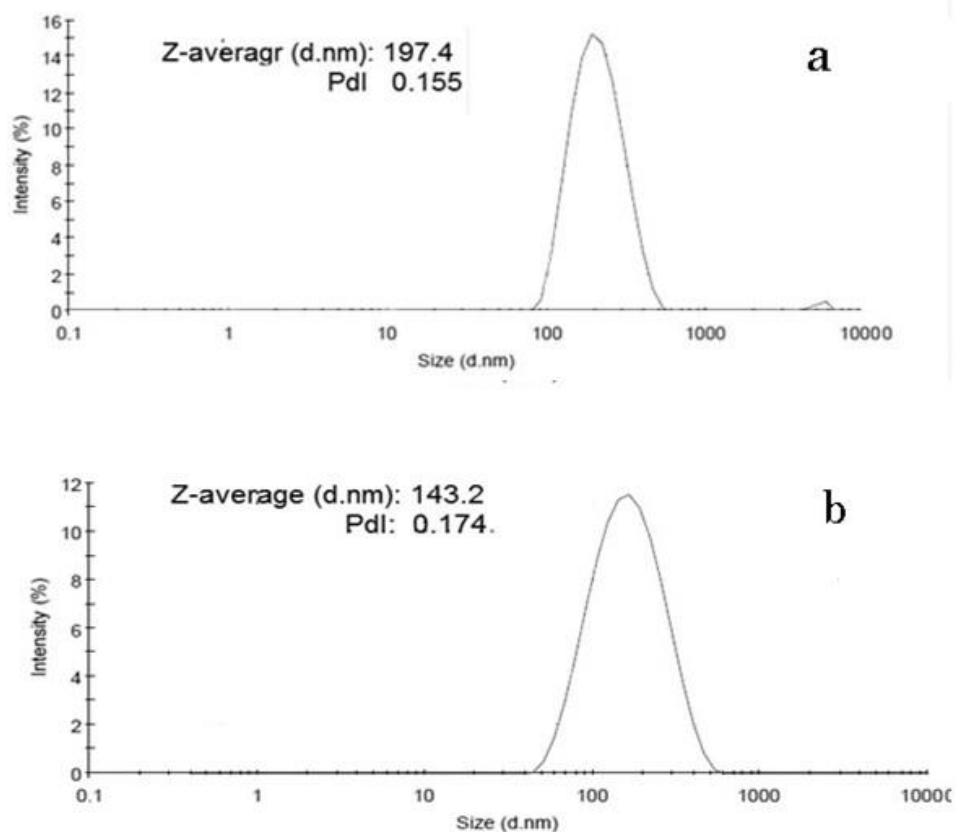
Ten  $\mu$ L of each bacterial suspension was then added, suitably diluted with the same broth to achieve a final bacterial concentration of  $10^5$  CFU/mL in each well. Microplates were then incubated at 37 °C for 24 h. Each experiment was performed three times; the measured antibacterial activity was expressed as the MIC range [20].

### 3. Results and Discussion

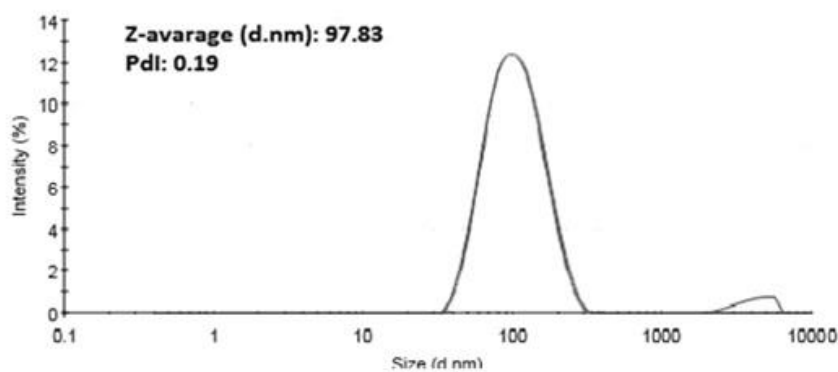
As it is shown in table 2 and figure 1, the mean particle size (Z-average) of liposomes formed by extrusion were about 198nm (formulation A) and 144 nm (formulation B). On the other hand, the results for liposomes formed by probe-sonication gave Z-averages of about 125nm (formulation A) and 97nm

(formulation B), indicating that the ultimate sizes of sonicated liposomes were smaller than those from extrusion and can be controlled by sonication duration [21]. The liposome size and distribution was found to decline with decreasing membrane pore size. It was found that by extruding through a filter with a pore size of 0.2  $\mu$ m and above, the liposomes produced were smaller than the pore size, whereas, when they were extruded through a filter with a pore size of less than 0.2  $\mu$ m, the resultant liposomes were slightly bigger than the nominal pore size [22]. On the other hand, in contrast to extruded liposomes, liposomes prepared by probe sonication were stable and maintained a nearly consistent size throughout the period of one month (figure 2), whereas extruded liposomes showed an increase in Z-average after 1 month. This difference may be due to the mechanisms of cavitation and fragmentation induced by ultrasonic irradiation [23]. Therefore, the resulting shear forces can be quite high and are likely sufficient to promote the formation of tense SUVs [20]. In comparison to formulation A, with a mean Z-average of approximately 198 and 128, formulations B, which contained CHEHMS rather than Chol showed smaller sizes. Therefore, formulation B was selected for further examinations.

As shown in table 3, the initial drug which was added to the lipid film was gradually increased to promote encapsulation efficiency, but the loaded drug in liposome remained constant. Although vancomycin as a hydrophilic molecule can be easily dissolved in the aqueous phase, it might not be able to



**Figure 1.** Size distribution of extruded liposomes. a: formulation A (Aex) and b) formulation B (Bex).



**Figure 2.** Size distribution of sonicated liposomes; formulation B (Bson).

easily diffuse through highly packed lipid layers which is formed during lipid film hydration method. It is also possible that low encapsulation efficiency of liposome is because of liposome small interior capacity,

which is not enough for entrapment of all of vancomycin molecules, efficiently [24]. As a result, some other liposome preparation methods may be more useful to efficiently load vancomycin into liposome interior cavity.

**Table 2.** Size distribution (Z-average and PdI) of prepared liposome formulations; A. Data are mean  $\pm$  SD. (DPPC:DOPE:Chol) and B (DPPC: DOPE:CHEMS) , after extrusion (A<sub>ex</sub>, B<sub>ex</sub>) and after probe sonication (A<sub>son</sub> , B<sub>son</sub>).

Formulation	Z-average (nm)	PdI	Z-average (nm) (after 1 month)
A <sub>ex</sub>	198.1 $\pm$ 3.11	0.02 $\pm$ 0.03	230.3 $\pm$ 2.34
B <sub>ex</sub>	144.3 $\pm$ 3.06	0.13 $\pm$ 0.02	163.4 $\pm$ 2.27
A <sub>son</sub>	125.17 $\pm$ 0.75	0.16 $\pm$ 0.01	126.6 $\pm$ 1.2
B <sub>son</sub>	97.84 $\pm$ 2.11	0.02 $\pm$ 0.02	98.13 $\pm$ 2.53

**Table 3.** Percent loaded drug and encapsulation efficiency (%EE) of purified sonicated liposomes.

(D <sub>0</sub> ) (mg/mL)	(D) (mg/mL)	% D/D <sub>0</sub>	% L/L <sub>0</sub>	% D <sub>0</sub> /L <sub>0</sub>	%D /L	%EE
10	0.86 $\pm$ 4.1	8.6	99.7 $\pm$ 1.42	1.13	0.10	8.84 $\pm$ 2.1
20	0.87 $\pm$ 2.2	4.4	110 $\pm$ 0.2	2.26	0.09	3.98 $\pm$ 1.2
50	0.88 $\pm$ 1.3	1.8	98.2 $\pm$ 0.32	5.65	0.10	1.77 $\pm$ 0.82
90	0.89 $\pm$ 2.4	1.0	99 $\pm$ 0.1	10.17	0.10	0.98 $\pm$ 1.2

\*D<sub>0</sub>: Initial drug concentration

D: Final drug concentration in liposomes

L<sub>0</sub>: Initial lipid concentration

L: Final lipid concentration

%EE: Encapsulation efficiency

As it is shown in table 4 and table 5, the MIC and MBC values for liposomal formulations on both Gram-positive and Gram-negative bacteria are more than free vancomycin solution. It seems that these liposomal formulations retained the drug and prevented it to exert its antibacterial activity. Retardant properties of liposomes have also been reported in the literature, e.g. for Cis-platin containing PC: Chol liposomes [25]. It has also been shown that cholesterol inclusion in liposome results in increased liposome stability and decreased liposome deformability. This stability is also related to drug retention by liposomes [26]. Estradiol liposome also exhibited some retardant properties [27]. Another study also showed that the drug absorption rate was relatively slower for treatment with liposomal salbutamol when

compared to free drug and shown to give a prolonged anti-asthmatic effect after oral administration when compared to free salbutamol solution [28]. This effect is also related to the charge of drugs as it has been shown that charged liposomes exhibited low release rates, presumably because it attracts the ionized part of drug and, decreasing its release [29]. Ghaffari et al. also showed that liposomes reduced permeation of clindamycin phosphate absorption from through human third-degree burn eschar [30].

The current data also show that the prepared liposomes are not able to fuse with the bacterial barrier and take the drug inside, although it has been claimed that CHEMS: DOPE liposomes (similar to the present liposomes) show fusogenic behavior [31].

**Table 4.** MIC and MBC values of vancomycin solution ( $\mu\text{g/mL}$ ).

Microorganism	MIC ( $\mu\text{g/mL}$ )	MBC ( $\mu\text{g/mL}$ )
<i>S. aureus</i>	2.4	2.4
MRSA	4.8	9.6
<i>E.coli</i>	156.2	156.2
<i>K. pneumonia</i>	625	625
<i>S. typhimurium</i>	625	625

**Table 5.** MIC and MBC values ( $\mu\text{g/mL}$ ) liposomal formulation (Sonicated;  $B_{\text{son}}$ ).

Microorganism	MIC ( $\mu\text{g/mL}$ )	MBC ( $\mu\text{g/mL}$ )
<i>S. aureus</i>	3.47	3.47
MRSA	6.95	20.85
<i>E.coli</i>	> 890	> 890
<i>K. pneumonia</i>	> 890	> 890
<i>S. typhimurium</i>	> 890	> 890

#### 4. Conclusion

One of the most challenging issues in the current health care system is emerging bacterial resistance to existing antibiotics. There are different mechanisms behind these resistances including inability of antibiotics to permeate through bacterial membranes, as is the case for vancomycin in Gram-negative bacteria. Based on our three decades' experience in membrane transport studies, we have decided to solve this problem by either membrane modification or application of nanocarriers. Our previous studies [32] have shown that PAMAM dendrimers increase the efficacy of vancomycin in bacteria. The present investigation, however, showed that despite dendrimers, and although we have used fusogenic liposomes, liposomes reduced vancomycin antibacterial activity. This is very

interesting and may be due to negligible release of this large antibiotic from liposomes (retention of vancomycin inside liposomal cavity or lipid-drug complexation), accompanied by inability of liposomes to enter bacterial membrane.

These data show that the issue of liposomes-bacteria requires further investigation. Moreover, more studies are required to prepare effective liposomes, which can potentiate antibacterial activity of vancomycin and also to clarify the interaction of liposomes with the bacterial barriers.

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