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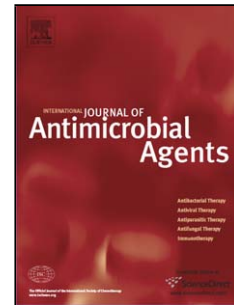
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## Encapsulation in fusogenic liposomes broadens the spectrum of action of vancomycin against Gram-negative bacteria

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## ABSTRACT

Many antibacterial agents, including the glycopeptides, are inactive against Gram-negative bacteria because of their inability to cross the outer membrane of these cells. Different chemical and technological approaches have been described to circumvent such limitation. In this study, we aimed to apply the strategy of fusogenic liposomes, up to now used to carry biological compounds and materials inside cells, to localise a glycopeptide antibiotic, vancomycin (VAN), to the periplasmic space, thus allowing it to exert its bactericidal activity. Small unilamellar liposome vesicles were prepared by an extrusion procedure (SUVETs) from a phospholipid–cholesterol hemisuccinate mixture known for its fusogenic properties with the eukaryotic cell membrane. VAN was loaded with high efficiency into these vesicles and in microbiological experiments *in vitro* was shown to be able to inhibit to a different extent the growth of wild and standard Gram-negative bacterial strains. Minimum inhibitory concentrations as low as 6 mg/L were observed, for instance against clinical isolates of *Escherichia coli* and *Acinetobacter baumannii*. In comparison, neither the free antibiotic nor VAN-loaded ‘classical’ (non-fusogenic) liposomes showed any activity against the same bacteria. Scanning and transmission electron microscopy studies allowed confirmation that the produced SUVETs were able to adhere to and fuse with the external membrane of *E. coli*. According to preliminary experiments, this technological strategy can be proposed as a potentially successful way to enlarge the spectrum of activity of VAN.

## 1. Introduction

The distribution and activity of a drug in the body is largely a function of its physicochemical properties. An alternative approach to affect the intrinsic biodistribution of antibacterial drugs is provided by their encapsulation in colloidal carriers, which hide and protect the drug molecules from peripheral degradation, delivering it to an inaccessible target site, possibly also in a controlled and predictable manner [1–3].

In this perspective of targeting, liposomes have been the most studied systems. They possess the typical features of colloidal carriers, are biodegradable and biocompatible, and their composition and properties can be finely modulated to improve their interaction with and/or penetration through cell membranes [4,5]. Liposomes have shown a particular validity in the treatment of infections by intracellular bacteria [6–8].

Scarce cell interaction and uptake is at the basis of the limited potency of many antibiotics against infections. Microorganisms in infected tissues are further protected by various biological structures in their cell or around the infection. For instance, Gram-negative bacteria possess an outer membrane (OM), rich in lipopolysaccharide (LPS) and proteins, that covers and protects the internal peptidoglycan wall, by which it is separated by an aqueous periplasmic space [9]. Permeation through the OM is governed by porins, water-filled open channels that allow the movement of hydrophilic molecules across the

membrane. The properties of porins vary considerably among wild-type bacterial species, and their functional structure, size and expression (and hence the ability of an antibiotic to be taken up by a bacterial cell) may change in strains with acquired resistance [9,10].

Antibacterial drugs can cross the OM by two main pathways: hydrophobic compounds enter by a passive route, whereas hydrophilic antibiotics diffuse through porin channels. The lipid and protein compositions of the OM have a strong influence on the sensitivity of bacterial cells to antibiotics, and intrinsic drug resistance involving modifications of these macromolecules has been often reported [9,11–13].

For instance, alterations in the composition and size of porins and/or in the LPS of the OM have been shown to alter the sensitivity profiles of bacteria to some fluoroquinolones [14],  $\beta$ -lactam antibiotics [10], erythromycin and even some of the more recent macrolides [15].

Glycopeptides are tricyclic macromolecular peptides with a complex chemical structure and a high molecular weight (1450–1500 Da). Because of their high molecular weight and size, they are unable to pass through porins in the OM to reach the cell wall area, which represents their site of action; therefore, Gram-negative bacteria are intrinsically resistant to this class of antibiotics [16].

Among the glycopeptides, vancomycin (VAN) is largely used in the clinical treatment of severe infections caused by multiresistant Gram-positive bacteria such as staphylococci, enterococci, diphtheroid bacilli and clostridia. VAN inhibits the synthesis of peptidoglycan, the major component of the bacterial cell wall. Its mechanism of action is unusual since it binds with its peptide portion the terminal D-alanine-D-alanyl peptide portion of the peptidoglycan precursor. This mechanism of action does not readily permit mutation to resistance [17,18].

Encapsulation into or association of antibiotics with colloidal carriers can effectively improve their interaction with pathogenic microorganisms. Among these carriers, liposomes are vesicular systems mainly formed by amphipathic phospholipids structured in ordered bilayers, with aqueous spaces inside that allow hosting of hydrophilic molecules. Their nature and structure resemble the cell membrane, thus opening the potentiality for an efficacious interaction between these carriers and cells. Such interaction has been typically classified into four processes, namely adsorption, endocytosis, lipid exchange and fusion [19]. The latter phenomenon has been studied in detail by many authors because it offers the possibility for introducing drugs inside cells more easily [20–22].

A peculiar class of phospholipid vesicles has been called ‘fusogenic’ liposomes. In general, their bilayers show enhanced ability to interact in their liquid crystalline phase with cell membranes, favouring the reciprocal mixing and



release of vesicle content inside cells. Strategies to achieve fusogenic liposomes essentially consist of incorporating in the liposome composition either inactivated Sendai virus envelope components [23–26] or particular fusogenic lipids that make the liposomes more fluid and able to promote the destabilisation of biological membranes [27–30].

In the present work, we have investigated the latter approach by preparing fusogenic liposomes containing VAN, with the aim of extending its antibacterial activity to Gram-negative organisms. In particular, we sought to verify the hypothesis that fusogenic vesicles, up to now essentially studied to improve the penetration of liposomal drugs into mammalian cells, could be applied to the specific condition of Gram-negative bacterial cells, where the presence of the OM resembles the eukaryotic cell membrane.

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) studies were also performed to visualise the interaction/fusion of these liposomes following incubation with Gram-negative bacterial cells.

## **2. Materials and methods**

### *2.1. Chemicals*

Dioleoylphosphatidylethanolamine (DOPE) and dipalmitoylphosphatidylcholine (DPPC) were purchased from Genzyme Pharmaceuticals (Liestal, Switzerland).

Vancomycin hydrochloride, cholesterol hemisuccinate (CHEMS) and cholesterol (CHOL) were purchased from Sigma-Aldrich Chimica Srl (Milan, Italy). Diethyl ether, phosphotungstic acid (PTA), glutaraldehyde and osmium tetroxide were purchased from Merck (Darmstadt, Germany). All other reactants and solvents were of analytical grade or higher (Sigma-Aldrich Chimica Srl).

## 2.2. Microbial strains

Ten wild strains of each of the following Gram-negative bacteria, isolated from clinical cases, were used: *Escherichia coli*; *Klebsiella* spp.; *Pseudomonas aeruginosa*; and *Acinetobacter baumannii*. The above clinical strains were identified by standard methods using control ATCC strains and correspond to the deposited type strains. *Escherichia coli* and *Klebsiella* spp. were identified by the API 20<sup>®</sup> kit, whilst *P. aeruginosa* and *A. baumannii* were identified using the API 20 NE<sup>®</sup> kit (bioMérieux Italia S.p.A., Bagno a Ripoli, Italy). Strains were enrolled in the experiment only when the result of the assay gave an 'excellent identification' score. Following identification, bacteria were stored at -80 °C in cryovials containing nutrient broth enriched with 20% glycerine.

As control bacteria, *P. aeruginosa* (ATCC 27853) and *E. coli* (ATCC 25922) were purchased from the American Type Culture Collection (Rockville, MD).

### 2.3. Liposome preparation and characterisation

Multilamellar liposome vesicles (MLVs) were first prepared by the reverse-phase evaporation technique [31]. Briefly, 15 mg of lipids (DOPE/DPPC/CHEMS in a 4:2:4 molar ratio) were dissolved in a round-bottomed glass tube with 2 mL of a 1:1 (v/v) chloroform–methanol mixture. The solution was evaporated to dryness under a dry nitrogen flow and the produced thin lipid film was further kept at ca. 35 °C under vacuum (T-50 oven; Büchi Labortechnik AG, Flawil, Switzerland) for 6 h to eliminate any solvent trace. To produce the liposomes, the lipid film was dissolved with 3 mL of diethyl ether; VAN (9 mg) was dissolved in 1 mL of a phosphate buffer solution (pH 7.4) and vortex-mixed for ca. 15 min with the ether solution to obtain an initial water-in-oil emulsion. To produce plain liposomes, 1 mL of the same buffer solution was added. The organic solvent was then removed off under vacuum (Rotavapor<sup>®</sup>; Büchi) to induce a phase inversion that gave an oil-in-water secondary emulsion. The water-bath temperature during the whole process was kept constant ca. 50 °C, i.e. a value higher than the phase transition temperature of DPPC. At the end, the liposome suspension was diluted to 3 mL with the same buffer solution. Conventional MLVs, used as control in the microbiological assay, were made with the same procedure starting from 10 mg of a DPPC/CHOL mixture (7:3, mol/mol).

To obtain the final desired monolamellar liposomes (SUVETs), the MLV suspension was manually extruded (LiposoFast<sup>™</sup> Basic; Avestin Europe

GmbH, Mannheim, Germany) 19 times through polycarbonate membrane filters (pore diameter 100 nm).

SUVET mean size was determined by photon correlation spectroscopy (PCS) using a Zetamaster apparatus (Malvern Instruments Ltd., Malvern, UK).

Experiments were carried out using a 4.5 mW laser diode operating at 670 nm as light source. Size measurements were carried out at a fixed scattering angle of 90°. To obtain the mean diameter and polydispersity index of the colloidal suspensions, a third-order cumulant fitting correlation function was performed by a Malvern PCS submicron particle analyser. The real and imaginary refractive indexes were set at 1.59 and 0.0, respectively. The following parameters were used for experiments: medium refractive index, 1.330; medium viscosity, 1.0 mPa s, and dielectric constant, 80.4. Each sample (50  $\mu$ L) was diluted with pro-injectione water to 10 mL to avoid multiscattering phenomena and placed in a quartz cuvette. Size analysis consisted of three series of ten measurements for each tested sample.

To determine the amount of VAN loaded in the liposomes, a 0.5 mL fraction of the SUVET was passed through a Sephadex G-50 column (Sigma) (eluent, phosphate buffer solution, pH 7.4) to remove the unencapsulated fraction of the antibiotic. The purified liposome suspension was treated with Triton X-100 (5% w/v), then filtered through 0.22  $\mu$ m pore size 13 mm nylon membrane filters (Whatman International Ltd., Maidstone, UK) and submitted to high-pressure

liquid chromatography to calculate VAN concentration, according to a published analytical method [32]. Drug concentration was expressed either as the entrapment efficiency, corresponding to the percent drug remaining encapsulated in the liposomes with respect to the amount initially added, and as drug loading, i.e.  $\mu\text{g}$  of VAN per mL of vesicle suspension.

#### *2.4. Microbiological assay*

Minimum inhibitory concentrations (MICs) were determined by the standard broth microdilution method [33]. Each microplate well was filled with 100  $\mu\text{L}$  of Müller–Hinton broth and then 100  $\mu\text{L}$  of VAN-loaded SUVET suspension (corresponding to 300  $\mu\text{g}$  drug/mL and 500  $\mu\text{g}$  lipids/mL) or a corresponding volume of unloaded SUVETs as negative control was added. To test the free drug, 100  $\mu\text{L}$  of an aqueous solution of VAN (300  $\mu\text{g}/\text{mL}$ ) was used. By following scalar dilutions with the same broth, the different drug concentrations were thus obtained. The control well consisted of 100  $\mu\text{L}$  of Müller–Hinton broth. Five microlitres of each bacterial suspension was then added, suitably diluted with the same broth to achieve a final bacterial concentration of  $10^5$  colony-forming units/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 (see Table 1).

### 2.5. Transmission electron microscopy

For electron microscopy preparations, 100  $\mu\text{L}$  of a suspension of DOPE/DPPC/CHEMS SUVETs was mixed with an overnight culture of a clinical isolate of *E. coli* ( $1 \times 10^8$  bacteria/mL) for 1 h at 37 °C under slow stirring. Bacteria in broth alone served as a control. A drop of the above suspension was layered on a Formvar-coated copper grid (Electron Microscopy Sciences, Fort Washington, PA) for 10 min at 37 °C. The grids were then negatively stained by dipping in 1% (w/v) PTA (pH 6.8) for 15 s. Observations were carried out using a Hitachi H-7000 transmission electron microscope (Hitachi High-Technologies Europe GmbH, Krefeld, Germany).

Some samples were processed for electron microscopy by a conventional method. Briefly, the bacteria–SUVET suspension was centrifuged and the pellet was re-suspended with 3% glutaraldehyde in 0.12 M phosphate buffer solution (pH 7.2) at 4 °C for 1 h. After fixation, the pellet was included in a cloth of fibrin, dehydrated through a graded series of acetone and embedded in Durcupan ACM (Fluka Chemika-Biochemika, Buchs, Switzerland). Ultrathin sections were double stained with uranyl acetate and lead citrate.

### 2.6. Scanning electron microscopy

A drop of bacteria–SUVET suspension was layered on a sterile cover glass and fixed with 3% glutaraldehyde in 0.12 M phosphate buffer solution (pH 7.2) at 4

°C for 1 h. The samples were post-fixed in 1% osmium tetroxide in the same buffer, dehydrated in ethanol, critical point dried and sputter coated with a 5 nm gold layer using an Emscope SM 300 (Emscope Laboratories, Ashford, UK). A Hitachi S-4000 (Hitachi High-Technologies America, Inc., Schaumburg, IL) field emission scanning electron microscope was used for the observations.

### 3. Results

To study the possibility of releasing VAN in the periplasmic space of Gram-negative bacterial cells, we selected a liposome composition already characterised in the literature for the preparation of fusogenic vesicles. A mixture of DPPC and DOPE, containing an amphiphilic derivative of cholesterol (i.e. CHEMS) [29], was used to prepare MLVs. Membrane extrusion of the latter produced the desired small unilamellar vesicles (SUVETs). The obtained SUVETs were submitted to a microbiological assay to compare the MIC values of free and encapsulated VAN against different Gram-negative bacterial strains. Experiments were performed in comparison with classical (non-fusogenic) DPPC/CHOL liposomes loaded with VAN as well as with empty (unloaded) DOPE/DPPC/CHEMS vesicles and the free antibiotic as controls.

SUVET liposomes were obtained by membrane extrusion of MLV suspensions. The latter were produced by a reverse-phase evaporation technique: in fact, the presence of VAN as the hydrochloride salt did not allow stable phospholipid

vesicles to be obtained using the classical thin-layer evaporation method (i.e. simple hydration of a lipid film with a buffered solution of the drug).

The MLV suspension was repetitively passed through a 100 nm polycarbonate membrane. The pores of the latter are almost cylindrical, and unilamellar or multilamellar vesicles larger than the pore diameter are reduced in size and lamellarity during passage through the pores, resulting in a final liposome size that corresponds to the mean size of the pores themselves [34]. As a consequence, uniform vesicles were obtained by this method with a mean size of  $103.23 \pm 2.87$  nm and a polydispersity index of 0.037, which indicates a very high particle size homogeneity. A mean entrapment efficiency of 65.8% was registered, corresponding to a VAN concentration of 2.055 mg/mL of liposome suspension.

In the microbiological assay, free VAN was inactive against all of the tested Gram-negative strains (MIC > 512 mg/L). Conversely, when the drug was loaded in the fusogenic DOPE/DPPC/CHEMS SUVETs, remarkable MIC values were measured (Table 1), as low as 6 mg/L for clinical isolates of *E. coli* and *A. baumannii*.

As control experiments, both unloaded and VAN-loaded non-fusogenic DPPC/CHOL SUVETs as well as unloaded fusogenic DOPE/DPPC/CHEMS



SUVETs (tested at a lipid concentration equivalent to a drug concentration of 512 mg/L in VAN-loaded vesicles) were all unable to affect bacterial growth.

#### 4. Discussion

The possibility of phospholipid vesicles fusing with the cell membrane has often been claimed at the basis of the success of liposomal formulations. For instance, encapsulation in liposomes allowed sub-MIC concentrations of tobramycin to act against Gram-negative and Gram-positive bacteria [35].

In this study, a 4:2:4 (mol/mol) DOPE/DPPC/CHEMS lipid mixture, already described in the literature to produce pH-sensitive liposomes [29], was chosen to prepare small unilamellar liposome vesicles able to interact and fuse with the cell membrane.

Kinetic studies showed that liposomes made of mixture of DOPE with other lipid components, such as oleic acid, distearoylphosphatidylglycerol or CHEMS, release their content into the cytoplasm after a short incubation time with cells [36–39]. In particular, the association of DOPE with CHEMS has in fact been recognised to impart vesicles a pH-dependent stability that allows selective fusion of liposomes with specific cell components to be achieved [38,40]. The exact mechanisms by which these liposomes traverse the cytoplasmic membrane barrier are not completely clear. However, it is likely that partial

fusion of vesicle bilayers with the cell membrane causes destabilisation of the latter, facilitating the release of liposome content into the cytoplasm.

The specific role of phosphatidylethanolamine or DOPE can be explained on the basis of the low hydration of these polar head-groups. The presence of DOPE in fact increases the lipophilicity of the liposomal membrane and reduces the energy of interaction among the lipid bilayers. The presence of DPPC was instead required to form stable liposomal bilayers, since the particular structure of DOPE led to an inverted hexagonal phase instead of a lamellar phase when the lipid was used alone [41].

In the present study, the fusogenic properties of DOPE/DPPC/CHEMS unilamellar vesicles have been exploited with the purpose of releasing the antibiotic not inside cells but specifically in the narrow area of periplasmic space of Gram-negative bacteria. As a consequence, the OM barrier can be bypassed and the antibiotic can operate its molecular activity at the level of the cell wall.

In the microbiological assay, free VAN displayed no activity against all of the tested bacterial strains. When the antibiotic was loaded in non-fusogenic DPPC/CHOL liposomes, no activity was observed against Gram-negative strains. Negative control tests confirmed that unloaded (empty) SUVETs, made both by DPPC/CHOL (non-fusogenic) or DOPE/DPPC/CHEMS (fusogenic), were also ineffective against the tested bacteria. In a separate experiment, the

extemporary addition of VAN to empty fusogenic liposomes, before addition of the suspension to culture wells, also did not produce any inhibitory activity.

However, the drug showed remarkable MIC values when loaded in fusogenic DOPE/DPPC/CHEMS SUVETs (Table 1). In particular, the activity shown against *A. baumannii* is particularly interesting since this microorganism is naturally sensitive to only a few antibiotics [42]. Even the least sensitive strain among those tested in this study, *P. aeruginosa*, showed an interesting MIC value (50 mg/L). The same order of activity was registered for the two reference ATCC bacterial strains used. Although it should be confirmed by specific assays, it is conceivable that the lower MIC values observed for *P. aeruginosa* and *Klebsiella* spp. were due to the production of mucus, which hinders contact with the liposomes.

The obtained in vitro results, however, supported the initial working hypothesis that the fusogenic properties of the chosen (phospho)lipid mixture were able to convey the active compound through the external membrane inside the periplasmic space, where its antibacterial activity can be exerted.

TEM analysis has often been used to confirm the interaction of liposomes with the bacterial cell membrane. For example, Omri and colleagues [43,44] have used TEM to show the ability of 'classical' DPPC or 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC)/CHOL liposomes to transfer aminoglycoside antibiotics

to resistant bacterial cultures, such as *P. aeruginosa* and *Burkholderia cenocepacia*. Similarly, Sachetelli et al. [45] used negatively charged vesicles with a low phase transition temperature to increase the release of tobramycin in the cytoplasm of resistant bacteria.

Thus, to obtain photographic evidence of the interaction of the prepared liposomes with the bacterial cells, SEM and TEM analysis were done on *E. coli* cultures incubated with DOPE/DPPC/CHEMS SUVETs. Figs 1–3 show the adherence of liposomes to bacteria and the ultrastructural modification following the fusion of some vesicles with the bacterial membrane, which led in some instances to a deformation of the cell membrane. In negative staining TEM analysis, liposomes (dark spheres) surrounding the bacterial cells are visible (Fig. 2A), and small or large liposomes fused with the OM of bacteria (Fig. 2B, arrows). Also using conventional visualisation (Fig. 3), the multilayered structure of the cell envelope was visible, with the arrow indicating the fusion of a large liposome with the OM of bacteria, which also appears deformed.

These results reinforce the supposition that the measured in vitro activity was due to a liposome-carried release of antibiotic inside the bacteria cells. Specific experiments with labelled liposomes have been planned to identify the localisation of the encapsulated probe inside bacterial cells after fusion with this kind of liposome. Furthermore, absence of any sign of bacterial cell injuries in

the microscopy analysis suggested that the tested liposome formulation was not cytotoxic.

The present liposomal formulation can be proposed for the local treatment of Gram-negative sustained infective conditions, such as burns where these bacteria have been largely found [46]. It is conceivable that the presence of eukaryotic cells and tissues will affect the selective fusion of these liposomes with the bacterial cells and this must require committed *in vitro* and *in vivo* studies. Moreover, in view of systemic use, the described formulation would need suitable tuning, for instance surface modification of liposomes with hydrophilic polymers, to attain a circulation time in the bloodstream long enough to reach the target sites.

However, the reported and somewhat expected results will prompt us to explore the feasibility of these fusogenic liposomes to improve the activity of other antibiotics against resistant microorganisms.

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**Competing interests**

None declared.

**Ethical approval**

Not required.

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**Fig. 1.** Scanning electron microscopy images of an overnight culture of (A) *Escherichia coli* and (B) the same strain incubated with fusogenic SUVETs for 1 h at 37 °C. Bar, 2  $\mu$ m. SUVETs, small unilamellar liposome vesicles prepared by an extrusion procedure.

**Fig. 2.** Transmission electron microscopy image of (A) liposome–bacteria interaction and (B) fusion (arrows) of an overnight *Escherichia coli* culture incubated with fusogenic SUVETs for 1 h at 37 °C as observed by negative staining. Bars, 100 nm (A) and 200 nm (B). SUVETs, small unilamellar liposome vesicles prepared by an extrusion procedure.

**Fig. 3.** Transmission electron microscopy micrograph of an ultrathin section of *Escherichia coli* cell in the presence of fusogenic SUVETs, as observed by the conventional method. Bar, 100 nm. SUVETs, small unilamellar liposome vesicles prepared by an extrusion procedure.

**Table 1**

Minimum inhibitory concentration ranges (mg/L) of VAN-loaded DOPE/DPPC/CHEMS SUVETs and free VAN against different Gram-negative bacterial strains <sup>a</sup>

	<i>Escherichia coli</i>	<i>Klebsiella</i> spp.	<i>Pseudomonas aeruginosa</i>	<i>Acinetobacter baumannii</i>	<i>E. coli</i> ATCC 25922	<i>P. aeruginosa</i> ATCC 27853
SUVETs	6–25	25–50	50	6–12.5	10.5	83.7
VAN	>512	>512	>512	>512	>512	>512

VAN, vancomycin; DOPE, dioleoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; CHEMS, cholesterol hemisuccinate; SUVETs, small unilamellar liposome vesicles prepared by an extrusion procedure.

<sup>a</sup> Ten wild strains for each species were tested.

