Antibiotic delivery by liposomes from prokaryotic microorganisms: Similia cum similis works better

Antibiotic delivery by liposomes from prokaryotic microorganisms: *similia cum similis* works better

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Running title: Antibiotic delivery by liposomes from prokaryotics

Abstract

To date the effectiveness of antibiotics is undermined by microbial resistance, threatening public health worldwide. ~~The possible failure of conventional antibiotics engenders the need for new drugs, with demanding investments of money, labor and time.~~ Enhancing the efficacy of the current antibiotic arsenal is an alternative strategy. The administration of antimicrobials encapsulated in nanocarriers, such as liposomes, is considered a viable option, though with some drawbacks related to limited affinity between conventional liposomes and bacterial membranes.

Here we propose a novel "top-down" procedure to prepare unconventional liposomes from the membranes of prokaryotes (PD-liposomes). These vectors, being obtained from bacteria with limited growth requirements, also represent low-cost systems for scalable biotechnology production. In depth physico-chemical characterization, carried out with dynamic light scattering (DLS) and Small Angle X-ray Scattering (SAXS), indicated that PD-liposomes ~~were~~ may be suitable for the employment as antibiotic vectors. Specifically, DLS showed that the mean diameter of loaded liposomes was ~ 200-300 nm, while SAXS showed that the structure was similar to conventional liposomes, thus allowing a direct comparison with more standard liposomal formulations.

Compared to free penicillin G, PD-liposomes loaded with penicillin G showed minimal inhibitory concentrations against *E. coli* that were up to 16-times lower. Noteworthy, the extent of the bacterial growth inhibition was found to depend on the microorganisms from which liposomes were derived.

216 words. Now 198, limit is 200

Keywords

Antibiotic delivery; Liposomes; Biolipids; Antimicrobial activity; Penicillin resistance

1. Introduction

Infectious diseases continue to represent one of the greatest health challenges worldwide, despite the advancements in understanding the disease mechanisms and extensive efforts in the design of new drugs. Antibiotics, the most important class of bioactive compounds against microbes, have been used to treat infections from immemorial time. However, from the very beginning of antibiotic utilization, their effectiveness was undermined by development of microbial resistance.[1-5] Two reports issued in 2014 by World Health Organization show that resistance against antibiotics hampers the prevention and treatment of a wide range of infections[6] and recommend measures for best practice prescription.[7] Indeed, fast bacterial adaptability to new environmental conditions and mobilization of resistome genes imply that all antibiotics in clinical use may have microbes able to resist them.[8-10] The failures of antibiotic therapy contribute to thousands of deaths annually.[11-12] To face problems related to the use of antimicrobial agents, high doses of antibiotics or development of new drugs are continuously required.[13] In this contest, a viable strategy is to enhance the efficacy of the current antibiotic arsenal. For instance, the administration of bioactive compounds encapsulated in nanoparticles,[14-17] and liposomes,[18-21] has been indicated as a promising approach. These vectors can protect antibiotics against deactivation and help to overcome physiological barriers, thus facilitating transport and allowing for slow release at the target site. This in turn improves drug bioavailability while limiting side effects.[22-25] In particular, liposomes and lipid-based nanoparticles exhibit low toxicity combined with high therapeutic efficacy, particularly for poorly soluble drugs.[26] Liposomes are able to interact with the bacterial outer membrane (OM) and lipopolysaccharides of Gram-negative bacteria, thus facilitating the internalization of drugs[27,28] at a concentration high enough to overcome transmembrane pumps that catalyze increased efflux of drug out of the bacterial cell.[16] As reported by several authors, liposome-OM fusion is often associated with minimum inhibitory concentration (MIC) reduction compared to free antibiotics.[19] For example, Mugabe *et al.*[29] demonstrated the liposome-bacterial membrane fusion by applying different techniques. The liposomal

formulation used by these authors contained aminoglycosides and polymyxin B and was able to reduce MICs by 4-16 times in a high-resistant strain of *P.aeruginosa*. Rukholm *et al.*[30] reported that liposomal containing gentamicin show better antipseudomonal activity than the free drug, with a 16-fold MIC reduction. Moreover, prevention of biofilm formation and/or its destruction[14,31] and treatment of intracellular bacterial infections could benefit from the use of nanovectors.[32,33] Therefore, in the perspective of enhancing delivery of currently used antibiotics, there is an urgent need for *ad-hoc* designed lipid vectors, highly compatible with the biological membranes and synthesizable through low cost processes. Liposomal carriers are usually prepared though a "bottom up" approach, consisting of the assembled single lipid components. Here we devised a "top-down" procedure to obtain unconventional liposome from microbial phospholipid, in the belief that the composition of lipid-based vectors plays a major role in the interactions with pathogen microorganisms.[34-36] The lipid fraction was thus extracted from bacteria and used to build novel liposome formulations (Prokaryote Derived-liposomes, PD-liposomes). Similar systems were prepared by Gupta *et al.* to encapsulate carboxyfluorescein as a model molecule for anticancer drug delivery.[37] More generally, the aptitude of vesicles produced by bacterial membranes to perform a variety of functions, including DNA delivery, has been pointed out in a recent review.[38] It is also known that Gram-negative bacteria naturally produce outer-membrane vesicles, which accomplish a variety of functional roles.[39,40] In this paper we chose Gram-negative purple non sulfur bacteria and cyanobacteria, which can be found in several ecological niches[41,42] and are already used for biotechnological processes.[43-45] Thanks to great versatility and limited growth requirements,[46,47] these bacteria produce large amounts of biomass that may represent a valuable and low-cost byproduct for scalable liposomes production. In depth physico-chemical characterization was performed to investigate the structural properties of PD-liposomes. Their ability to overcome resistance against antibiotics was compared ~~with~~ to standard liposomes (Std-liposomes). Penicillins and *Escherichia coli* were used as antibiotics and test organism, respectively. Penicillins belong to β-lactam antibiotics, widely used for their high effectiveness, low cost, ease of

delivery and minimal side effects.[48] Indeed, the emergence of resistance to penicillins places high demand for ways to improve the effectiveness of this important class of antibiotics.[49] We thus propose PD-liposomes to contrast reduced uptake and enzymatic degradation of penicillins and show the effectiveness of these unconventional carriers in helping penicillins to overcome the OM barrier in *E. coli*.

2. Materials and methods

2.1. Chemicals

Ampicillin trihydrate (96-100% purity, anhydrous basis, henceforth called Amp) (PubChem CID:23565), Penicillin G (98 % purity, henceforth called PenG) (PubChem CID:5904) and all other chemicals, i.e. chloroform (PubChem CID: 6212), ethanol (PubChem CID:702), methanol (PubChem CID:887), acetone (PubChem CID:180), dimethyl sulfoxide (DMSO) (PubChem CID:679), sulfuric acid (PubChem CID:1118), hydrogen peroxide (PubChem CID:784), ascorbic acid (PubChem CID:54670067), ammonium molybdate (VI) tetrahydrate (PubChem CID:61578), sodium chloride (PubChem CID:5234), acetonitrile (PubChem CID:6342), and tridecafluoroheptanoic acid (HFBA) (PubChem CID:67818), –were purchased from Sigma-Aldrich.

3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride (DC-Chol) (PubChem CID:16219102) and cholesterol (Chol) (PubChem CID:5997) were from Avanti Polar Lipids (Alabaster, AL); 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) (PubChem CID:6437081), 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) (PubChem CID:6437392) and soybean phosphatidylcholine (with 770 average MW) were from Lipoid GmbH (Ludwigshafen am Rhein, Germany).

2.2. Bacterial strains and growth conditions

The microorganisms used to prepare bacterial lipid vectors were (i) the unicellular *Cyanothece* sp., strains CCY0110 and VI22, isolated from marine and saline

habitats respectively, and (ii) the purple non-sulfur photosynthetic bacterium, *Rhodopseudomonas palustris*, strain 42OL, isolated from a pond containing wastewaters of a sugar refinery. All the bacterial strains belong to the specialized culture collection of the Department of AgriFood Production and Environmental Sciences (University of Florence, Italy). The *Cyanothece* strains are N_2-fixing gram-negative bacteria able to produce extracellular slimes of polysaccharidic nature. In particular, VI22 strain, excrete extracellular polysaccharides which are structured as thick capsules surrounding the cells, while the CCY0110 releases the majority (~75%) of the polysaccharides in the surrounding medium and does not form outermost structures.[50] Inversely, the *R. palustris* strain 42OL is a Gram-negative bacterium not able to accumulate exo-polysaccharides.[51] *Cyanothece* sp., strains CCY0110 (henceforth indicated as CCY) and VI22 were grown in ASN-III medium[52] and enriched seawater medium (AMA),[41] respectively; *R. palustris*, strain 42OL was grown anaerobically in RPN medium.[53] The *E. coli* XL1Blue strain (Stratagene, La Jolla, CA, USA), with or without pUC18 plasmid,[54] was used to test the antimicrobial activity of antibiotic preparations. *E. coli* XL1Blue cells were cultured aerobically at 37°C in Luria Broth (LB) complex medium.[55]

2.3. Preparation of conventional liposomes (Std-liposomes)

Two formulations were used as the reference carriers for antibacterial molecules: (i) the cationic DOPC:DOPE:Chol:DC-Chol (4:3:2:1 mole ratio) liposomes, henceforth called StdL1, and (ii) the zwitter-ionic soybean-PC:Chol (4:1mole ratio) liposomes, henceforth called StdL2. The literature on liposomes used as carriers ~~of~~ for antibiotics presents a large variety of compositions, with preference for cationic (i.e. synthetic) components added to phosphatidylcholines and phosphatidylethanolamines. Here we followed this indication in (StdL1), but we also chose to compare cationic liposomes with a zwitterionic formulation obtained from natural source (StdL2).

Solutions of the starting lipids in chloroform/methanol (3/1 v/v) were mixed in a round bottom vial and the appropriate volume of penG dissolved in DMSO/acetone (2/3, v/v) was added to obtain a starting antibiotic amount of 800 ppm. In all

samples the total lipid content was 10 mg/mL. The solvent was then evaporated and the dry film was kept 30' under vacuum prior to rehydration with milliQ water. These samples were submitted to 8 freeze-thaw cycles (plunging in liquid N_2, vortexing and warming in a water bath at 50 °C) for optimal homogenization. Finally, liposomes were downsized by extrusion through polycarbonate membranes with 200 nm pore diameter (27 passages).

2.4. Bacterial phospholipid extraction and preparation of PD-liposomes

Bacterial cultures were harvested at mid- to late-growth phase by centrifugation (20 min, 2000 g, 20 °C), added to ethanol and left in the heating cabinet at 30 °C for 48 h to allow solvent evaporation. Dry material (50 mg) ~~of dry material~~ were as used for each lipid extraction. The lipid fraction was extracted with 5 mL of Folch solution (chloroform:methanol 2:1, ~~by volume~~v/v), followed by three washing steps with 1 mL of aqueous 0.9% NaCl, during which the upper liquid phase and residual dry material were removed.[5657] In the case of antibiotic-loaded PD-liposomes, 600 µL of either Amp or PenG solutions were added to the lipid fraction extracted by Folch solution. PD-liposomes thus obtained from CCY0110, VI22 and 42OL bacterial strains were denominated CCY-lipo, VI22-lipo and 42OL-lipo, respectively. After repeated vortexing, polidisperse/multilamellar liposomes were obtained. These were then downsized by sonication (five cycles of 4 minutes each at 70% power with a Bandelin Electronic Sonoplus D2070, Bandelin Electronic UW2070). For plain and antibiotic loaded PD-liposomes sonication was preferred to extrusions, since the solution of lipids extracted from bacterial membranes appeared less homogeneous than standard lipids, thus needing more vigorous homogenization.

2.5. Quantification of phospholipids extracted from bacteria

Total phosphorus was determined by the procedure of Chen *et al.*[58] with few modifications. Briefly, dried bacteria were dissolved in 25 mL of methanol:chloroform 1:2 (v/v). This solution (175 µL) ~~of this solution were~~ was heated in a water bath (70 °C, 5 min). Then 450 µL H_2SO_4~~,~~ were added and the

samples ~~were~~ heated in vaselin oil (190 °C, 50 min). After the first 20 min, 150 µL H_2O_2 were added to each sample. Finally 3.9 mL of distilled H_2O and 0.5 mL of ammonium molybdate (VI) (2.5 %, w/w) were added, the sample tubes ~~were~~ sealed by screw caps and the solutions ~~were~~ kept in a water bath (70 °C, 7 min) to form a deep blue complex. Absorbance at 820 nm was measured ~~with~~ on a UV/VIS spectrophotometer (Lambda 35, Perkin Elmer). These procedures ~~was~~ were ~~repeated~~ performed in triplicate for each bacterial strain.

2.6. Quantification of the antibiotics associated with liposomes

The quantitative measurement of uploaded antibiotics was performed by liquid chromatography-electrospray mass spectrometry (HPLC-ESI MS) using a Surveyor HPLC pump coupled to a LTQ linear ion trap mass spectrometer via electrospray interface (Thermo Instruments, San Jose, CA, USA). Samples were diluted 1:50 (by volume) with ethanol prior to injecting in the HPLC column in order to disrupt liposomes and avoid possible interference or masking effects due to residual bilayer fragments. The HPLC column was a Phenomenex Gemini C18, 150 x 2 mm, 0.5 µm, operating at 0.35 ml/min flow rate. The HPLC solvents were water (A) and acetonitrile (B), both containing 0.005% HFBA. A gradient elution was performed as follow: at time 0 min B 5%, then B was increased to 60% in 7 min, then to 90% in 1 min and left at 90% for 3 min; the column was then reconditioned for 9 min at starting conditions. The MS was operating in positive ion mode, with 10 µl injection volume. MS parameters were optimized for each analyte. Two runs were performed for each sample: first, a full scan was recorded in the mass range 95-360 m/z (for Amp) and 135 to 400 m/z (for PenG); then a MS spectrum was acquired. For Amp the ion 174 m/z (product ion of 350 m/z, the $M+H^+$ precursor ion) was isolated and fragmented by collision induced fragmentation (CID) and full product ion spectrum recorded in the range 50-200 m/z; for PenG the ion 176 m/z (product ion of 335 m/z, the $M+H^+$ precursor ion) was isolated and fragmented by CID and full product ion spectrum recorded in the range 50-350. An external calibration was applied with a calibration curve in the concentration range of interest by integrating the chromatographic peak area of the specific MS product

ion of each analyte (98 m/z for PenG and 118 m/z for Amp).The encapsulation efficiency was calculated by the ratio (expressed as %) between the starting antibiotic amount and the mass spectrometry quantification. For Std-liposomes the loading rate of PenG was 20.9 % in both StdL1 and StdL2. The results obtained for PD-liposomes were higher and are reported in the results and discussion section.

2.7. Dynamic Light Scattering (DLS) and Small Angle X-ray Scattering (SAXS) experiments

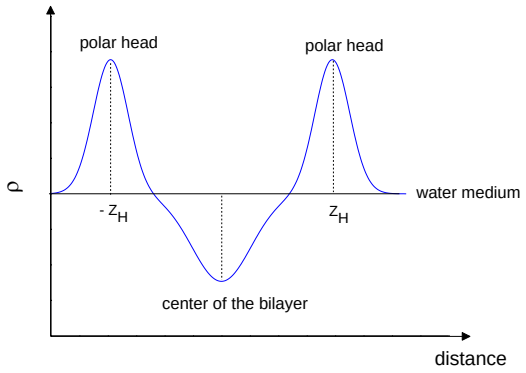
Dynamic Light Scattering was performed on a Malvern Zetasizer Nano S (Malvern Instruments, MA) equipped with a 4-mW, He-Ne laser operating at 633 nm and with back-scattering detector (173°). The time autocorrelation function was analyzed by the cumulant method to obtain the mean diameter and the polydispersity index (PDI), which is related to the size uniformity of scattering objects. Small values of PDI (<0.2) indicate a homogeneous population, while PDI >0.4 indicates high heterogeneity. For each sample a minimum of 11 runs (10 s each) were recorded with three repetitions to ensure reproducible results. When smaller distributions coexisted with the main population peak, contributions below 5% to the integrated scattered intensity were discarded.

SAXS was performed at the ID02 beamline of the ESRF (Grenoble, France). Samples were placed in 2.0 mm capillaries and 5 curves recorded at different points were averaged to obtain the scattered intensity I(q) as a function of the moment transfer $q = (4\pi/\lambda)\sin\theta$ (where 2θ is the scattering angle). The q-range covered was 0.094-5.47 nm^{-1}. I(q) diagrams were fitted with the Global Analysis Program (GAP), written by Dr Georg Pabst,[59,60] which allows to obtain structural details of lipid membranes, and electron densities in the polar/apolar regions. Specifically, the SAXS pattern of bilayer-based structures is modeled by:

$$I(q) = \frac{(1 - N_{d\ i})_f S_f(q) P(q) + N_{d\ i} P_f(q)}{q^2} \quad (1)$$

where N_{diff} is the fraction number of single-bilayer vesicles, S(q) is the structure factor describing the inter-particle interactions and P(q) is the absolute square of

the bilayer form factor. The electron density is described by three-Gaussians (Scheme 1), representing the polar heads, placed at ± z_H, and the hydrocarbon core. The standard deviation of these distributions are σ_H and σ_c, respectively. The terminal methyl group in the bilayer center corresponds to the minimum of the electron density. r_H is the amplitude of the hydrophobic tails.



Scheme 1. Electron density profile in a typical phospholipid bilayer obtained by the GAP software

2.8. Measurement of antimicrobial activity

E. coli XL1Blue strain, with and without the pUC18 plasmid carrying the *bla* gene encoding a periplasmic β-lactamase, was used to test the bacterial inhibitory activity of antibiotic-loaded liposome preparations in comparison with antibiotic in solution and plain liposomes mixed with soluble antibiotic. The inhibitory activity was determined differently depending on the kind of liposome tested. For PD-liposomes the broth microdilution method in standard microtiter plates was used. In this case, *E. coli* XL1Blue cells were grown in LB and collected at the exponential growth phase to be diluted in LB 2x until OD_{600nm} = 0.05, corresponding to a cell density of approximately $2x10^6$ cfu/ml. Two fold serial dilutions of the additives were prepared and added to an equal volume of bacterial suspension in LB 2x in a final volume of 250 µl in the microtiter well to obtain the desired antibiotic concentration.

Microtiter plates were incubated at 37°C, with shaking at 100 rpm; the plates were read at 590_{nm} in a microtiter plate reader (Immunella S, GDV, Rome, Italy) at time zero and after 24h of incubation. The MIC was considered the lowest antibiotic concentration of an antimicrobial agent preparation that completely inhibited growth (no absorbance increase in the microtiter well). For Std-liposomes (StdL1 and StdL2) optical density determination of the cultures was not possible due to strong turbidity of the liposome solutions, therefore bactericidal activity was determined. Bacterial cells with and without antibiotics were grown in 0.5 ml of LB medium in 2 ml Eppendorf tubes; after 24 hours growth at 37°C, cultures were serially diluted, plated on agarified LB without antibiotic and incubated 24 hours at 37°C. The entity of microbial killing was determined by counting survivors as colony forming units (CFUs). The reduction in CFU from the initial inoculum was taken as a measure of bactericidal activity. For each test, at least two independent experiments were performed in duplicate. Growth control consisting of a bacterial inoculum in LB medium with no test compounds, and in LB medium with plain liposomes at the same concentration at which they were present in the tests, and sterility controls were included in the study. Antibiotic susceptibility interpretations were made according to National Committee for Clinical Laboratory Standards.[61]

3. Results and discussion

3.1. Design of antibiotic carriers

The choice of the lipids to be used in Std-liposome formulations (StdL1 and StdL2) was motivated by literature reports on the role played by different lipids in the stability and delivery efficacy toward bacterial culture.[62-65] In particular, flexible and long (i.e. dioleoyl) chains can accommodate bulky molecules such as antibiotics, while cholesterol is used to build stable bilayers. To test cationic versus non charged liposomes, we chose the cholesterol derivative DC-Chol for StdL1 liposomes, whereas the zwitterion formulation StdL2 was based on a natural and biocompatible lecithin mixture like soybean-PC. Std- and PD-liposomes were

prepared from the lipid material indicated in the experimental section in order to have a total lipid concentration of 10 mg/ml in all samples. To calculate the antibiotic/phospolipid molar ratio and allow direct comparison with Std-liposomes, an average molecular weight of 700 g/mol was assumed for PD-phospholipids. This choice was motivated by the need to have an estimate of lipid amount in our formulations and was justified by the finding that in PD-liposomes the bilayer thickness was comparable to that of standard lipids with chains of 14-16 carbon atoms (see below the discussion of SAXS results). The assumption of an average 700 g/mol molecular weight gave a phospholipid content of 60 ± 3% w/w for all the three bacterial strains studied in this work.

3.2. Structural characterization of plain and loaded PD-liposomes

As liposomes prepared from bacterial lipids were shown to be valuable carriers to increase the delivery of PenG, we proceeded to their structural characterization. Dynamic Light Scattering is a straightforward and suitable tool to evaluate the mean size and size distribution of isolated nano-objects in solution. In the case of liposomal drug delivery these properties are known to influence liposome stability, drug entrapment and delivery efficacy. The results obtained for plain and antibiotic-associated liposomes are reported in Table 1.

All solutions contained scattering objects with a fairly large size distribution, indicating that polydisperse liposomes were formed by the lipid mixtures extracted from bacterial strains. The mean diameter of plain liposomes depended on the spontaneous curvature of lipid bilayers and followed the order VI22-lipo < 42OL-lipo< CCY-lipo. After the incorporation of Amp and PenG, lower PDI were observed, which was indicative of more uniform size distributions. However, while liposomes prepared from cyanobacteria membranes (CCY-lipo and VI22-lipo) were smaller after antibiotic loading, those obtained from *R. palustris*, tended to grow upon antibiotic association. This indicated that a different loading modality was characteristic of the different membrane structure. Further structural properties could be obtained by the analysis of SAXS profiles. In particular this technique is able to establish if the lipids and the procedure used in the preparation are

consistent with compact sphere (with no water core) or with vesicles. As indicated by DLS data, the overall size of PD-liposomes was too large to be accessed by SAXS, however this technique was able to provide detailed information on the bilayer structure, that could not be obtained by DLS.

Table 1. Size of plain and antibiotic loaded liposomes obtained by DLS

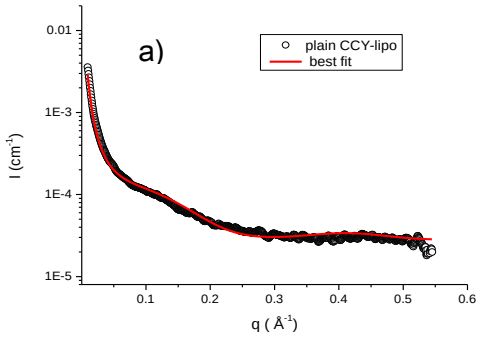
Liposome type	Antibiotics	% uploaded[a]	Mean diameter (nm)[b]	PDI
CCY-lipo	-		340±30	0.61
CCY-lipo	Amp	44.4	250±20	0.38
CCY-lipo	PenG	34.0	200±20	0.38
VI22-lipo	-		290±40	0.69
VI22-lipo	Amp	29.3	180±20	0.38
VI22-lipo	PenG	27.5	210±50	0.48
42OL-lipo	-		240±50	0.55
42OL-lipo	Amp	44.2	300±50	0.48
42OL-lipo	PenG	33.7	320±50	0.49

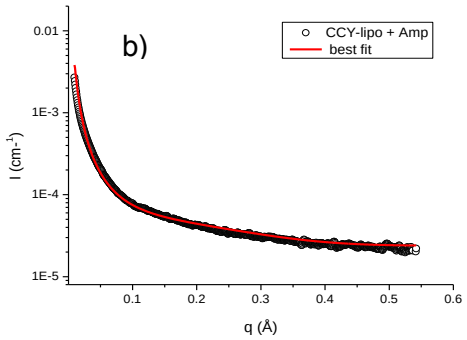
[a] The starting amount of antibiotics was 800 ppm

[b] The mean diameter was calculated based on the scattered intensity and the size range refers to 95% of the population. PDI, indicates the polydispersity index, as explained in the text.

The SAXS diagrams of plain and antibiotic-loaded vectors derived from CCY110 bacteria are reported in Figure 1(a-c); their fitting proved that these systems have the structure of unilamellar liposomes. The best fit parameters are listed in Table 2. The bilayer head-to-head thickness ($2Z_H$ = 46 Å) of plain CCY-lipo was only slightly larger than the values measured for DOPC/DOPE liposomes ($2Z_H$ = 39.6 Å) and DMPC liposomes ($2Z_H$ = 34.7 Å).[66] Moreover, this value was close to the thickness reported for DPPG bilayers at 25 °C ($2Z_H$ = 44 Å),67 which is in line with the widespread use of glycerolipid to build model bacteria membranes. The insertion of antibiotics in CCY-liposomes caused a contraction in the bilayer, but its electron densities were only slightly varied. This change, being detected throughout the

membranes and not in a particular region, suggests that the antibiotic molecules were interacting with both the polar heads and the hydrophobic core.





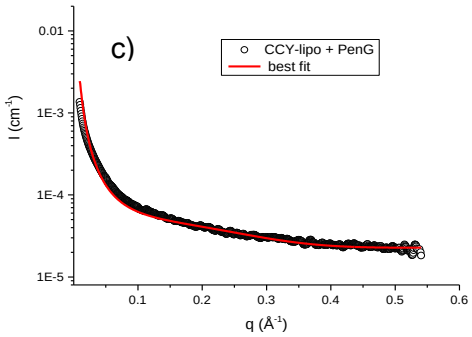


Figure 1. Experimental (symbols) and fitted (continuous lines) SAXS intensity diagrams of plain and antibiotic-loaded vectors derived from CCY110 bacteria.

Similar results were obtained for 42OL-liposomes (Figure 2), therefore we concluded that the insertion modality of Amp and PenG in the membranes derived from these capsule-free bacteria was similar. The best fit parameters obtained for these samples are also reported in Table 4.

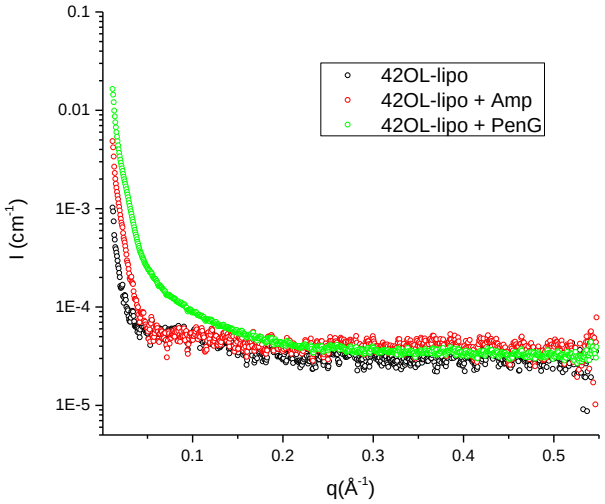


Figure 2. SAXS profiles of plain and antibiotic-loaded vectors obtained from *Rhodopseudomonas palustris* bacteria (42OL-liposomes).

The liposomes prepared from VI22 bacteria had a slightly different structure, as it could already be inferred from fitting the diagram of plain VI22-lipo (Figure 3). Indeed the polar layer of VI22-lipo was markedly thicker that other PD-liposomes (see Table 2), probably as a consequence of some molecules composing the outer polyglycan envelope being retained in the extracted lipid mixture.

Loading VI22-lipo with antibiotics appeared to increase the thickness of the outer layer, though a complete fitting of the scattering pattern was not possible and only

the position of the first maximum could be reproduced. A part of this difficulty could be also ascribed to low SAXS intensity. As an example to illustrate this point, Figure 4 shows the experimental diagrams of samples VI22-lipo + PenG.

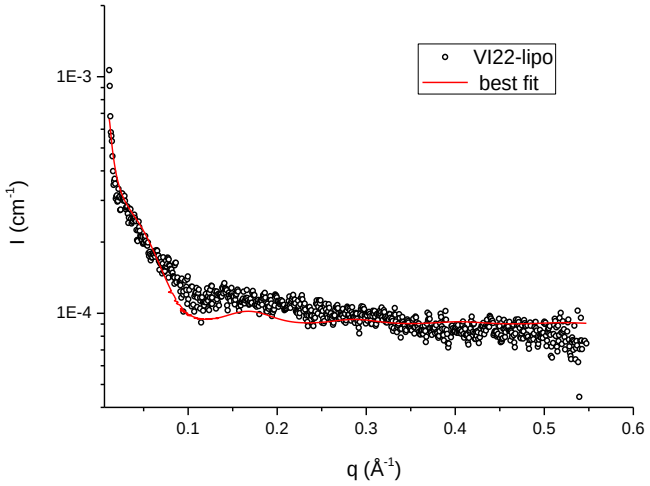


Figure 3. Experimental (symbols) and fitted (continuous lines) SAXS intensity diagrams of plain liposomes prepared from VI22 bacteria.

It is worth noticing that none of the systems studied in this work showed quasi long-range order, which allowed to infer that multilamellar liposomes were not present in PD-liposome samples. Indeed, the SAXS signal of multi- or oligo-lamellar structures is much narrower than the pattern of monolamellar liposomes, so that their presence is unambiguosly revealed by Bragg peaks, even if there is coexistance of theses two types of aggregates and monolamellae are by far the major component (e.g. 98-99%).

In summary, the structural investigation of PD-liposomes proved that these novel carriers have the shape of monololamellar vesicles and, though with large polydispersity, their main characteristics are comparable to those of standard liposomes. The insertion modality of antibiotics depended on the membrane

composition. In particular, the presence of a thick polysaccharide layer derived from VI22 bacteria, inhibited high loading rates in VI22-lipo.

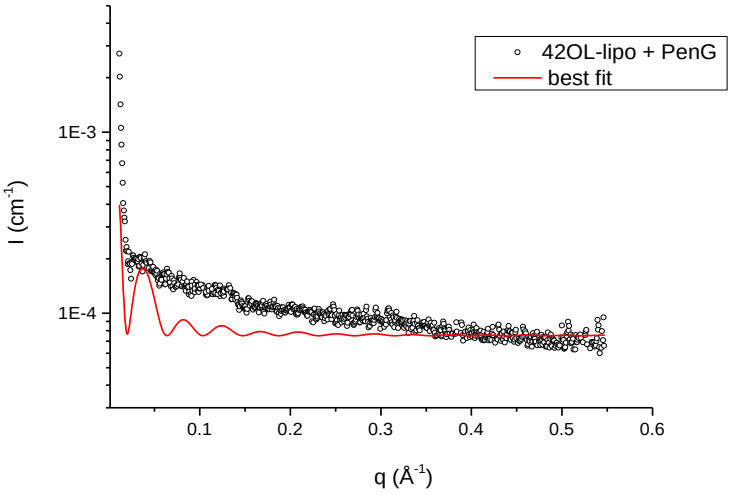


Figure 4. Experimental SAXS intensity diagrams of liposomes from VI22 bacteria loaded with Penicillin G and fitting with the GAP software showing that the structural complexity of these liposomes can only be partially unraveled by core-shell analysis

Table 2. Structural and electron density parameter of plain and antibiotic-loaded liposomes obtained by the fitting of SAXS intensity diagrams

Liposome type	Antibiotics	Z_H	s_H	r_C	s_C
CCY-lipo	-	23.0	0.10	-1.1	0.50
CCY-lipo	Amp	11.5	0.12	-1.2	0.70
CCY-lipo	PenG	13.0	0.12	-1.3	0.65
VI22-lipo	-	54	0.55	-0.7	0.38
VI22-lipo	Amp	80	0.18	-0.1-	0.55
VI22-lipo	PenG	75	0.22	-0.1	0.70
42OL-lipo	-	21.0	0.09	-0.8	0.42

42OL-lipo	Amp	17.0	0.09	-0.6	0.7
42OL-lipo	PenG	12	0.10	-0.8	1.0

3.3. Bacterial susceptibility to different antimicrobial preparations

Antibiotics cross the outer membrane (OM) of Gram-negative bacteria by diffusing through protein channels known as porins, OmpF in the case of *E. coli*.[68,69] In the periplasmic space penicillins target and inhibit penicillin binding proteins (PBP) with transpeptidase activity, preventing the formation of new pentapeptide bridges among peptidoglycan chains and making the growing bacteria highly susceptible to cell lysis and death. Two primary mechanisms of resistance to β-lactams are the decreased uptake or forced efflux from the cell and the production of β-lactamase enzymes that degrade β-lactam antibiotics.[46] To evaluate if our liposome formulations could help to increase the antibacterial effectiveness of penicillins against *E. coli,* we tested different concentrations of PD-liposomes, and Std-liposomes, against ~~equal~~ corresponding concentrations of free antibiotics. Firstly, the bactericidal activity of PenG-loaded Std-liposomes, in an antibiotic concentration range ~~of concentrations~~ that in the microtiter plate reached the maximum values of 44 and 55 μg/mL for StdL1 and StdL2, respectively, was compared to that of equivalent concentrations of free antibiotic. PenG- loaded StdL1 and StdL2 did not inhibit the growth of *E. coli* XL1Blue strain (showing ~ 10^2 increase in CFU from the initial inoculum), while free penicillin-G showed bactericidal activity (with an about 40 times decrease in CFU from the initial inoculum at 100 μg/mL). On the contrary, PenG-loaded PD-liposomes were more effective than free antibiotic in inhibiting *E. coli* growth with a reduction of penicillin G MIC by 2-fold, 8-fold and 16-fold when the antibiotic was loaded on CCY-lipo, VI22-lipo and 42OL-lipo, respectively (Table 3). We then performed further susceptibility tests only with bacterial liposomes. To exclude any independent contribution of the bacterial liposomes in the above reported inhibitory effect, we also tested free PenG mixed with amounts of unloaded-liposomes identical to those of PenG-loaded liposomes at MIC values; the MIC values of these mixtures were

lower than that of PenG-loaded liposomes and identical to those with free penicillin G (Table 3). Unlike PenG loaded liposomes, CCY-lipo, VI22-lipo and 42OL-lipo loaded with Amp, with a MIC value of 14.5 μg/ml, were as active as free Amp (data not shown), suggesting that the antimicrobial activity of Amp was not enhanced by PD-liposomes.

Table 3. MIC values (μg/mL) of free PenG, free PenG plus unloaded PD-liposomes, and PenG-loaded PD-liposomes against *E. coli* XL1Blue.

Free PenG	Free PenG with PD-liposomes			PenG-loaded PD-liposomes*		
	CCY-lipo	VI22-lipo	42OL-lipo	CCY-lipo	VI22-lipo	42OL-lipo
58	58	58	58	29	7.2	3.6

* The initial concentration of PenG of the PD-liposome stock solutions in these experiments were 272 μg/mL, 29 μg/mL and 17 μg/mL for CCY-lipo, VI22-lipo, 42OL-lipo, respectively.

E. coli XL1Blue possesses a not inducible chromosomal *amp*C gene ~~costitutively~~constitutively expressing low level of β-lactamase.[70,71] To investigate a possible protecting role of liposomes from β-lactamase attack in the periplasmic space, we tested Amp- and PenG-loaded liposomes against *E. coli* XL1Blue carrying the multicopy pUC18 plasmid with the *bla-TEM1* gene encoding a class A β-lactamase, that confers resistance to penicillins and early cephalosporins (Salverda et al., 2010). Results, reported in Table 4, showed that antibiotic-loaded liposomes, at the PenG and Amp concentrations that, starting from the stock solution used in these experiments, were the higher reachable in microtiter plates, and that, as above reported, were inhibitory for the *E. coli* XL1Blue strain (MIC values: 14.5 μg/mL for Amp with any of the three types of PD-liposomes and 29, 7.2 and 3.6 μg/mL with PenG in CCY-lipo, VI22-lipo and 42OL-lipo, respectively), did not inhibit the growth of *E. coli* XL1Blue (pUC18).

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Table 4. MIC values in μg/mL of free Amp and PenG, and Amp-loaded and PenG-loaded PD-liposomes against *E. coli* XL1Blue (pUC18) strain.

Free PenG	PenG-loaded PD-liposomes*			Free Amp	Amp-loaded PD-liposomes**		
	CCY-lipo	VI22-lipo	42OL-lipo		CCY-lipo	VI22-lipo	42OL-lipo
>1000	>136	>14.5	>8.5	>1000	>199	>200	>168

* The initial concentration of PenG in the PD-liposome stock solutions are reported in Table 1.

** The initial concentration of Amp in PD-liposomes were 398 μg/mL, 400 μg/mL and 337 μg/mL for CCY-lipo, VI22-lipo, 42OL-lipo, respectively.[4]

Conclusions

Using unconventional (prokaryote derived) lipid material to formulate liposomes was proven~~d~~ to be an efficient strategy for delivery of antibiotics in comparison with standard formulations. As a first ~~noticeable~~ encouraging result~~s~~, we observed MIC reduction against *E. coli* cells with all the PD-liposomes loaded with PenG with respect to the free antibiotic. Specifically, liposome~~s~~ 42OL-lipo, from *R. palustris,* ~~was~~ were the most effective ~~formulation~~ (16-fold MIC reduction), followed by VI22-lipo (8-fold MIC reduction) and CCY-lipo derived from *Cyanothece* spp (2-fold MIC reduction). The physico-chemical characterization with SAXS proved that the structural features of these newly prepared liposomes were those of unilamellar vesicles, though in the case of antibiotic loaded VI22-lipo the usual core-shell structure accounted only partially for the observed i~~I~~ntensity diagram. This peculiarity was attributed to the presence of a thick polysaccharide capsule surrounding the cells of the original VI22 bacteria, ~~-~~where some of the components

~~of which~~ may have been retained in the extracted lipid mixture and became part of VI22-lipo. However, as PenG-loaded VI22-lipo showed a considerable ~~amount of~~ antimicrobial activity against *E. coli* XL1Blue cells, the potential presence of these components did not seem to interfere with the mechanism of release of the antibiotics into the periplasmic space of *E. coli* cells. DLS showed that there were no huge differences in the size of loaded liposomes among the three bacterial strains, the mean diameter ranging from ~ 200 ~~nm~~ to ~ 300 nm. We found no correlation between the liposomal ~~e~~ size and antimicrobial activity. 42OL-lipo ~~was~~ were the largest of the three and the more effective ~~as~~ regarding the antimicrobial activity. CCY-lipo and VI22-lipo were smaller than 42OL-lipo and similar in size, but differed substantially ~~for~~ in antimicrobial activity. Instead, the different microbial origin of the PD-liposomes could influence their antimicrobial activity, presumably by influencing the interaction with the *E. coli* OM and the consequen~~tly~~ release of the antibiotic into the periplasmic space.

The lack of increase in the antimicrobial efficacy of CCY-, VI22- and 42OL-liposomes loaded with Amp with respect to free Amp (for all antibiotic formulation the MIC was 14.5 μg/mL) could be explained by the greater OM permeability to Amp as compared to PenG. In fact, the permeability of Amp was reported to be four times higher than PenG, a behavior attributed to differences in the gross physicochemical properties (liposome size, charge and lipophilicity) of these two molecules, that influenced their passage through the porin channel.[72,73] Accordingly, we found *E. coli* XL1Blue strain to be more resistant to free PenG than to free Amp, with exactly 4-fold difference in MIC values (58 μg/ml and 14.5 μg/ml, respectively). Therefore, also the loaded antibiotic can play a role in the evaluation of the efficiency of such vectors, suggesting that each new formulation must be targeted to both the molecules and microorganisms under consideration.

Noteworthy, antibiotic loaded PD-liposomes did not exert any inhibition on the *E. coli* XL1Blue strain overexpressing the β-lactamase encoded by the *bla-TEM1* gene on the multicopy pUC18 plasmid. This complete lack of antimicrobial~~ic~~ activity ~~would~~ could be a result ~~by~~ of the enzymatic degradation of PenG and Amp by the β-lactamase after they have reached the periplasmic space. If this is ~~most likely~~ the

case, then PD-liposomes did not exert any protection against the β-lactamase activity. The antibiotics would be released into the periplasmic space in an unprotected, naked form. This release mode is similar to that demonstrated for standard liposomes, and involves the interaction and fusion with the bacterial OM (citazione 29). Overall, our data strongly suggest that, unlike the conventional liposomes $StdL_1$ and $StdL_2$, PD-liposomes CCY-lipo, VI22-lipo and 42OL-lipo can help PenG to enter the bacterial cell through the OM and exert its inhibitory activity on transpeptidase enzymes in the periplasmic space. These encouraging results deserve further investigations on the potential that liposomes of bacterial origin can have as systems of drug delivery to microorganisms. Thanks to their similarity to bacterial membranes and to microbial membrane vesicles that many prokaryotes produce to delivery virulence factors and degradative enzymes to microbial cells in their environment,[74] PD-liposomes could be tested not only to counteract intrinsic mechanisms of antibiotic resistance, such as that of Gram-negative bacteria to β-lactams, but also for preventing or inhibiting microbial biofilm formation.

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