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Influence of Lipid Composition on Physicochemical and Antibacterial Properties of Vancomycin-Loaded Nanoscale Liposomes

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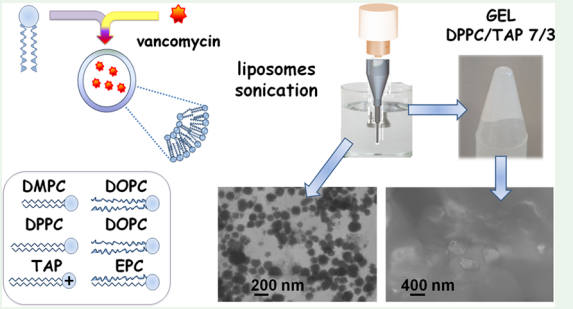
ABSTRACT: The bacterial resistance against antibiotics has reached an alarming level, leading to an increase in morbidity and mortality. One of the possible approaches to tackle this problem of major concern for the global community is to renovate "old" antibiotics to make pathogens susceptible to their pharmacological activity. Vancomycin (VAN) is a glycopeptide used for the treatment of Gram-positive bacterial infections such as the ones caused by methicillin-resistant *Staphylococcus aureus*. Nevertheless, some strains of this bacterium developed high-level VAN resistance. To revert VAN resistance and decrease its side effects, we included this antibiotic in different liposomal formulations. Nanoscaled liposomes were formulated with pure phospholipids differing in the presence of unsaturation and/or in the length of the acyl chains. DPPC liposomes were also tested in a mixture with cholesterol hemisuccinate (at 40 molar percentage) and/or a cationic saturated lipid (at 30 molar percentage) to make them pH-responsible and/or positively charged. Two techniques for liposome preparation, thin film hydration and reverse phase evaporation, were employed and compared. The key element of this investigation is a systematic and rational variation of liposomes composition and VAN loading technique to modulate the physicochemical properties of the formulations. The availability of a "revised" broad-spectrum VAN could increase its therapeutic options in the case of bacterial infections and, at the same time, limit the failure of the treatment due to the beta-lactam resistance in bacteria.



KEYWORDS: *chemical structure−activity relationship, reverse phase evaporation, antibacterial activity, vancomycin, liposomes, gelation*

1. INTRODUCTION

The extensive and often inappropriate use of antibiotics (about 100,000 tons are manufactured annually worldwide)[1] resulted in multidrug resistance in bacteria, *i.e.*, the selection of pathogenic bacteria resistant to multiple drugs.[2] In some cases, the antimicrobial treatment is inefficacious and the infectious disease can bring death.[3] As a consequence, there is the need to explore and find alternative antibacterial therapies based on newer generation (sometimes expensive) antibiotics or on the restoration of "old" antibiotics, re-engineered with the aim of optimizing their effectiveness. The inclusion of these molecules in liposomes can affect their biodistribution and pharmacokinetics, minimize their toxicity (and then allow higher drug dosing) and favor their accumulation and internalization in target cells,[4,5] thus enlarging their spectrum of action. Vancomycin (VAN) is a water-soluble glycopeptide that sterically inhibits peptidoglycan synthesis and maturation with a subsequent reduction of cellular mechanical strength.[6] VAN is the first option in the treatment of methicillin-resistant *S. aureus* (MRSA) infections, one of the most challenging among those that afflict public health.[7] However, the emergence of resistant bacteria has reduced the susceptibility of MRSA to VAN,[8] and, in some cases, its side effects decrease the efficacy of the treatment.[9] Literature reports show that the use of liposomes as a VAN delivery system can revert the resistance of MRSA to VAN while reducing its side effects.[10−15] Moreover, MRSA can persist in alveolar macrophages contributing to the failure of the treatment of pneumonia with VAN.[10] Considering that liposomes tend to accumulate into macrophages,[16] they can be considered an ideal VAN delivery to increase its efficacy against MRSA. It is clear that the molecular structure of liposome components[17−20] and the methodology used for their preparation and loading play a pivotal role in determining the

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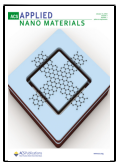

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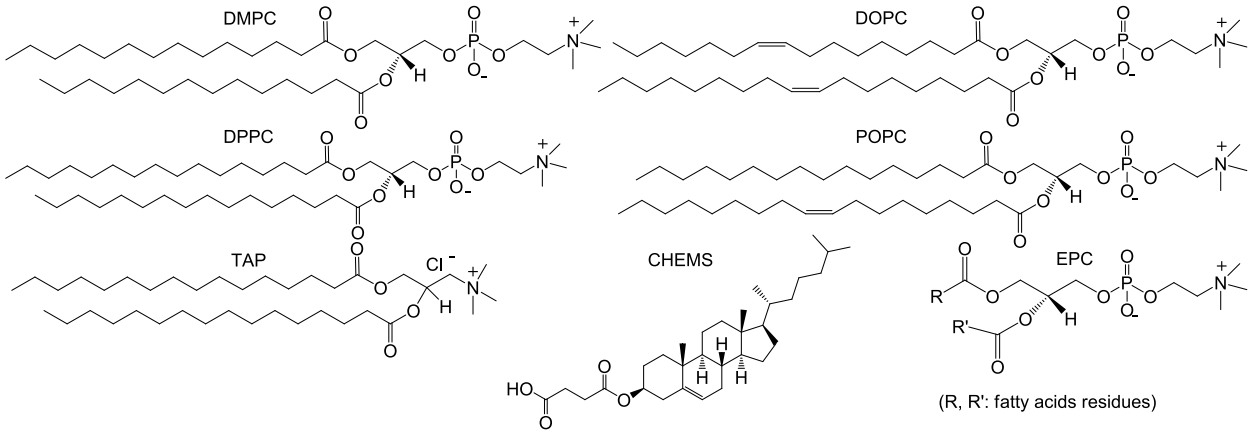
Chart 1. Liposomes Components



physicochemical characteristics of the aggregates and, as a consequence, their biological fate.[21−26]

Here, we report an investigation on the loading of VAN in nanoscaled liposomes formulated with different phospholipids (PCs) featuring different alkyl chain lengths and extents of saturation/unsaturation, namely, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) 1,2-dioleooyl-*sn*-glycero-3-phosphatidylcholine (DOPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC), and a crude mixture containing about 60% of unsaturated phospholipids extracted from egg yolk (EPC) widely used in liposomes preparation (Chart 1).

Two passive loading methodologies, thin film hydration (TFH, Figure 1A) and reverse phase evaporation (RPE, Figure 1B), were explored to include VAN into liposomes.

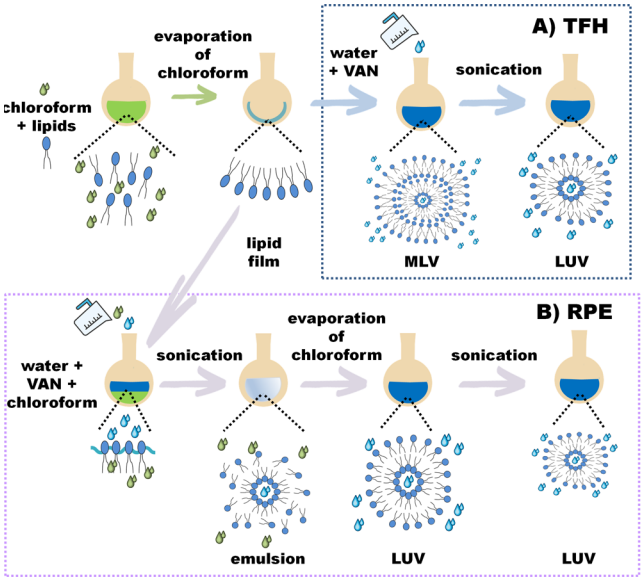


Figure 1. Schematic illustration of (A) TFH and (B) RPE liposome preparation methodology.

DPPC, the lipid that formed the best formulation in terms of dimension and entrapment efficiency (EE) was chosen to prepare mixed nanoscaled liposomes together with (i) 40 molar percentage of cholesterol hemisuccinate (CHEMS) to increase vesicles stability[27] and impart them a fusogenic behavior (making them pH sensitive)[28] or (ii) 30 molar percentage of a saturated cationic lipid (1,2-dipalmitoyl-3-trimethylammonium-propane, TAP) to make nanovesicles positively charged and favor their interaction with negatively charged target cells. A ternary mixture of lipids (DPPC/CHEMS/TAP at 4/3/3 molar ratio) was also prepared to evaluate the combined effect of CHEMS and TAP on liposome properties. In addition to the EE, the physicochemical properties of the aggregates were investigated by differential scanning calorimetry (DSC), dynamic light scattering (DLS), transmission electron microscopy (TEM), and small-angle X-ray scattering (SAXS) measurements. The antimicrobial activity of the best VAN-containing formulations against a methicillin-resistant *Staphylococcus aureus* (MRSA) strain and Gram-negative (*E. coli*) organisms was also investigated to get preliminary data on their potentiality for the treatment of bacterial infections. The methodical variation of lipids and technique for VAN loading and liposomes preparation was aimed at deepening our knowledge of the characteristics of liposome components that can be considered crucial for an efficient VAN entrapment and delivery. In previous works, we demonstrated that it is possible to control the delivery of a drug to bacterial cells by tuning liposome composition. In particular, we put in evidence that even subtle variations of the molecular structure of lipids can significantly affect liposomes physicochemical properties[29] and, as a consequence, their ability to interact with target cells.[30−33]

2. EXPERIMENTAL SECTION

2.1. Materials. DMPC, DPPC, DOPC, POPC, TAP, and CHEMS were purchased from Avanti Polar Lipids (Alabaster, AL). EPC (~60%), VAN hydrochloride, phosphate-buffered saline tablets (PBS, 0.0027 M KCl; 0.137 M NaCl; pH 7.4), dialysis tubing cellulose membrane (cutoff = 14,000), chloroform, methanol, methyl *t*-butyl ether, diisopropyl ether, diethyl ether, and ethyl acetate were purchased from Sigma-Aldrich (Milan, Italy). All reagents and solvents were used without further purification. The American Type Culture Collection reference bacterial strain, *Staphylococcus aureus* ATCC4300 with an MRSA profile and *Escherichia coli* ATCC 25922 were used for the susceptibility test (Liofilchem, Italy).

2.1.1. Instrumentation. Liposomes were sonicated using a Hielscher UP100-H ultrasonic processor with a microtip probe (7 mm) or Falc LBS ultrasonic bath, and they were centrifuged using an ALC PK110 centrifuge with fixed-angle equipment. DLS measurements were performed using a Malvern Zetasizer Nano ZS, equipped with a 5-mW He−Ne laser operating at 633 nm, while UV measurements were carried out on a DU 800 UV−vis single beam spectrophotometer (Beckman Coulter. DSC measurements were

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conducted using a Mettler Toledo DSC 3 calorimeter (Mettler-Toledo International Inc., Columbus, OH, USA). SAXS measurements were performed at SAXSLab Sapienza with a Xeuss 2.0 Q-Xoom system (Xenocs SAS, Grenoble, France), equipped with a microfocus Genix 3D X-ray source with Cu anode (λ = 0.1542 nm), a two-dimensional Pilatus3 R 300 K detector which can be placed at variable distance from the sample and an additional Pilatus3 R 100 K detector at fixed shorter distance from the sample to access larger scattering angles (Dectris Ltd., Baden, Switzerland).

2.2. Methods. *2.2.1. Liposomes Preparation Using the TFH Technique.* A proper amount of lipid (DMPC, DPPC, DOPC, POPC, or EPC) in combination or not with TAP and/or CHEMS was solubilized in $CHCl_3$ in a round-bottom flash, and then the solvent was removed using a rotavapor. A lipid film was obtained and was left under reduced pressure (≈0.4 bar) for at least 6 h. Then, it was hydrated with PBS 150 Mm containing VAN (3 mg/mL) and vortex-mixed in order to obtain multilamellar vesicles (MLVs). This dispersion was sonicated for 8 min (72 W, cycles 0.5 s) obtaining unilamellar liposomes. The final concentration of liposomes was 10 mM in lipid content.

Unentrapped VAN was removed through dialysis. The external PBS solution was exchanged two times (100-fold the liposomes dispersion volume) during 4 h.

2.2.2. Liposomes Preparation Using the RPE Technique. A lipid film composed of 3×10^{-5} moles in a total of DMPC, DPPC, DOPC, POPC, or EPC (in the presence or in the absence of TAP and/or CHEMS) was prepared as reported above. Then, it was solubilized in 3 mL of organic solvent (methyl t-butyl ether, diisopropyl ether, diethyl ether, and ethyl acetate in the presence or not of 1 mL of $CHCl_3$) and 1 mL of a VAN solution (9 mg/mL) in PBS 150 mM was added. This two-phase solution was emulsionated using a bath sonicator for 5 min at room temperature and the organic solvent was slowly removed using a rotavapor. The temperature was set to 25 °C and the pressure was reduced to 50 mbar every 5 min, starting from 550 mbar until 130 mbar. At this point, 2 mL of PBS 150 mM was added, and the dispersion was vortex-mixed. After that, it was returned to rotavapor at 150 mbar until the suspension was homogeneous. The final concentration of the liposomal dispersion was 10 mM in lipid content and 3 mg/mL in VAN content. Unentrapped VAN was removed through centrifugation (36,000*g*, 4 °C, 1 h). After removing the supernatant (containing the untrapped VAN), the pellet was resuspended in PBS 150 mM. Some formulations (DPPC, DPPC/TAP 7/3, and DPPC/TAP/CHEMS 4/3/3) were sonicated using a tip sonicator for 8 min (72 W, circles 0.5 s).

2.2.3. Evaluation of VAN EE. The EE of VAN was evaluated by UV−vis experiments. A solution composed of 0.5 mL of methanol and 0.5 mL of the liposome suspension before and after dialysis was analyzed and the EE was given by the percentage ratio between the absorption value at 280 nm after and before dialysis or centrifugation.

2.2.4. DLS Measurements. The aggregate distributions of the hydrodynamic diameters (D_H) were evaluated on 1 mM liposomal suspension at 25 °C soon after their preparation, and over time upon storage at room temperature, by the non-negative least-squares algorithm analysis of the measured DLS autocorrelation functions. The analysis provided the distributions of the diffusion coefficients D from which D_H was obtained by using the Stokes−Einstein relationship $D_H = kT/3\pi\eta D$, where kT is the thermal energy and η the solvent viscosity. Intensity-weighted distributions were the averages of three consecutive measurements of the same samples.

2.2.5. SAXS Measurements. SAXS measurements were performed on 10 mM liposome dispersions prepared according to the RPE procedure (before sonication) soon after their preparation and 2 weeks later. The samples were loaded into vacuum-tight quartz capillary cells with a thickness of 1.5 mm and measured in the instrument sample chamber at reduced pressure (∼0.2 mbar) in a thermostat holder at 25 °C. Measurements with different sample−detector distances were performed so that the overall explored q region ($q = 4\pi\sin(\theta)/\lambda$, where 2θ is the scattering angle) was 0.045 $nm^{-1} < q < 33$ nm^{-1}, or 0.045 $nm^{-1} < q < 10$ nm^{-1} when using capillary cells with a smaller opening. The two-dimensional scattering patterns were subtracted for the dark counts and then masked, azimuthally averaged, and normalized for transmitted beam intensity, exposure time, and subtended solid angle per pixel by using the FoxTrot software (version 3.4.9) developed at SOLEIL. The one-dimensional I vs q profiles were then subtracted for the buffer and cell contributions and put in absolute scale units (cm^{-1}) by dividing for the known thickness. The different angular ranges were merged using the SAXS utility tool.[34]

2.2.6. STEM Measurements. Morphology of the aggregates prepared by RPE was evaluated on 10 mM liposomes sonicated or not. A scanning electron microscope (ZEISS GeminiSEM 500) with an annular detector, aSTEM, was used to observe their morphologies and dimensions. Briefly, 10 μL of the investigated liposome suspensions was air-dried onto a copper grid for electron microscopy covered by a thin amorphous carbon film.

2.2.7. DSC. DSC measurements were carried out on MLVs (150 mM in total lipids) in case of the TFH technique on liposomal pellet obtained after centrifugation (150 mM in total lipids) and on sonicated liposomes (10 mM) prepared according to RPE. All preparation procedures were described before.

Two heating scans were recorded at a rate of 5 °C/min followed by two heating scans at a rate of 1 °C/min. Temperatures were determined with an accuracy of ±0.1 °C and ΔH values with an accuracy of ±0.5 kJ/mol. These experiments were performed in duplicate, obtaining three reproducible thermograms each time.

2.2.8. In Vitro Antimicrobial Susceptibility Tests. Susceptibility tests for different liposome formulations, with and without VAN, were determined by the conventional broth microdilution method in 96-well plates with a bacterial inoculum of 5×10^5 CFU/mL, as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2020).[35] In addition, *Staphylococcus aureus* ATCC 43300 and *Escherichia coli* ATCC 25922 were grown in cation-adjusted Mueller-Hinton medium (CAMHB) for 18 h at 35 °C. The minimum inhibitory concentration for the different formulations was determined as the lowest agent concentration at which no growth was observed. All experiments were performed in triplicate.

3. RESULTS AND DISCUSSION

Nanovesicles were formulated with different saturated or unsaturated lipids alone or in a mixture with CHEMS and/or a cationic lipid, TAP, applying two different methodologies, TFH or RPE. TFH was the first method described for liposomes preparation[36] and it is still largely employed thanks to its simplicity. RPE requires a multistep procedure based on the formation of inverted micelles or water-in-oil emulsions for obtaining liposomes. Generally, using RPE higher EE of hydrophilic molecules (like VAN) is obtained with respect to TFH. On the other hand, traces of organic solvent can remain and affect the stability of the loaded payload and/or of the aggregates.[37] Nanoscaled liposomes of DPPC, DMPC, and DOPC prepared by TFH followed by sonication were monodisperse and featured a D_H of about 100 nm, as previously reported;[24,25] similar results were obtained with liposomes composed of EPC or POPC or with mixed liposomes and in the presence of VAN for all the formulations. The only exception is the formulation DPPC/TAP 7/3: in this case, gelation of the suspension occurred upon sonication in the presence or in the absence of VAN (Figure 2). The formulations are quite stable even if they showed an increase of polydispersity over time in some cases (Tables S1−S3).

Dimensions of the same formulations at a 1 mM concentration prepared according to RPE showed different distributions depending on the compositions (Figure S1 A).

Liposomes formulated with saturated lipids with or without CHEMS prepared by RPE showed a D_H between 120 and 150

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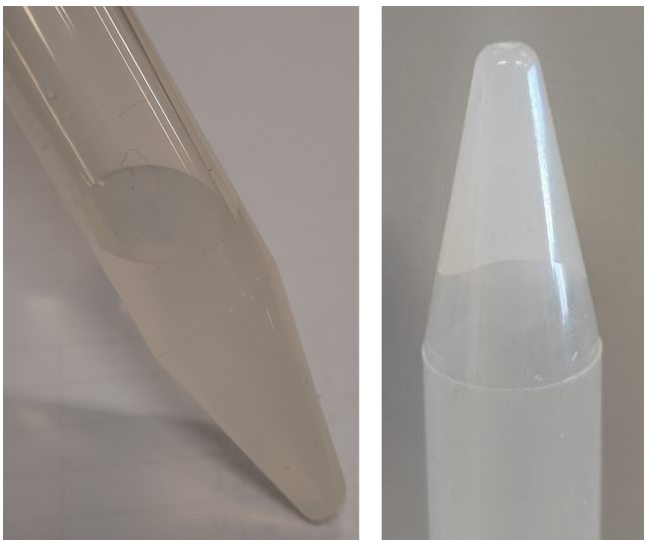

Figure 2. DPPC/TAP 7/3 liposomes 10 mM prepared by TFH before (on the left) and after sonication (on the right). Similar results were obtained by preparing the same formulation according to the RPE procedure.

nm and were monodisperse, similar to what was observed in the case of TFH. The same results were obtained in the presence of VAN (Table S4). On the other hand, liposomes prepared with unsaturated lipids were polydisperse. To verify if this result could be ascribed to the organic solvent used in their preparation, we replaced methyl *t*-butyl ether with diisopropyl ether, ethyl acetate or diethyl ether (each with or without chloroform), but we obtained polydisperse liposomal suspensions in all cases. Sonicated nanoscaled liposomes prepared with RPE showed a reduction of about 20% of the dimensions in the case of aggregates containing saturated lipids (Figure S1 B) and, like in the case of TFH, gelation of DPPC/TAP 7/3 samples. Sonication also promoted a lowering of polydispersity for the liposomes prepared with unsaturated lipids, providing monomodal populations with D_H values of about 100 nm (Figure S1 B), as observed for other formulations investigated after sonication. The D_H values of the peak of the distributions are reported in Table 1. The results unquestionably confirm that sonication or extrusion is mandatory to obtain single-population liposomes when formulated with unsaturated lipids.[37,38] All the saturated formulations prepared according to the RPE technique were stable for at least 2 months at room temperature (Tables S5−S7).

We assessed the lamellarity of the VAN-containing formulations obtained with RPE before sonication by SAXS measurements. The SAXS profiles show an initial intensity decay close to the power law q^{-2} expected for flat structures, in agreement with the presence of bilayers and the overall size of the vesicles being beyond the maximum dimension accessible in the available q range (<100 nm). The characteristic oscillation around 1 nm^{-1} given by the electron density profile across the individual bilayer can show interference peaks due to a multilamellar structure factor in the case of multilamellarity. In addition, a peak in the wide-angle regime (q 12−19 nm^{-1}) corresponding to characteristic distances of 0.4−0.5 nm (Figure S2) could be related to the lateral packing among the hydrophobic tails within the bilayers, and it can be sharper or broader in the case of a higher or lower degree of order of the lipid chains. It is reasonable to assume that if the lipid membrane is in the ordered gel phase, where the hydrocarbon chains are fully extended and closely packed, a sharper peak should be observed, whereas the width should increase and the position moves to lower q values (implying larger packing distances, $d = 2\pi/q$) if the membrane is in a more disordered liquid crystalline phase above the melting temperature.

Our results show that, in case of DOPC and POPC formulations, multilamellar liposomes were clearly observed (Figure 3A), with characteristic interlamellar spacings of 6.4 and 6.5 nm, respectively. EPC liposomes were largely unilamellar soon after their preparation, even if some weak peaks modulating the main maximum could be appreciated in positions 0.63, 0.88, 1.00, and 1.22 nm^{-1}, suggesting a certain heterogeneity in possible liquid crystalline populations. After 2 weeks, the SAXS profile showed an intense diffraction peak at 1.016 nm^{-1}, an index of a consistent increase in the multilamellar population (Figure 3B). Evidently, the peculiar composition of EPC (mixture of unsaturated phospholipids and other lipid components like phosphatidylethanolamines, triglycerols, and cholesterol) reduces the stability of the liposomes it forms bringing them to rearrange over time. On the other hand, in the case of liposomes composed of saturated lipids (DMPC and DPPC) mainly unilamellar aggregates (stable over 2 weeks) were obtained (Figure 3C, D).

We also analyzed the gel obtained upon sonication of DPPC/TAP liposomes and also in this case we observed the presence of a single bilayer (Figure 3E). In particular, a higher signal is shown in the low-q SAXS profile for DPPC/TAP liposomes compared to that of DPPC only (Figure S3). Several factors can contribute to the larger signal-to-noise ratio and a trivial one could be due to the arrested gel state of the DPPC/

Table 1. Main Peak D_H Values of the DLS Intensity Weighted Diameter Distributions of Investigated Nanoscaled Liposomal Formulations Prepared by RPE before and after Sonication in the Absence of VAN[a]

	before sonication		after sonication	
formulation	D_H (nm)	PDI	D_H (nm)	PDI
DPPC	129 (±14)	0.21	110 (±11)	0.15
DMPC	122 (±16)	0.28	95 (±12)	0.17
DOPC	637 (±150)	0.71	100 (±40)	0.33
POPC	757 (±100)	0.62	120 (±33)	0.27
EPC	830 (±140)	0.55	140 (±27)	0.32
DPPC/CHEMS 6/4	120 (±21)	0.13	100 (±8)	0.10
DPPC/TAP 7/3	153 (±13)	0.36	-	-
DPPC/CHEMS/TAP 4/3/3	151 (±16)	0.31	120 (±12)	0.18

[a]Estimated standard deviations are reported in parentheses.

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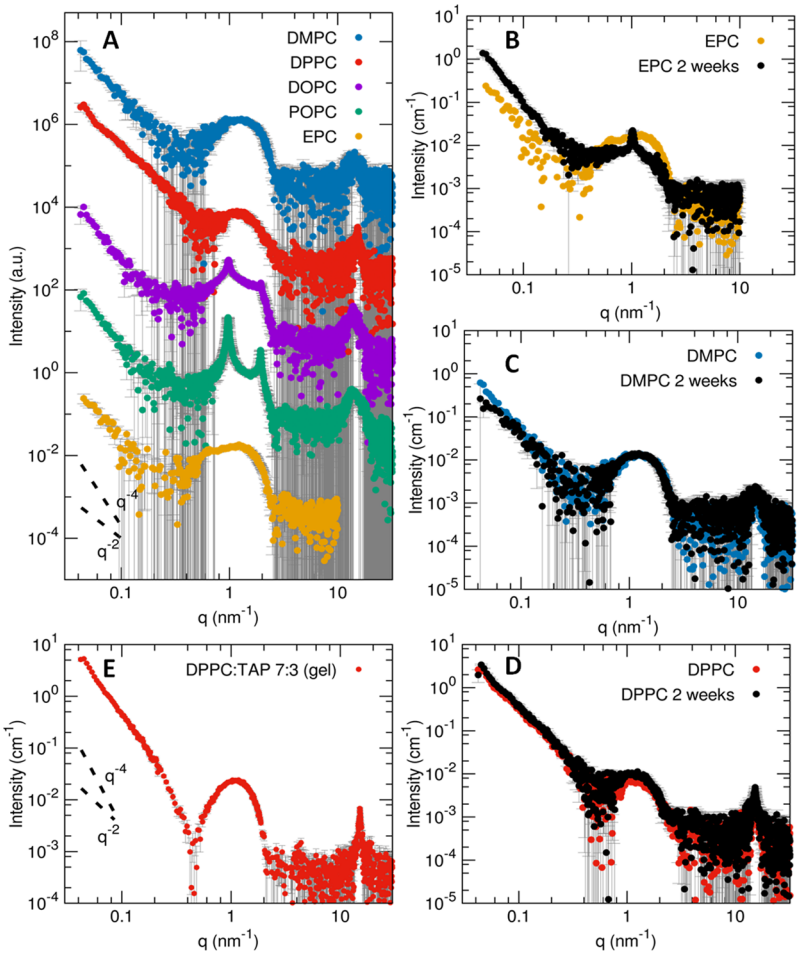

Figure 3. (A) SAXS profiles of VAN-containing liposome formulations before sonication. The curves have been shifted on the intensity axis by a suitable factor to avoid data overlap and help visualization. (B–D) Comparison between the scattering profiles of EPC, DMPC, and DPPC formulations measured soon after preparation and after 2 weeks. (E) SAXS profile of DPPC/TAP liposomes after sonication.

TAP sample which prevents sedimentation in the capillary, increasing the effective lipid volume fraction in the scattering volume. The initial slope for the DPPC/TAP sample deviates more visibly from the expected slope for a flat structure (it is closer to q^{-3} than q^{-2}), and this could be related to the fact that a model of the isolated bilayer is less applicable for this sample in which we expect larger scale interconnections to justify the arrested state. Most relevantly, the SAXS signal of bilayers is mainly due to the electron density profile across the membrane and can change significantly even with small perturbations of the balance between the volume occupied by the regions with electron density that is lower (tails) or higher (heads) than the aqueous solvent. Even if a realistic representation of such a profile requires several contributions,[39,40] a very simple model that already captures the main features of the SAXS signal is a head–tail lamellar model (just two electron density values for tails and heads). The profiles obtained assuming the same imposed values of the scattering length densities for hydrophobic tails (8×10^{-4} nm^{-2}) and polar heads (12×10^{-4} nm^{-2}) show different best-fitting values for the tail and head domain thicknesses (Table S8). Alternatively, if the tail region parameters are kept the same for DPPC/TAP and DPPC, then an increase in the best-fitting head domain thickness and a decrease in the corresponding scattering length density are observed to improve the agreement.

In general, the gelation of a liposomal suspension can be induced by the addition of long-chain alcohols or differently substituted glycerol.[41,42] It is based on the opening of the vesicles (formulated with at least two components) to give homogeneously distributed sheetlike structures among water layers that act as a network. Obviously, this phenomenon is strictly linked to liposome composition. It occurs in a narrow composition range and generally is observed in liposomes featuring highly packed rigid bilayers.[43] Also, surface charge and surface charge density are determinants for liposomes gelation because they affect the intrabilayer interaction between headgroups in the aqueous network. Our results indicate that, in the case of DPPC/TAP 7/3 liposomes, upon the opening of the closed aggregates induced by sonication, lipids experience optimal conditions for gel formation in terms of either rigidity and surface charge of the bilayer. The addition of CHEMS makes liposome gelation impossible. It is reasonable to suppose that this effect is due to the reduction of liposomal surface charge due to the presence of CHEMS rather than to a reduction in acyl chain mobility. In fact CHEMS, differently from cholesterol, tightens the bilayer in the gel phase but not in the liquid-crystalline one.[44] Considering that upon sonication liposomal dispersion reaches temperatures well above the main transition temperatures of the formulation, the effect of CHEMS on the rigidity of the bilayer during sonication should be neglectable.

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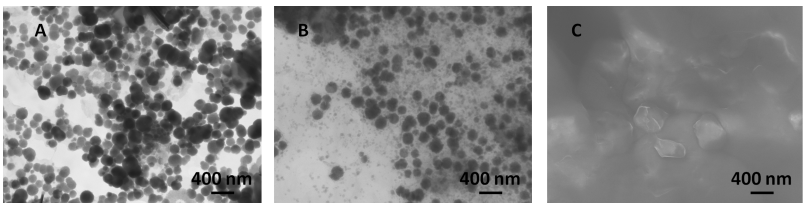

Figure 4. STEM images of 10 mM (A) not sonicated DPPC/TAP/CHEMS 4/3/3 liposomes, (B) not sonicated DPPC/TAP 7/3 liposomes, and (C) sonicated DPPC/TAP 7/3 liposomes.

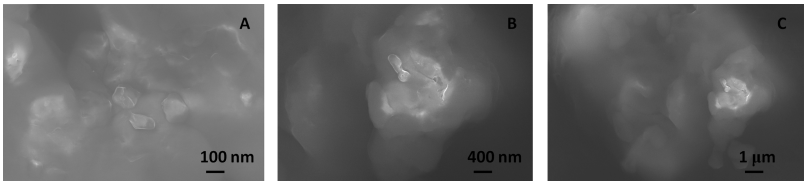

Figure 5. STEM images collected with different magnifications (A, 150×; B, 200×; C, 300×) of the gel obtained sonicating 10 mM DPPC/TAP 7/3 liposomes.

We investigated the nanovesicle morphology by electron microscopy measurements. In Figure 4A, we reported an image obtained investigating DPPC/TAP/CHEMS 4/3/3 before sonication as an example. Similar images were collected with sonicated nanoscaled liposomes or other formulations, with the exception of sonicated 10 mM DPPC/TAP 7/3 liposomes. Our results indicate the presence of nanoliposomes that feature dimensions that are in good agreement with those observed by DLS measurements. In Figure 4B, C, STEM images of 10 mM DPPC/TAP 7/3 liposomes before and after sonication are reported. It is clearly evident that the aggregates present in the sample disappear upon sonication due to gelation.

Applying different magnifications on the same region of the sonicated sample allowed us to put in evidence variations in the thickness of the gel layer (Figure 5).

Based on the obtained results, we decided to further investigate nanoscaled liposomes composed of DPPC alone or in a mixture with CHEMS and/or TAP for their potential as VAN delivery systems. The EE observed using TFH was very low (not above 3 molar percentage, which was less than 90 μg/mL), whereas using RPE the EE raises to about 15 molar percentage (≈450 μg/mL) without significant differences as a function of liposomes composition. We verified through a dialysis experiment that the amount of entrapped VAN in the case of liposomes prepared according to RPE did not diminish upon sonication (data not shown). The obtained results are not surprising. In fact, it is well known from the literature that the EE of hydrophilic drugs in liposomes is very high if they are prepared according to the RPE procedure, whereas in the case of TFH, the higher EE is observed with hydrophobic compounds.[45] This evidence can be explained considering that the microemulsions that form during sonication and solvent evaporation bring about the encapsulation of a relatively high amount of water and, consequently, of the solute dissolved within. In particular, a higher EE was obtained for liposomal VAN using RPE with respect to TFH also in a previous study reported in the literature.[46]

To deepen our knowledge on the physicochemical properties of the bilayer, we carried out DSC measurements of the formulations, empty or loaded with VAN, prepared by TFH or RPE. As expected, the presence of CHEMS at 40 molar percentage abolished the main transition, and no peaks were observed in the thermograms. The thermograms and the calorimetric data obtained for the other samples prepared with TFH or RPE in the presence or in the absence of VAN are reported in Figure S4 and in Table 2.

Table 2. Calorimetric Data Obtained for DPPC or DPPC/TAP 7:3 (Not Sonicated) Liposomes Prepared according to TFH or RPE in the Presence or in the Absence of VAN[a]

formulation	T_m (°C)	ΔH (kJ/mol)
DPPC RPE	41.0	−19
DPPC RPE with VAN	39.2	−22
DPPC TFH	41.7	−33
DPPC TFH with VAN	41.2	−26
DPPC/TAP 7:3 RPE	54.0	−10
DPPC/TAP 7:3 RPE with VAN	52.4	−20
DPPC/TAP 7:3 TFH	54.4	−26
DPPC/TAP 7:3 TFH with VAN	53.7	−20

[a]Error in the case of the obtained temperatures and ΔH are within 0.5 °C and 1 kJ/mol, respectively (averaged over three measurements).

Lipids in void nanoliposomes (containing TAP or not) prepared with TFH are more efficiently packed with respect to those in liposomes obtained through RPE as clearly indicated by ΔH values, confirming that the preparation method can have a significant impact on lipid organization. In the case of DPPC liposomes, the presence of VAN abolishes the pretransition and induces a slight reduction of the T_m. The preparation method used influences, besides lipid packing, the effect of VAN. In nanovesicles prepared according to RPE loaded with VAN, a broadening of the peak and shoulder is clearly observed. This evidence indicates that VAN, bearing sugars and many polar moieties, strongly interacts with the polar headgroups of lipids. The fact that a shoulder appears when VAN is included in liposomes suggests that probably the sugar moieties of VAN affect lipids organization. In fact, at a temperature next to T_m, sugars dehydrate lipids, inducing a partial delayed melting[47] and membrane thinning with the formation of a disordered, glassy state in the hydrophobic core of the bilayer.[48] The formation of this glassy phase could explain the reduction of ΔH value, mainly dependent on van der Waals interactions among lipid chains. On the other hand, when VAN was loaded in liposomes prepared according to TFH, the shape of the peak did not change. This result could

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be related to the fact that in the latter case lipid packing in the bilayer is more efficient with respect to liposomes prepared with RPE, so the presence of VAN in the aqueous phase in this case has a reduced effect. Another possible explanation is related to the higher amount of liposomal VAN in aggregates formed with RPE with respect to TFH. The increase of ΔH value indicates that anyway, the presence of the drug disturbs lipid organization but not in a significant way like observed with the RPE method. It has to be noticed that in the case of liposomes prepared with TFH that the amount of VAN entrapped in the aggregates is lower than that of the corresponding liposomes prepared with RPE, so the extent of the effect of the drug could also be reduced for this reason.

From the SAXS data, it can be indeed observed that the diffraction peak width decreases in the order of increasing main T_m (Table S9) and DPPC liposomes (which at 25 °C is below the T_m) show the sharpest peak. Moreover, the addition of TAP into the DPPC liposome formulation increases the peak sharpness even more, and this correlates with the higher T_m measured by DSC, indicating a more ordered and rigid membrane.

The addition of a 30 molar percentage of TAP brings to a net increase of the T_m (more than 10 °C) and of ΔH values and the broadening of the peak, indicating that this cationic lipid exerts a marked effect on lipid organization. Also in these samples, the presence of VAN induces a decrease of T_m with respect to empty nanoliposomes, even if in this case there does not appear any shoulder in the thermograms. This difference is reasonably to be ascribed to weaker interactions among alkyl chains of DPPC and TAP than those among alkyl chains of DPPC alone. The effect of the sugar moieties of VAN on lipid packing is less relevant. Similar results were observed investigating the gel obtained upon sonication of DPPC/TAP liposomes (Figure S5). This evidence confirms that the bilayers are still present in the gel and that its thermotropic properties do not change even if it is not organized in vesicles.

The antimicrobial activity of VAN loaded in DPPC, DPPC/CHEMS 6/4, and DPPC/CHEMS/TAP 4/3/3 nanoscaled liposomes was tested (after sonication) on *S. aureus* and *E. coli* and was compared to the one of free VAN. VAN delivery efficiency of DPPC/TAP 7/3 formulation could not be assessed because its turbidity before sonication made the evaluation impossible. Anyway, considering its gelation after sonication, it has good potential as a drug delivery system in topical treatment as reported in the literature for other liposomal gels.[49−52] All the formulations were ineffective on *E. coli*, but it is not surprising considering that (i) it is a Gram-negative bacteria difficult to penetrate because of its outer membrane and (ii) free VAN is not active on these pathogens at acceptable MIC. VAN loaded in DPPC nanoliposomes did not show any biological activity whereas DPPC/CHEMS 6/4 and DPPC/CHEMS/TAP 4/3/3 liposomes showed a MIC (1.4063 and 1.2891 μg/mL, respectively) comparable to the one of free VAN (1 μg/mL). The corresponding empty formulations did not show any bactericidal activity. This result indicates that these mixed fusogenic formulations can be suitable carriers for the delivery of VAN and could enlarge its therapeutic spectrum, even if it is impossible to assess the specific contribution of CHEMS and TAP. This in turn enlarges the perspective of these formulations as delivery systems of other active principles that could not be used as-is.

4. CONCLUSIONS

One possible approach to face the increase in the failure of antimicrobial therapies is to use proper drug delivery systems that are able to revert the resistance of pathogens to the active principle. At this aim, different nanoscaled liposomal formulations composed of saturated or unsaturated phospholipids and mixed pH-sensitive and/or cationic aggregates were prepared according to the TFH or RPE procedure and fully characterized to evaluate their potentiality as VAN delivery systems. The systematic approach used in this investigation was aimed at deepening our knowledge on the effect of variations in molecular properties of liposome components associated with the preparation and VAN loading technique employed. Our results provide evidence that the molecular structure of lipids, affecting lipid organization and the charge of the aggregates, is crucial in determining liposome properties and their biological efficacy as drug delivery systems. Moreover, our findings demonstrate that, besides the choice of the proper lipid components, the methodology used for liposome formulation can play a pivotal role in determining lipid organization and entrapping efficiency and, as a consequence, their success as a delivery system. The evidence that some mixed formulations feature the same bactericidal activity as free VAN makes them exploitable as VAN drug delivery systems.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsanm.3c05419.

DLS distribution images, stability data of loaded liposomes prepared according to TFH and RPE techniques, WAXS spectra and parameters, SAXS spectra and parameters, thermograms, and calorimetric data (PDF)

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Notes

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