Inclusion of oligonucleotide antimicrobials in biocompatible cationic liposomes: A structural study

Inclusion of oligonucleotide antimicrobials in biocompatible cationic liposomes: A structural study

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ABSTRACT

Transcription factor decoys (TFD) are short oligonucleotides designed to block essential genetic pathways in bacteria and defeat resistant infections. TFD protection in biological fluids and their delivery to the site of infection require formulation in appropriate delivery systems. In this work, we build on a classical phosphatidylcholine/phosphatidylethanolamine (POPC/DOPE) scaffold to design TFD-loaded cationic liposomes by combining the DNA-complexing abilities of a bolaamphiphile, (1,10-(dodecane-1,12-diyl)-bis-(9-amino-1,2,3,4-tetrahydroacridinium) chloride (12-bis-THA), with the biocompatible cationic lipid ethyl-phosphatidylcholine (DPePC). The goal is to perform a structural study to determine the impact of the bolaamphiphile and TFD incorporation on the liposome structure, the capacity for TFD encapsulation, and the colloidal stability in saline media and cell culture environments. The systems are characterized by means of dynamic light scattering, small-angle X-ray scattering, and f-potential measurements, to provide a clear picture of the liposome structure. Circular dichroism (CD) spectroscopy is used to assess the compaction of the oligonucleotide in a psi form, while steady-state fluorescence and fluorescence correlation spectroscopies give insight into the entrapment rate and distribution of the TFD in the liposomes. We found that the combination of the two cationic species, 12-bis-THA and DPePC, allows encapsulation of 90% of the TFD. Results of CD experiments revealed that the TFD is condensed, therefore likely protected from the lytic action of serum nucleases. Finally, the systems showed colloidal stability in aqueous dispersion with ionic strength comparable to biologically relevant media.

INTRODUCTION

The emergence of an increasing number of antimicrobial resistant bacterial strains has been growing into a serious global threat, which calls for the design of novel therapeutic approaches. Among others [1,2], transcription factor decoys (TFD) represent one such approach. TFDs are short oligonucleotides consisting of a specific base sequence, designed to imitate the binding site to transcription factors, i.e. proteins that contain specific DNAbinding sites and regulate the transcription process [3]. TFDs, by acting as decoys, can block some essential genetic pathways in bacteria and defeat infection from antibiotic-resistant strains [4]. The active TFDs, highly hydrophilic and negatively charged, cannot be simply injected into the host's bodily fluids, as they would likely face colloidal instability or digestion by nuclease enzymes, and they would not be able to cross the bacterial cell wall, also negatively charged. Therefore, an efficient delivery system must be properly formulated in order to protect the biologically active oligonucleotide and to carry it to the site of its therapeutic action. Previous work has demonstrated that the bolaamphiphile 1,10-(dodecane-1,12-diyl)-bis-(9-amino-1,2,3,4-tetrahydroacridinium) chloride, from now on referred to as 12-bis-THA, thanks to its two cationic tetrahydroacrydinium heads, can condense TFDs into very strong complexes, termed nanoplexes. In these complexes, the TFD is compacted in a denatured state [5] which cannot be recognized and attacked by the DNA-digesting serum enzymes [6]. The 12-bis-THA/TFD nanoplexes have proven in vivo activity against some antibacterial-resistant strains, such as the methicillin-resistant Staphylococcus aureus, MRSA [7]. Moreover, 12-bis-THA exhibits an intrinsic, non-specific antimicrobial action by strongly interacting with and disrupting the bacterial cell wall, similarly to its structural analogue dequalinium [8] which is used as a topical antibacterial agent. Albeit very promising, these nanoplexes are characterized by the tendency to precipitate rapidly in saline media, which suggests the need to reformulate the complex into a delivery system possessing colloidal stability in bodily fluids. A large number of studies has emerged in the last two decades concerning cationic liposomes (small unilamellar vesicles, SUVs) as efficient carriers in non-viral gene delivery systems [9,10] thanks to the electrostatic-driven association between cationic lipids and DNA. However, a major drawback of these vectors lies in the cytotoxicity of the most common cationic lipids (for example, DOTAP and DOTMA) used for their formulation [11], which are sometimes employed in concentrations as high as 1:1 wt ratio with respect to the neutral ''helper" lipids (e.g. phosphatidylcholines, phosphatidylethanolamines, cholesterol) [10]. We have shown in previous work [12] that TFDs can be incorporated in cationic liposomes, obtained through the adhesion of 12-bis-THA to the surface of a lipid scaffold composed of 1-palmitoyl-2-oleoylsn-glycero-3-phosphocholine and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (POPC and DOPE). POPC is a common constituent of mammalian cell membranes [13], while DOPE is a non-lamellar-forming helper lipid known to enhance transfection [14]. Despite their ability to transfect to the bacterial cytoplasm, such formulations presented a non-negligible cytotoxicity, which precludes their possible clinical translation. In the present work, we propose the formulation of biocompatible liposomes using POPC, DOPE, and the

cationic 1,2-dipalmitoylsn- glycero-3-ethylphosphocholine chloride (DPePC): ethyl-phosphocholines derive from the natural zwitterionic phosphatidylcholines but carry an ethyl ester on the phosphate group, resulting in a positive net electrostatic charge [15–17]. Lipoplexes based on ethyl-phosphocholines have been proposed as efficient transfection vectors [18–20] and they have demonstrated a substantially better cytotoxic profile compared to systems based on other cationic lipids such as DOTA and DOTAP [21]. Liposomes, compared to lipoplexes [22], possess the advantage of colloidal stability and controlled size. In this work 12-bis-THA is also included in the liposomal formulation to ensure TFD condnsation and favour interaction with the bacterial cell wall [23]. Combining dynamic light scattering, f-potential measurements, fluorescence correlation spectroscopy, and small-angle X-ray scattering, we investigate the structural and colloidal properties of these lipoplexes, depending on composition and preparation protocol, to correlate them with oligonucleotide encapsulation efficiency and distribution, evaluated through fluorescence and circular dichroism spectroscopies. Finally, the colloidal stability of such systems in media with high ionic strength is monitored over time. Indeed, colloidal stability in saline media is crucial with regards to the shelf-life of the final formulation (for example, fluids for intravenous administration) and its integrity in biological environments, as well as for practical issues such as the reliability of preliminary in vitro biological assays.

MATERIALS AND METHODS

The lipids POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) and DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) were acquired from Avanti PolarLipids (Alabaster, AL). DPePC (1,2-dipalmitoyl-sn-glycero-O-ethyl-3-phosphocholine, chloride salt) was a Genzyme product. 12-bis-THA (1,10-(dodecane-1,12-diyl)-bis-(9-amino-1,2,3,4-tetrahydroacridinium) chloride, MW= 635.6 g/mol) was kindly provided by Procarta Biosystems Ltd. (Norwich, UK) and originally synthesized by Shanghai Chempartners co. (Denmark) with >98% purity. The TFD, an oligonucleotide consisting of 77 base pairs (MW = 47,626 Da), was manufactured and purified by HPLC at AxoLabs (Kulmbach, Germany) as described elsewhere [5,12]. The TFD and its Alexa-488-labelled version were kindly provided to us by Procarta Biosystems Ltd. (Norwich, UK). The OliGreen reagent and TE buffer kit was supplied by ThermoFisher Scientific, Inc. Rhodamine 110 (for FCS calibration) and sodium taurocholate hydrate (> 97.0%, for TLC) were purchased by Sigma Aldrich (St. Louis MO). Sucrose (ultrapure bioreagent) was a J.T. Baker product. Triton X-100 for molecular biology was purchased from Fluka. Ultrapure water was obtained by means of a Millipore water purification system.

According to this preparation protocol, the lipids POPC, DOPE, and the cationic species (DPePC + 12-bis-THA) were weighed in the 63/27/10 wt% ratio, dissolved in chloroform and thoroughly mixed. The total amount of lipids was such as to obtain 5 * 10-3 mol/L in the final liposomes, and the corresponding average molar

ratios between zwitterionic and cationic lipids (vz/c) were vz/c ca 9. Samples were obtained by varying the DPePC:12-bis-THA weight ratio (the sum of the two being always 10 wt% of the mixture). The molecular weights of these four species are similar, so that weight ratios can be taken as efficient representatives of the mole ratios for the scopes of this work. The solvent was evaporated using a gentle N2 flow, and lipid films were obtained, which were further dried by vacuum pumping for at least 8 h. These films were hydrated (with either warm water or TFD solution), and the mixture was vortexed to obtain a suspension of multilamellar vesicles. Eventually, the suspension was extruded ten times at 40 C through a polycarbonate membrane (pore size = 100 nm) to obtain SUVs.

In this protocol, 12-bis-THA was not in the initial dry film, but it was incorporated in pre-extruded liposomes as follows. POPC, DOPE and DPePC were weighed in a 63/27/(10-x) wt% ratio, where x represents the 12-bis-THA %wt to be added in the second step. Indeed, as in the previous case, different DPePC:12-bis-THA ratios were employed (the sum of the two being always 10 wt% of the mixture). The three lipids were dissolved in chloroform and thoroughly mixed; after vacuum-pumping to remove chloroform, and addition of either warm water or TFD aqueous solution, the suspension was extruded ten times at 40 °C through a polycarbonate membrane (pore size = 100 nm) to obtain SUVs. Separately, the bolaamphiphile was dissolved in methanol, and a dry film of it was obtained by evaporating the solvent under a light N2 flux followed by vacuum-pumping. The appropriate amount of preextruded liposomal dispersion was poured on top of such 2-bis- THA dry film, then the sample was vortexed and kept in orbital stirring for about 10 h, in order to allow incorporation of 12-bis- THA onto the liposomal surface.

The concentration of 12-bis-THA in the liposomes was determined by HPLC analysis, using a Zorbax Eclipse XDB-C18 column (150 x 4.6 mm, 5 mm). The chromatography was carried out in isocratic conditions at a flow rate of 1 mL/min. KH2PO4 (20 mM)/acetonitrile/triethylamine (60:40:0.5 v/v/v) at pH = 3.8 was used as mobile phase. The detection of 12-bis-THA was performed by means of its UV absorption at k = 254 nm.

The final concentration of the TFD in the liposomes, as well as its distribution in the bulk, in the lumen, and bound to the bilayer, was assessed by fluorescence spectroscopy, using the Quant-iTTM OliGreen DNA-staining dye (Life technologies). Indeed, this dye undergoes a dramatic enhancement of its fluorescence when bound to DNA. Samples were inserted in quartz cuvettes and the fluorescence spectra were recorded in the 500–700 nm range on a LS50B spectrofluorimeter (Perkin-Elmer, Italy) (kex = 480 nm; ex/em slits = 10 nm). Following the OliGreen protocol (available at the supplier's website), an intensity vs. [TFD] standard curve was obtained by plotting the maximum of fluorescence emission (kem = 520 nm) as a function of the TFD concentration. Five standard solutions were prepared, containing 0, 10, 100, 500, and 1000 ng/mL TFD respectively, while the concentration of the dye was always a 200-fold dilution of the stock reagent, as indicated by the supplier. A mixing time of exactly 3 min was observed, taking care of not exposing the sample to the light, after which three fluorescence spectra were acquired and averaged. Intensity values at 520 nm

were corrected for the signal enhancement due to the interaction between OliGreen and 12-bis-THA. Appropriate standard curves were obtained taking into account the effect of Triton X-100 and sodium taurocholate, respectively, to be used for TFD quantification in the samples where either of such reagents was added.

The total TFD concentration ([TFD]tot) in the finished liposomes was determined by diluting the dispersion in a 30 mM sodium taurocholate solution, so that the TFD concentration would fit in the range of the standard curve (an appropriate dilution factor was therefore calculated for each sample depending on its theoretical [TFD]). Sodium taurocholate disrupts the liposomes and breaks the bond between 12-bis-THA and the TFD. In this way, we could retrieve the total amount of TFD, which could differ from the theoretical one if, for example, part of the sample was lost in the extrusion process. The unbound TFD ([TFD]free) was determined by mixing the standard OliGreen dye with the whole liposomal dispersion, simply diluted according to the standard curve requirements. In this situation, OliGreen cannot react with the TFD contained in the liposomal cores or strongly bound to 12-bis-THA in the bilayer. The unavailable TFD is usually also likely to be protected from degradation in serum; for this reason, we termed it ''encapsulated TFD". We calculated this simply by: [TFD]encaps = [TFD]tot – [TFD]free. The determination of the TFD inside the lumen was achieved by liberating the entrapped oligonucleotide. For this scope, the lipid bilayers were disrupted via the addition of Triton X-100 (1 wt%). In this scenario, the fluorescent dye reacts with the unbound TFD plus the TFD released from the cores with Triton X-100, but not with the TFD still bound to 12-bis-THA. Therefore, we calculated [TFD]lumen = [TFD]liberated – [TFD]free.

Circular Dichroism (CD) measurements were performed using a Jasco J-715 spectropolarimeter. The solutions were contained in quartz cells with optical path lengths of 1 cm, 0.5 cm, or 1 mm depending on the sample. CD spectra were recorded at room temperature in the 200–400 nm range.

Dynamic light scattering (DLS) analysis was performed using a Brookhaven BI9000-AT digital autocorrelator, equipped with a green laser (k = 532 nm; Torus, mpc3000, LaserQuantum, UK) and an APD detector placed at 90°. The samples, contained in glass test-tubes, were immersed in a thermostatic cell and the temperature was kept fixed at 25 C. In a Dynamic Light Scattering (DLS) experiment, we exploit the time correlation function of the random fluctuations in scattered intensity due to the particles' Brownian motion in solution [24, 25]. By fitting the autocorrelation function with the appropriate algorithms, one can derive the diffusion coefficient of the particles and, by assuming a spherical shape, their average hydrodynamic diameter (DH) through the Stokes-Einstein law

Fluorescence correlation spectroscopy (FCS) measurements were performed with a Laser Scanning Confocal

Microscope Leica TCS SP2 (Leica Microsystems GmbH, Wetzlar, Germany) with a ISS module (ISS, Inc.1602 Newton Drive Champaign, IL, USA) equipped with two APD (Avalanche Photodiodes) with 500–530 nm and

607–683 nm BP and with a 63 x water immersion objective. An aqueous solution of Alexa 488-fluorescently-labeled TFD (3.3 x 10-3 mg/mL) in the absence and in the presence of POPC/DOPE liposomes (1.3 mg/mL) and 12-bis-THA (4.0 x 10-2 mg/mL) was placed in the measurement chambers (Lab-Tek Chambered # 1.0 Borosilicate Coverglass System, Nalge Nunc International, Rochester, NY, USA) to a final 300 lL volume. The fluorescently-labeled TFD was excited with the 488 nm line of an Argon laser, and its fluorescence emission was acquired between 500 nm and 530 nm. In FCS, the autocorrelation function of the fluorescence intensity G(τ) is calculated from the fluctuations of the signal around its average value,δ (t) = I(t) - <I (t)> , as [26,27]:

$$G(\tau) = \frac{\delta I(t)\delta I(t+\tau)}{\langle I(t)\rangle^2}$$

The models employed for the analysis of the autocorrelation functions (ACFs) take into account the shape and the exact size of the detection volume. This is approximated as 3D-ellipsoidal Gaussian shape with axial (z0) and lateral (w0) defining parameters, determined through calibration procedure carried out with a reference fluorescent dye having a known diffusion coefficient (we employed a standard solution of Rhodamine 110, D = 430 mm2 s-1 in water at 25 C). For a three-dimensional Brownian diffusion mode in a 3D Gaussian volume shape, the ACFs profiles can be analysed according to the following equation:

$$G(\tau) = \frac{1}{\langle c\rangle \pi^{3/2} w_0 z_0} \sum_i f_i \left(1 + \frac{4D_i\tau}{w_0^2}\right)^{-1} \left(1 + \frac{4D_i\tau}{z_0^2}\right)^{-1/2}$$

Small-angle x-ray scattering (SAXS) experiments were conducted at the Austrian SAXS beamline (Elettra Synchrotron, Trieste, Italy). The samples were enclosed in a steel cell containing a 1 mm thick quartz Mark capillary. Samples were irradiated with a CuKa X-ray beam at an 8 keV energy, with typical irradiation times of 30 s. All scattering patterns were acquired at room temperature, on a Mar300-image-plate detector (MarResearch, Norderstedt, Germany). For each sample, 3 spectra were acquired and averaged. The sample-to-detector distance was 1308 mm, leading to a Q-range of 0.0067–0.46 Å-1. Data reduction and background subtraction were carried out with the software IGOR Pro (Wavemetrics, Inc.) [28]. Data modelling was performed with the software GAP (by Prof. Georg Pabst, Graz University, Austria) [29]. The experimental intensity of unilamellar vesicles was modelled on the basis a pure form factor function [30]:

$$F(Q) = \sqrt{2\pi}\left[2\sigma_H \exp\left(\frac{-\sigma_H^2 Q^2}{2}\right)\cos(Qz_H) + \sigma_C \tilde{\rho} \exp\left(\frac{-\sigma_C^2 Q^2}{2}\right)\right]$$

which is the Fourier transform of the electron density profile of the lipid bilayer. The electron density profile was modeled using three Gaussian profiles [30]: two Gaussians were used to represent the headgroups,

centered at zH and of width rH, while one Gaussian of width rC represented the terminal methyl group at the center of the bilayer. The respective electron densities were qH and ~q, with qH = 1. For the mixtures of oligolamellar and unilamellar vesicles, the model used to fit the experimental intensities was a linear combination weighted on the fraction Ndiff of positionally uncorrelated bilayers and the fraction of interacting bilayers, for which an interlamellar structure factor S(Q) arised:

$$I(Q) = \frac{(1 - N_{diff})S(Q)P(Q) + N_{diff}P(Q)}{Q^2}$$

Here, P(Q) is the square of F(Q) (Eq. (6)). The structure factor used was the one obtained with the Modified Caillé Theory [31].

The colloidal stability of liposomes was assessed in four saline media: RPMI 1640 (with sodium bicarbonate, without L-glutamine; purchased by Sigma Aldrich), LB Broth (2.5% solution, Miller's modification; purchased by Sigma Aldrich), sodium chloride 150 mM, and sodium citrate 150 mM. Stability assays were carried out by diluting the aqueous liposome dispersions 10x in each medium and incubating the samples in the dark at room temperature. The formation of a precipitate was verified through naked-eye observation after 24 h.

RESULTS AND DISCUSSION

Nucleotide molecules interact with cationic liposomes through an ensemble of electrostatic and hydrophobic forces and particularly via the association between the negatively charged phosphate backbone and the positively charged lipid headgroups [32]. Therefore, a possible strategy to maximize the encapsulation of oligo- or polynucleotides into cationic liposomes is to hydrate the lipid film directly with the solution of the DNA or RNA of interest [33], where charge compensation would be the driving force for association. Indeed, let us consider briefly what would happen in the absence of such positive/negative charge interaction by taking a typical dry film composed of zwitterionic lipids as an example. Upon hydration with a nucleotide solution, vortexing, and extrusion, the nucleotide would distribute in the dispersion (inside and outside the liposomes) on a purely statistical basis. The total volume of solution enclosed by the vesicles, i.e. the lumen, is easily calculated via some geometrical considerations. For a typical 4 mg/mL liposomal dispersion, assuming monodisperse lipid vesicles with diameters of 100 nm, an average lipid molecular weight of 750 g/mol, and the average molecular area 0.5 nm2 [34,35], this would result in a total lumen volume of 1.3%. This means that hydrating e.g. a phosphatidylcholine dry film with a TFD solution, in the absence of specific interactions, would result in almost all the TFD being localized outside the vesicles. Conversely, when a cationic species is present in the initial dry film, as the latter is swollen with a TFD aqueous solution, the oligonucleotides interact with the positive headgroups in the forming bilayer. When working in large excess of cationic species with respect to TFDs, all of the oligonucleotide is expected to associate with the lipid

bilayer. As bilayers close up into vesicles, assuming an identical partition of the cationic lipid in the inner and outer leaflets, the theoretical amount of TFD included in the lumen should therefore be roughly 50%. The remaining TFD would be associated with the cationic headgroups exposed on the vesicle surface. In this situation, the ‘‘inner TFD” is most likely screened from recognition as exogenous DNA by nuclease enzymes, while the ‘‘outer TFD” is at risk of being attacked and undergo rapid degradation in serum [33]. An effective way to avoid nuclease attack is to protect the ‘‘outer TFD” in a condensed form, which is usually obtained upon complexation by cationic agents [36]. With these grounds, we designed a liposomal carrier that would encapsulate a maximum amount of biologically active oligonucleotide and simultaneously protect it in serum. We prepared lipid films from a mixture composed of POPC (1-palmitoyl-2-oleoyl-snglycero- 3-phosphocholine) and DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) as zwitterionic lipids, containing a 10%wt of cationic lipids. Weight ratios were then: POPC/DOPE/(cationic species) = 63/27/10%wt, where ‘‘cationic species” is a mixture of the biocompatible DPePC (1,2-dipalmitoyl-sn-glycero-O-ethyl-3- phosphocholine, chloride salt) and the bolaamphiphile 12-bis- THA ((1,10-(dodecane-1,12-diyl)-bis-(9-amino-1,2,3,4-tetrahydroa cridinium) chloride). The corresponding average molar ratios between zwitterionic and cationic lipids (vz/c) were vz/c 9. For simplicity, it is worth noting that the molecular weights of these four species are similar, so that weight ratios can be taken as representatives of the mole ratios for the scopes of this work. The bi-cationic bolaamphiphile 12-bis-THA demonstrated, in previous studies, (i) a high affinity for lipid bilayers [12] and (ii) the ability to efficiently condense the TFD [5]. Moreover, a study on the interaction of 12-bis-THA/TFD nanoplexes with model membranes based on cardiolipin [23], an anionic lipid abundant in bacterial membranes [37], suggested a specific targeting action of 12-bis-THA towards the bacterial cell wall. In this picture, the role of 12-bis-THA appears fundamental to ensure both TFD protectionand active targeting. We proceeded via the preparation of a POPC/DOPE/DPePC scaffold and we investigated two different protocols to obtain the incorporation of 12-bis-THA. The bolaamphiphile could be added either by direct mixing in the initial lipid mixture, or by decoration of the surface of pre-formed liposomes. For each protocol, three samples were prepared by varying the weight ratio of DPePCand 12-bis-THA, namely 2:1, 5:1, and 10:1. In all cases, the dry lipid films were hydrated with an aqueous TFD solution, where the oligonucleotide content was such as to determine the following positive/negative charge ratios: Z+/- = 18, 30, and 46 (respectively, for the 2:1, 5:1, and 10:1 samples).The dispersions, once extruded through membranes with 100 nm pores, appeared translucent and stable, without precipitate or signs of phase separation, thus they were analysed by means of f-potential measurements and dynamic light scattering. The hydrodynamic diameters of liposomes were obtained by cumulant analysis of the autocorrelation functions [38], which allowed to extract the diffusion coefficients of the dispersed particles; these were then converted into sizes by the Stokes-Einstein equation

The results are shown in Figure 1: graphs a and b pertain to the samples obtained by co-extrusion, while graphs c and d relate to the surface-decorated liposomes (analysed after the incorporation of 12-bis-THA).

For each composition, the plots show the dependency of the size and the f-potential on the (DPePC+12-bis-THA)/TFD charge ratio Z+/-. For comparison, the data obtained for liposomes containing only DPePC as the cationic species are also presented; notice that the same DPePC sample appears in both sets of graphs, as the two different preparation methods refer to liposomes containing added 12-bis-THA. All liposomes, irrespective of the preparation method, had diameters ranging between 80 and 140 nm. Polydispersity indexes, obtained from the second order cumulant, ranged between 0.08 and 0.18 (data not shown), consistent with relatively narrow size distributions. Liposomes prepared by co-extrusion presented in general smaller sizes than those obtained by surface decoration, suggesting that 12-bis-THA may decrease the elasticity of the lipid membrane. This hypothesis is corroborated by the fact that the extrusion process for this set of samples was particularly slow, and higher extrusion pressures were required, compared to the case where 12-bis-THA was not present in the initial lipid mixture [39]. f-potentials were positive for all samples, as expected for liposomes containing 10%wt of cationic species. The diameters of co-extruded liposomes seemed correlate with the weight ratio of the cationic amphiphiles, as a higher DPePC content led to larger vesicles; surprisingly, f-potential values followed a counterintuitive trend, becoming less positive with increasing Z+/-. On the other hand, both size and f-potential of the decorated vesicles correlated with Z+/-, with the largest diameter corresponding to the lowest f-potential and Z+/- values, as expected according to the DLVO theory for charged colloids [40]. In order to gain better insight on the structural features originating such differences between the two sets of samples, we undertook a deeper investigation of the systems.

In order to understand whether all of the oligonucleotide introduced in the samples was retained after the extrusion process, the effective TFD content of each formulation was evaluated with the DNA-staining fluorescent probe OliGreen. The method was first validated by performing the assay on the neat 12-bis-THA/TFD nanoplexes in water. First of all, a standard curve was obtained by reacting growing concentrations of the pure TFD with OliGreen. Then, the fluorescence intensity was measured in a dispersion of the nanoplexes with Z+/- = 10; the intensity value, corrected by the signal enhancement due to 12-bis-THA interference, corresponded to 3 ± 0.5% of the theoretical TFD amount in solution, showing that when the TFD is complexed by 12-bis- THA, it is unavailable for reaction with the fluorescent dye. This behaviour is indicative of the strong association between 12-bis- THA and the oligonucleotide, and it has been reported for a similar system involving dequalinium, plasmid DNA, and the DNA-staining dyes ethidium bromide and SYBR Green [6]. Nevertheless, we know from previous work that sodium taurocholate (NaTC) disrupts the nanoplexes, liberating the TFD in its renatured form [5]. Therefore, after the addition of NaTC 30 mM to the system,

the fluorescence assay yielded 100 ± 10% of the expected fluorescence intensity. We also verified, by means of DLS, that the detergency properties of NaTC could provide the destruction of the lipid vesicles (data not shown). In light of these results, the total TFD concentration in each of the liposomal formulations previously

described was determined by performing the OliGreen assay after introduction of 30 mM NaTC, to disrupt both the SUVs and the 12-bis-THA/TFD complexes. The results are shown in Fig. 2 as histograms, representing the experimentally obtained TFD concentration as a percentage with respect to the theoretical amount. The data concerning liposomes obtained by co-extrusion show a clear trend: the highest TFD recovery was 55 ± 5% of the initial oligonucleotide in the 10:1 sample, while for the other formulations the recovery percentages were even lower. This clearly hints at the loss of oligonucleotide in the extrusion process, as experienced in previous work [12]. These samples were analysed by HPLC to quantify the amount of 12-bis-THA present after extrusion, revealing that it was 80%, 67%, and 79% (respectively for the 2:1, 5:1, and 10:1 samples; error bar = ±2%) of the initial molar concentration. The amounts of missing TFD and 12-bis-THA do not seem to correlate directly. However, we could hypothesize the formation of a large and/or rigid complex between 12-bis-THA and the TFD (and maybe part of the lipids), the stoichiometry of which is at the moment unknown, that adsorbs on the polycarbonate filters used for extrusion. As a consequence, the theoretical Z+/- values in the plots of Fig. 1a and b do not correspond to the real Z+/- of the liposomes. Considering the actual amounts of recovered TFD and 12-bis-THA for each formulation and assuming that no DPePC is lost, we can estimate that the real Z+/- values should follow the order (10:1) < (5:1) < (2:1), which is well in agreement with the trend of the f-potentials in Fig. 1b and, accordingly, with the hydrodynamic sizes in Fig. 1a. In the surface-decorated liposomes, the OliGreen assay (Figure 2) showed a complete recovery of the initial oligonucleotide amount. As expected, no TFD was lost since 12-bis-THA was not present in the initial lipid film. The surface decoration protocol is thus to be preferred, as it achieves both the complete incorporation of 12-bis-THA (confirmed by HPLC analysis yielding 100% ± 2% recovery for each sample) and the efficient loading of the TFD in the liposomes. Since 12-bis-THA is added to the pre-formed liposomes, andthese do not undergo further purification, one could wonder whether any free 12-bis-THA/TFD nanoplexes are formed by a hypothetical preferential interaction of the bolaamphiphile with the possible unbound TFD in solution. In order to rule out this possibility, we performed a fluorescence correlation spectroscopy (FCS) study of the systems. Our aim was to verify if, in the presence of DPePC liposomes and 12-bis-THA, a competition between the interaction of the TFD with the cationic liposomes and the – possibly free – cationic bolaamphiphile is present. A FCS experiment was performed on the diffusion of TFD in a sample where POPC/DOPE liposomes and 12-bis-THA are present in a 30:1 mol ratio. This is a limiting case, where no TFD binding competition can be reasonably expected by the zwitterionic liposomes. The highest affinity of the TFD should be for 12-bis-THA and the formation of nanoplexes. In Figura 3a, a control FCS curve is displayed for the TFD freely diffusing in aqueous solution. The curve can be analysed according to a two-components diffusion model (Eq. (4)), consisting of a fast diffusion component (D = 370 lm2 s−1), due to the presence of a residual percentage of Alexa-488 free dye (unbound to the TFD), and a slower diffusion component (D = 60 ± 20 lm2 s-1) consistent with the free diffusing TFD [41–43]. In Fig. 3b a representative FCS curve of the TFD in the presence of POPC/DOPE liposomes and 12-bis-THA is displayed. From the fitting

it results that all the TFD is bound to objects with a diffusion coefficient of D = 3.4 ± 0.2 lm2 s-1. According to the Stokes-Einstein equation (Eq. (3)), this yields a hydrodynamic diameter of 128 ± 8 nm, consistent with the size of liposomes, but smaller than the size of nanoplexes (180 nm [23]). Thus, even in a condition where theinteraction of 12-bis-THA with the TFD is highly favoured, we demonstrate that a single self-assembly of liposomes/TFD/12-bis-THA is formed, without the presence of free nanoplexes in solution.

Besides determining the amount of total recovered TFD, the Oli-Green method was used to quantify the oligonucleotide in the different compartments of the liposomes obtained by surface decoration. Indeed, in one such dispersion, the TFD can be found: (i) freely dispersed in the bulk solution, (ii) weakly adsorbed at the outer leaflet headgroups (not condensed), however this is unlikely for high Z+/- values, (iii) strongly adsorbed and condensed by 12-bis-THA at the outer lipid leaflet, and (iv) contained in the aqueous lumen. Among these, TFD types (i) and (ii) are usually not protected by nuclease attack, types (iii) and (iv) on the contrary are likely to be protected. The procedure designed to quantify the oligonucleotide in the different liposomal compartments is described in the following and presented in Figure 4 along with the results, which are expressed as the percentage of TFD amount with respect to the theoretical concentration. (a) Reacting the OliGreen dye with the liposomal dispersion as such yielded the unbound TFD concentration (i.e. the sum of TFD types (i) and (ii) described above), as the fluorophore can only ‘‘see” the non-condensed TFD. (b) Next, the lipid bilayers were disrupted via the addition of Triton X-100 (1 wt%), which can solubilize lipid membranes but, as a non-ionic detergent, is unable to displace the TFD/12-bis-THA complex. In this way, the OliGreen dye could react with the oligonucleotide released from the cores plus the TFD that was already free. The net lumen [TFD] was easily determined by difference with respect to the unbound TFD. (c) Finally, the addition of 30 mM NaTC disrupted both the lipid bilayers and the TFD/12-bis-THA complexes, rendering all the TFD in the systems available for reaction with the dye (total TFD, see also Figure 2): the difference between this amount and the unbound TFD yielded the encapsulated TFD (i.e., the sum of types (iii) and (iv)). As displayed by the histogram plot in Fig. 4, the unbound TFD was around 10% of the initial amount regardless of the DPePC:12-bis-THA ratio. Circa 50% was contained in the liposomal cores, in agreement with the expected partitioning of the TFD between inner and outer lipid leaflets, as we assumed earlier. Most importantly, 90% of the oligonucleotide introduced is encapsulated, i.e. either contained in the lumen or condensed by 12-bis-THA headgroups at the bilayer. The TFD thereby encapsulated is very likely to be protected from DNA-degrading agents. In order to determine whether the positive/negative charge ratio could affect the vesicle structure or the distribution of the TFD in the different liposomal compartments, we varied the Z+/- values in the formulation containing the least amount of bolaamphiphile, i.e. the sample with DPePC:12-bis-THA = 10:1, as this is ideally the formulation presenting the lowest toxicity. These experiments also allowed us to assess the TFD loading capacity of the liposomes, which is another key parameter in drug formulation. Therefore, Z+/- was varied between 23 and 2.3, corresponding to TFD concentrations of 7 mg/mL, 18 mg/mL, 35 mg/mL, and 70 mg/ mL. The last sample presents Z+/- = 2.3 which is close to overall

neutrality and corresponds an excess negative charge with respect to 12-bis-THA: this means that part of the TFD may not be efficiently encapsulated. For all such systems, the determinations of size, f-potential and TFD distribution were carried out as described previously, and the results are summarized in Figure 5. As expected, f-potentials were positive but decreased with increasing TFD content, while the hydrodynamic diameters of the liposomes increased due to the swelling induced by the growing TFD concentration. The fluorescence assay yielded over 90% protected TFD even in the Z+/- = 2.3 liposomes. Therefore, the investigated system can encapsulate at least 60 mg/mL TFD. These results, combined with the data discussed earlier, reinforce the idea that the structure of the vesicles depends on the overall Z+/- ratio and not on the DPePC:12-bis-THA ratio. These systems were tested for colloidal stability by means of light scattering intensity readings, carried out over one week after the preparation. As shown in Fig. 6, no meaningful variations in these liposomal dispersions were noticed, except for the one with Z+/- = 2.3 which registered a decrease in scattered light intensity, indicating precipitation of the largest objects in suspension. This suggests that loading more than 60 mg/mL TFD will probably lead to an important destabilization of the system, and this TFD concentration represents the maximum encapsulation capacity of these liposomes. Finally, we assessed the condensation of the TFD by 12-bis-THA through circular dichroism (CD) spectroscopy analysis of the DPePC:12-bis-THA = 2:1 liposomes, prepared in a water/sucrose 50/50 wt% solution to match the lipids' refraction index. Here, the 2:1 sample was chosen because it has the highest TFD content among those we investigated, which gives better chances of obtaining a good compromise between CD signal intensity and light absorbance by 12-bis-THA. The spectrum, normalized by TFD concentration and optical path length, is presented in Figure 7a, where it is compared to the spectra obtained for POPC/DOPE/DPePC/TFD liposomes and for the free TFD. The pertaining UV–vis absorbance spectra are shown in Figure 7b. The CD spectrum of the liposomes without bolaamphiphile is perfectly superimposed to that of the neat TFD, presenting the typical bands of DNA in B-conformation [5,44]. The difference occurring in correspondence of the positive band at k 220 nm is not related to the supramolecular structure of the oligonucleotide, but it most likely originates from optical effects depending on sucrose or scattering from liposomes. This demonstrates that DPePC alone is unable to condense this oligonucleotide at the used concentrations. The CD signal of the TFD is still present in the liposomes containing 12-bis-THA, but the intensity of the bands is about 40–50% lower than in the other two samples. This is explained by the presence of the non-condensed TFD inside the lumen, which is indeed about half the total TFD as discussed earlier (see Fig. 4). Therefore, in the present systems we can modulate the structure and the properties of the formulations by tuning the concentrations of oligonucleotide and of the biocompatible cationic lipid DPePC, while the amount of 12-bis-THA can be kept to the minimum necessary to ensure TFD encapsulation and protection against nucleases.

The bilayer structure of liposomes with DPePC:12-bis-THA = 2:1 prepared in typical saline buffers was probed through SAXS experiments. The 2:1 sample was chosen as it contains the highest amount of bolaamphiphile

and is therefore likely to present the most marked, if any, structural modifications. Samples were prepared by hydrating the dry films with the following TFD solutions: NaCl 150 mM, sodium citrate (Na3Cit) 150 mM, and PBS buffer 10 mM. In each case the TFD concentration was 10 mg/mL, such that Z+/- = 19. Liposomes extruded in PBS buffer precipitated shortly after extrusion, therefore this system was not studied by SAXS. Fig. 8 displays the scattering curves acquired for liposomes in water, in NaCl and in Na3Cit, as well as the model fit curves obtained with the software GAP [29] (kindly provided by Prof. Georg Pabst, University of Graz, Austria). For the liposomes in pure water, the SAXS pattern was consistent with unilamellar vesicles and could be treated as arising from pure diffuse scattering, i.e. I(Q) = P(Q)/Q2, where P(Q) is the squareof the form factor F(Q) (Eq. (6), Materials and Methods). For the liposomes in NaCl and Na3Cit, the scattering patterns showed quasi-Bragg peaks arising from interactions between adjacent bilayers, as is typical with oligolamellar vesicles. These however coexisted with unilamellar vesicles in suspension, which was more evident in Na3Cit solution than in NaCl. Such effect is related to the higher ionic strength in the citrate buffer, since this property scales with ion valence [45] and citrate exists as a mixture of doubly and triply negative species at pH close to neutrality [46]. This determines a more efficient electrostatic screening of the headgroup compared to a NaCl solution at the same molarity, thereby reducing more efficiently the repulsion between adjacent bilayers and promoting the formation of oligolamellar vesicles [47]. In both cases, the function used to fit the experimental scattering intensities was a linear combination of the two models (for noninteracting and for interacting bilayers) weighted on the relative amounts of the two types of vesicles (Eq. (7)). The results of data modelling are summarized in Table 1. It is evident from Fig. 8 that the best fit function for Na3Cit liposomes is unable to match the experimental intensity over the entire Q-range. This issue probably arises from the fact that the model used cannot handle bilayer asymmetry, and this reflects in the higher standard deviations for the calculated structural properties; however, the quasi-Bragg maxima and the main minima are all correctly identified. We calculated that, for liposomes in NaCl and Na3Cit solutions, the unilamellar vesicles contribute for, respectively, 94% and 90% to the total scattering intensity, while the remaining contribution is due to bi-lamellar vesicles. This means that SUVs represent most of the vesicles in suspension and that the structural modifications due to the buffers are minimal. Data modelling allowed us to determine the center zH and the width rH of the Gaussians representing the headgroups, from which we calculated the bilayer thickness as:

$$d_B = 2(z_H + 2\sigma_H)$$

The results indicate a slight increase in thickness of the lipid bilayers in saline media, however the variation (of the order of 1 Å) is too small to be physically significant. The most important result here is that, despite an increased tendency towards the formation of oligolamellar structures, the bilayer of POPC/DOPE/ DPePC/12-bis-THA/TFD is unmodified. The liposomes are stable in saline media at physiological ionic

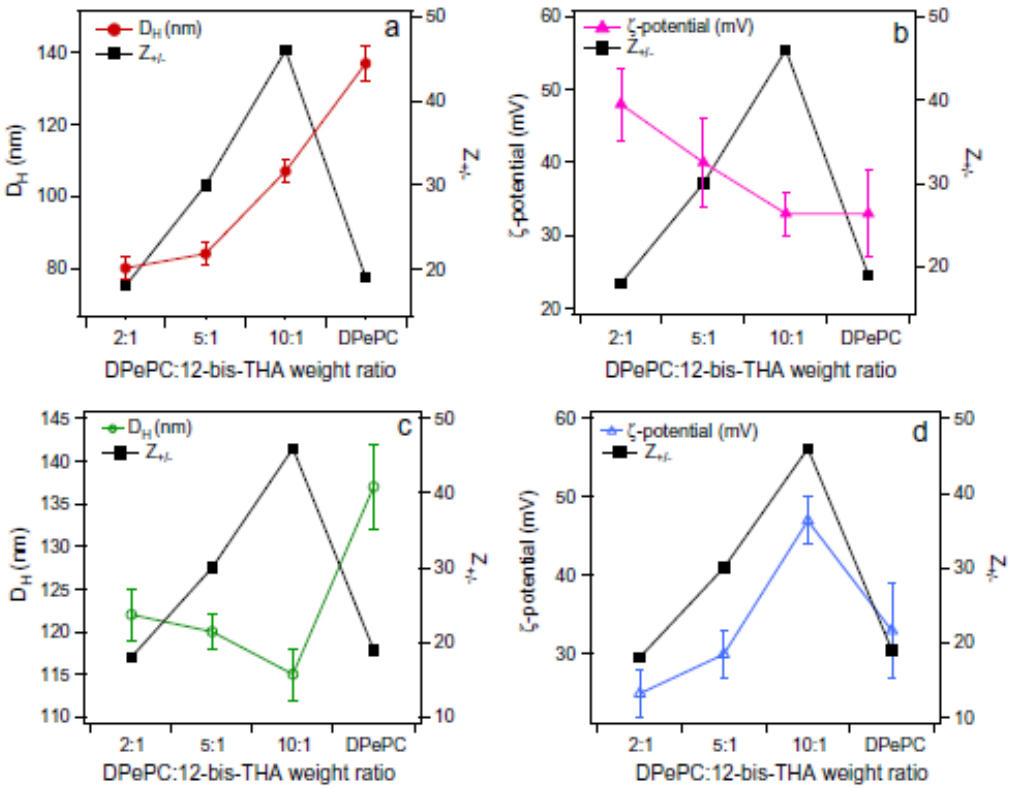
strength, and they are therefore suitable for biomedical applications. The colloidal stability of liposomes was monitored over time in different types of saline media. These tests are of paramount importance not only in view of a prospective final formulation (e.g. fluid for intravenous administration) and the stability in the patient's bodily fluids, but even to ensure the reliability of the common biological assays performed in vitro. For this reason, we chose two common cell culture media, RPMI 1640 (used in cytotoxicity tests on eukaryotic cells) and LB Broth (used to grow bacterial colonies for antibacterial activity assays), and two saline buffers, NaCl and sodium citrate (Na3Cit) at physiological ionic strength. Stability assays were carried out by naked-eye observation after diluting the aqueous samples 10x in each medium. This procedure well reproduces the protocol adopted for biological assays, albeit the latter are performed at much lower concentrations of liposomes (typically down to 0.01 mg/mL lipids, corresponding to a 500 fold dilution). The results relative to observations in the first 24 h are summarized in Table 2; no further variations of the systems were observed in the following days. The formulations were stable in all media except LB Broth, where the 2:1 liposomal dispersion precipitated and the others gave rise to an opalescent solution. These results once again support the suitability of the DPePC/12-bis-THAbased liposomes as stable oligonucleotide carriers. However, the precipitation of the 2:1 formulation in the medium used for antibacterial activity assays could discourage its further development, due to the poor reliability of said assay in these conditions, which also goes hand-in-hand with the possible increased toxicity due to the high 12-bis-THA content. In the case of the 5:1 and 10:1 formulations, the appearance of the opalescence took place slowly over the first 24 h, and it should not affect the antibacterial activity tests if these are carried out shortly after dilution of the formulations in the culture medium.

CONCLUSIONS

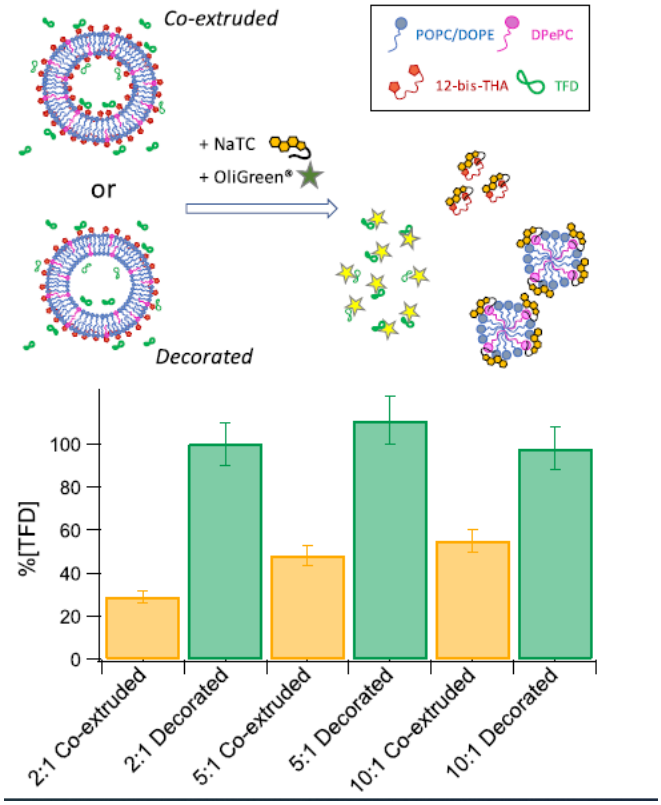
The growing number of antimicrobial-resistant bacterial strains faces us with a potentially dangerous health crisis [48]. Transcription factor decoys are a promising new class of therapeutics against resistant infections [4,23], but they pose the challenge of efficient drug formulation to preserve colloidal stability and transfection ability. By building on previously described experimental approaches [12,23], we designed a cationic liposomal carrier based on a zwitterionic lipid (POPC/DOPE) scaffold and the positively charged lipid DPePC. The latter was particularly chosen because, as demonstrated in several works, ethyl-phosphocholines endow liposomes and lipoplexes of a benign cytotoxic profile [20,21], as opposed to the known toxic side effects of formulations based on more commonly used cationic species such as DOTAP and DOTMA [11,21]. Furthermore, we exploited the cationic bolaamphiphile 12-bis-THA [5] to ensure TFD condensation and active targeting towards bacterial membranes [23]. The experiments here reported have shown that the most efficient way to obtain POPC/DOPE/DPePC/12-bis-THA liposomes is by decorating the surface of pre-extruded POPC/DOPE/DPePC vesicles with a small amount of 12-bis-THA. A structural study carried out by means of fluorescence and circular dichroism spectroscopies has allowed us to estimate the degree of encapsulation and the distribution of a model TFD at different locations in our liposomal carriers. We could

determine that these liposomes can encapsulate up to 60 mg/mL TFD of oligonucleotide, which is quite in line with the current literature concerning the colloidal carriers for transfection of DNA and RNA to eukaryotic cells [17,19,33,49]. Moreover, this concentration is even quite high if one considers that TFDs are effective at much lower concentrations than DNA or RNA, as a single TFD-transcription factor interaction can impede the transcription of a single gene [23]. Importantly, the TFD in our liposomes is encapsulated in a fashion that should efficiently protect it against serum nuclease digestion, i.e. either encapsulated inside the liposomal lumen or condensed in a (reversibly) denatured psi form in the headgroup region of the lipid bilayer. Dynamic light scattering and f-potential measurements have demonstrated that the liposomal vectors here obtained possess the optimal size and electrostatic charge characteristics to interact with bacterial cell membranes and deliver the oligonucleotide [12]. Importantly, the preparation of these liposomes in saline buffers and their investigation by visual inspection, dynamic light scattering and small-angle X-ray scattering, have demonstrated that they are stable and retain the normal bilayer structure even in media with high ionic strength, reminiscent of biological environments. Among the three samples investigated, the formulation containing the highest 12-bis-THA concentration was found unstable in LB Broth, a medium used to support bacterial cultures. This result, in addition to the possible toxicity induced by the bolaamphiphile, discourages further development of this formulation. For these reasons, the liposomes containing a 10:1 ratio between DPePC and 12-bis-THA should constitute the focus of future work. In this scenario, we have laid the basis for a promising oligonucleotide vector targeting bacteria responsible for resistant infections. Nevertheless, several further steps are to be taken, such as the surface engineering of the cationic liposomes in order to protect them from rapid clearing by the reticuloendothelial system, for example by the addition of a ‘‘stealth” PEG shell [50]. In this way, by building on the information acquired through the present investigation and previous data, we have the opportunity to move rationally and systematically towards a real pre-clinical formulation exploiting the innovative approach of TFDs against antimicrobial-resistant infectious diseases.

FIGURES



Figuire 1) Size and f-potential characterization of TFD-loaded POPC/DOPE/DPePC/12-bis-THA liposomes with variable amounts of 12-bis-THA. (a and b): prepared via the coextrusion method; (c and d): prepared by surface decoration. DH (nm) = hydrodynamic diameter; Z+/- = theoretical mlar ratio of positive (DPePC, 12-bis-THA) and negative(TFD) charges. The lines between markers are not fit curves but only a guide for the eye. Error bars represent the standard error on the average of 5 repeat measurements.



Figigure 2) Quantification of the total TFD content in liposomes obtained with the two proposed protocols, expressed as % of the theoretical concentration. Orange bars are relative to the co-extruded liposomes, while green bars are relative to the decorated liposomes. The ratios 2:1, 5:1, and 10:1 refer to the DPePC:12-bis-THA weight ratios. The drawing above the histogram is a pictorial representation of the experimental protocol.

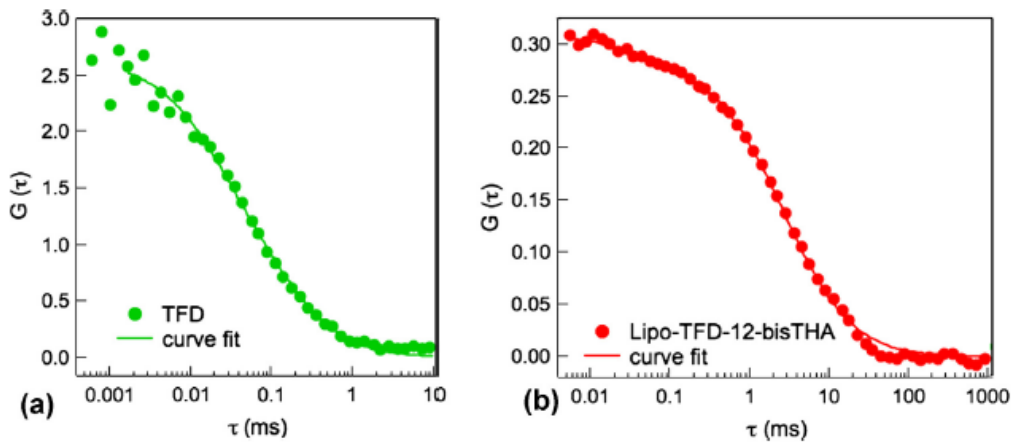


Figure 3) Representative FCS curves measured for (a) Alexa-488-labeled TFD and for (b) Alexa-488-labeled TFD incubated with POPC/DOPE liposomes and 12-bis-THA. The markers represent the experimental curves, while the continuous lines represent the model fits.

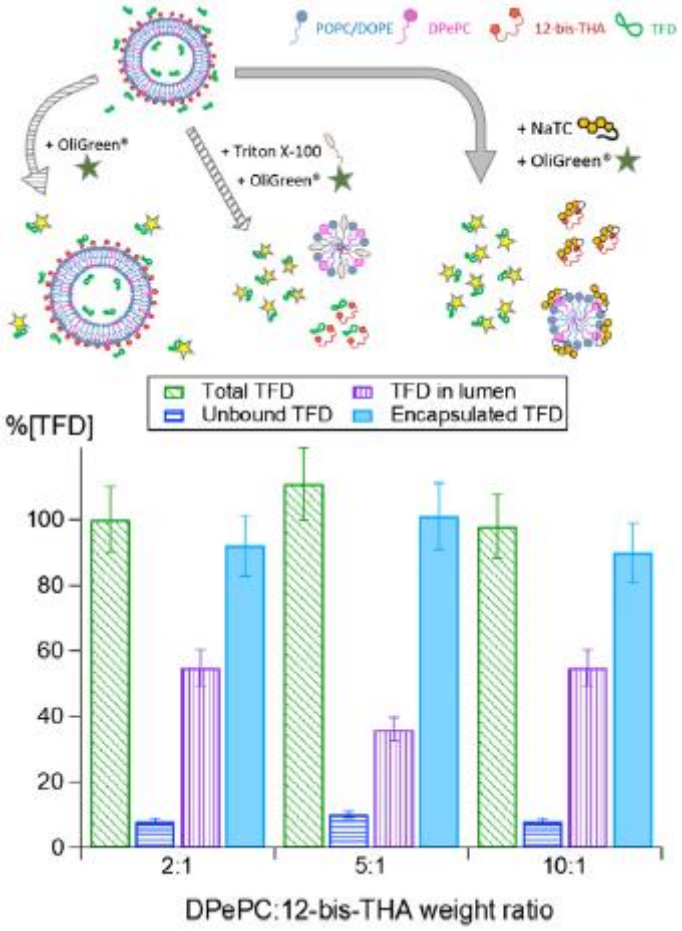


Figure 4) Quantification of the TFD concentration in the different compartments of liposomal dispersions, expressed as % of the theoretical concentration.

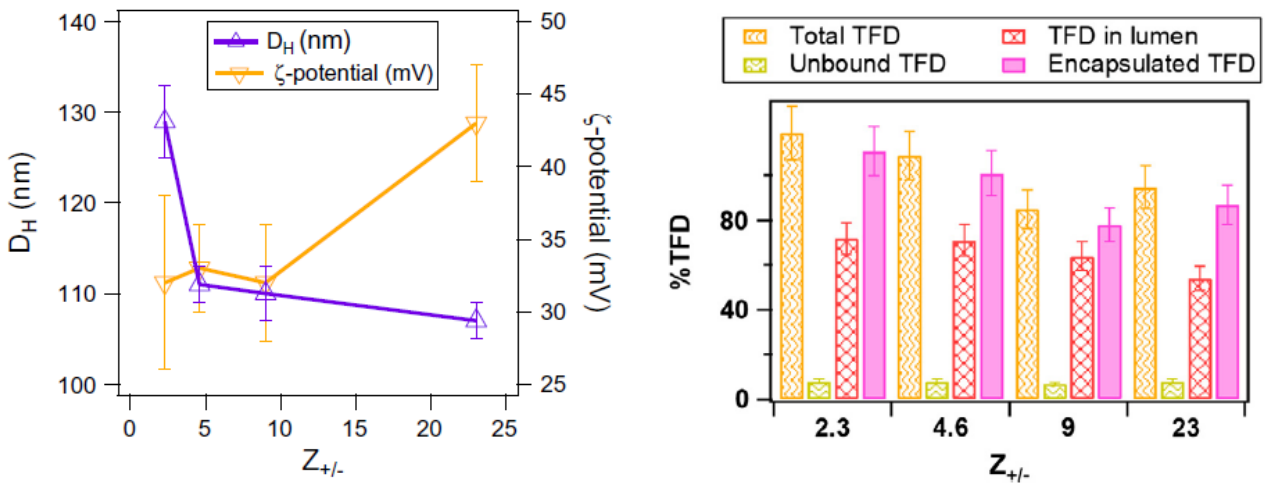


Figure 5) Physical-chemical characterization of POPC/DOPE/(DPePC:12-bis-THA = 10:1) liposomes with varying TFD concentration. Left: Hydrodynamic diameters (DH, nm) and f-potentials (mV). Right: Quantification and distribution of TFD.

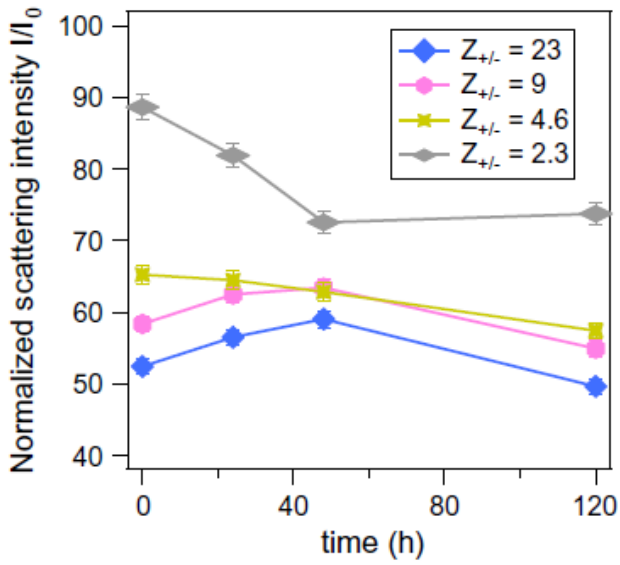


Figure 6) Trend of normalized light scattering intensity over time (120 h) for POPC/DOPE/(DPePC:12-bis-THA = 10:1)/TFD liposomes with increasing amounts of loaded TFD (=decreasing Z+/-). For each sample, the scattering intensity was normalized to that of pure toluene to account for laser fluctuations and detector efficiency variables.

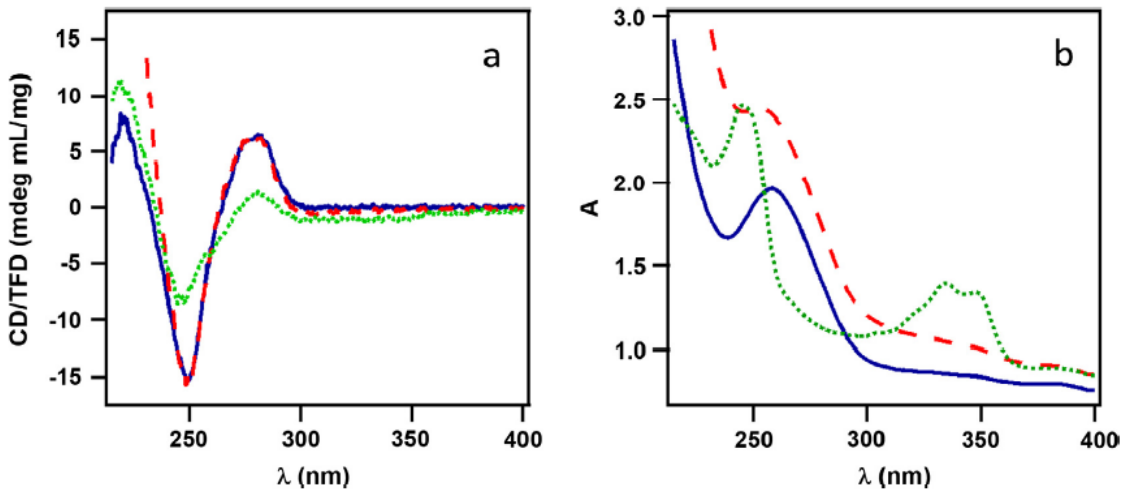


Figure 7) Normalized CD (a) and absorbance (b) spectra of: free TFD (blue solid line), POPC/DOPE/DPePC/TFD liposomes (red dashed line), and POPC/DOPE/(DPePC:12-bis-THA = 2:1)/TFD liposomes (green dotted line).

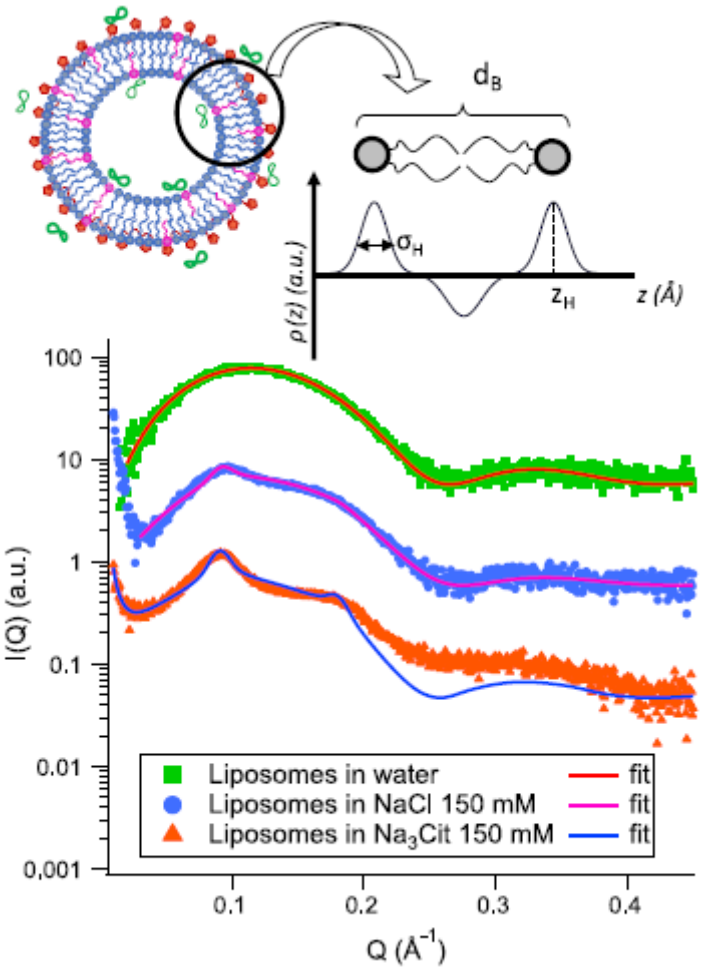


Figure 8) Small-angle X-ray scattering of POPC/DOPE/(DPePC:12-bis-THA = 2:1)/TFD liposomes in water and in saline buffers. Top: Scheme representing the region of the liposomal nanostructure probed by SAXS, a typical electron density profile, and the salient properties relative to the model used to fit the experimental data. Bottom: experimental SAXS curves and best fit function.

TABLES

	Ndiff	N. of lamellae	zH (Å)	rH (Å)	d (Å)	dB(Å)
Water	1*	1*	18.22(±0.02)	3*	-	48(±1)
NaCl	0.937(±0.002)	2	17.64(±0.90)	3.7(±0.7)	67.4(±0.2)	50(±1)
Na3Cit	0.895(±0.003)	2	19(±1)	3(±1)	69.2(±0.1)	50(±1)

Table 1) Results of SAXS data modelling (Fig. 8) and calculation of some structural properties. d (Å): lamellar spacing; zH (Å) and rH (Å): center and width, respectively, of the Gaussians representing the headgroups; Ndiff: ratio of uninteracting bilayers (i.e. ULVs); N. of lamellae: average number of interacting lamellae; dB (Å): bilayer thickness. * Constrained parameter during fitting.

DPePC:12-bis-THA	RPMI	LB broth	NaCl 150 mM	Na3Cit 150 mM
2:1	Isotropic	Precipitate	Isotropic	Isotropic
5:1	Isotropic	Opalescent	Isotropic	Isotropic
10:1	Isotropic	Opalescent	Isotropic	Isotropic

Table 2) Colloidal stability of liposomal samples in saline media (observations noted 24 h after preparation)