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COMMUNICATION

Cubosomes for ruthenium complex delivery: formulation and characterization†

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An amphiphilic ruthenium-based molecule (DOPURu) with potential antineoplastic activity has been synthesized, and its aggregation behavior in the presence of phospholipids has been investigated. A very rich variety of aggregates has been found, spanning from vesicles to cubic bicontinuous phases. Cubosomes here presented represent one of the first systems with potential use for medical therapy.

We present the first example of lipid-based bicontinuous cubic structures containing an amphiphilic NAMI-A-like ruthenium complex as potential antitumor agents. Cubic phases are recognized to be among the best candidates for *in vivo* drug delivery applications, protein crystallization matrices, and soft nanoporous materials,[1] and have great importance even in food science,[2] as well as in biology.[3] To date, practical applications of cubic phases, or of their dispersions, have been hampered because typical systems forming cubosomes are constituted of unsaturated monoglycerides (uMGs), as glycerol monooleate, that exhibit hemolytic properties at low to moderate concentrations *in vivo*.[4] Therefore, it is mandatory to find more biocompatible lipid compositions able to form bicontinuous cubic phases. Here, we propose phospholipid-based cubosomes, constituted of 1,2-dioleoyl-*sn-glycero*-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn-glycero*-3-phosphoethanolamine (DOPE), and a new synthesized amphiphilic ruthenium-based molecule, baptized DOPURu, whose basic structure is inspired by those of phospholipids. At the best of our knowledge this system represents one of few examples existing in the literature of biocompatible and pH-stable cubic phases.[5]

We have synthesized a new amphiphilic unimer, named DOPU, able to form stable complexes with ruthenium (DOPURu).[6] The molecular structure of this unimer in strictly inspired by that of NAMI-A that is recognized as one of the most promising drugs in oncology therapy. In fact, NAMI-A and KP1019 have already completed Phase I of clinical trials,[7] exhibiting high selectivity toward solid tumors' metastases and a lower toxicity if compared to the traditional platinum-based drugs.

Although a rather large number of studies on ruthenium complexes have been published in the recent years,[8–10] no example of nanovectors carrying ruthenium complexes for anticancer therapy is reported in the literature, except for a recent communication by some of us.[6] We showed the ability of DOPURu to form multilamellar vesicles in binary aqueous systems.[6] With the aim to improve the biocompatibility of the formulation as well as to modulate the quantity of the metal transported, the aggregation behavior of DOPURu has been now studied in the presence of DOPE and DOPC in equimolecular amount. Our research, carried out by means of a multi-technique approach (SANS, DLS, CryoTEM, EPR spectroscopy) has allowed showing the formation of a rich variety of aggregates. Among these, the formation of bicontinuous cubic liquid crystalline phases is of particular interest.[11]

The synthesis of DOPURu is schematically represented in Scheme 1, where the new unimer DOPU (**2**) has been obtained from the uridine derivative (**1**)[12] by straightforward introduction of a pyridine function on the uracile base. The hydrophobic part of the molecule consists of two oleoyl chains attached to the 2 and 3 positions of the sugar ring. In order to provide stealth characteristics to the supramolecular aggregates, a hydrophilic PEG-350 residue has been added at position 5.[13,14] The ligand unimer DOPU has been coordinated to Ru(III) by displacing a dimethyl sulfoxide (DMSO) molecule from the suitable precursor $[RuCl_4(DMSO)_2][H(DMSO)_2]$.[15] The reaction has been performed in a fully deuterated mixture of acetone and DMSO, by mixing DOPU and the metal complex in a 2:1 ratio. According to this stoichiometry, removal of the solvents in vacuum gives rise to the amphiphilic metal complex $[RuCl_4(DMSO)(DOPU)]^-$, accompanied by the protonated unimer $[(DOPU)H]^+$ as counterion. Coordination of the pyridine moiety has been revealed by the expected large high-field shift of its protons signal, which appears as a broad peak at −0.5 ppm in the ^1H NMR spectrum (Fig. S5†).[16] In the $[RuCl_4(DMSO)(DOPU)]^-$ complex the coordination of the paramagnetic metal ion Ru(III) presents a pseudo-octahedral structure with axial symmetry, as revealed by the EPR spectrum in chloroform (Fig. S4†).[6]

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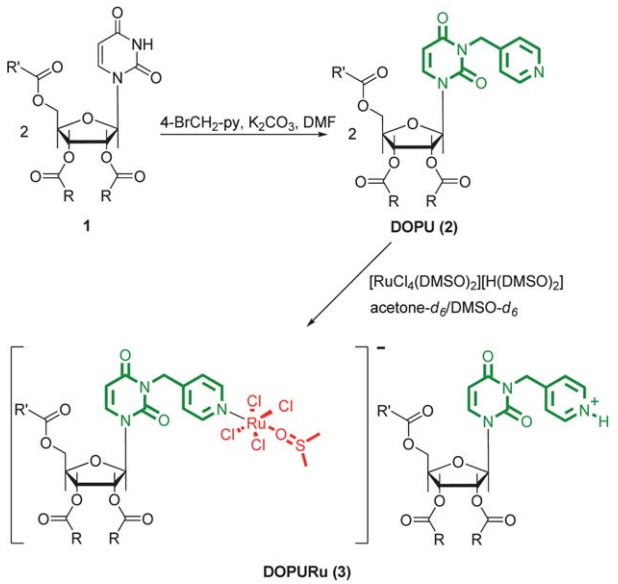

Scheme 1 Schematic synthesis of the new unimer DOPURu. In the scheme, R′ = $CH_3O(CH_2CH_2O)_8CH_2$–; R = *cis*-$CH_3(CH_2)_7CH{=}CH(CH_2)_8$–.

The (DOPC/DOPE)/DOPURu aggregates were prepared by the lipid thin film hydration method as detailed in the ESI†.

The DLS technique was used to characterize the hydrodynamic size of the aggregates (see Fig. S1 in the ESI†).[17–19] Diffusion coefficients along with hydrodynamic radius R_h obtained through the Stokes–Einstein equation have been collected in Table S1†. We found the presence of large aggregates formed by our new synthesized molecules, whose size is ranged between 70.0 nm of DOPURu structures and 130.0 nm of pure (DOPC/DOPE) supramolecular aggregates.

A clear picture of the morphology of the aggregates has been obtained through Cryo-TEM images. For (DOPC/DOPE)/DOPURu mixed systems at intermediate ratios (namely 60/40 and 50/50) the Cryo-TEM investigation revealed that these samples contain vesicles (uni- and multilamellar) and cubosome-like particles. Fig. 1 and S6† show the most representative Cryo-TEM images of the aggregates. The most striking feature of these samples is that ordered cubosome-like particles are present (see Fig. 1A–C).

The structure of cubosomes consists of a three-dimensional curved bicontinuous lipid bilayer, separating two congruent networks of water channels. These particles vary in size and sometimes seem to be intergrown with vesicles (see Fig. 1C). Some cubosomes have cubic morphology and in most cases the edges are more disordered compared to the interior of the aggregate. Fast Fourier transforms of selected areas of the cubosomes reveal the presence of the characteristic body-centered cubic phase belonging to the primitive *Im*3*m* space group (q^{229}) with a mean lattice parameter of $\cong 9 \div 11$ nm. In addition to the cubosome particles, multilamellar vesicles of different sizes are also present in this sample (see Fig. 1D,E). These latter ones have a diameter between 300 and 500 nm, although some very larger ones (~1700 nm) are also visible. In some multilamellar vesicles, cubic-like phases are present within the lamellar region (see Fig. 1F). Fast Fourier transforms of the lamellar regions yielded a repeat distance of around 4.3 nm (see Fig. 1 D,E, d and e). The fact that cubic-like phases are present within the lamellar phase of multilamellar vesicles (Fig. 1D and F) lends itself to the idea that the cubosomes are formed by reorganization of the lamellar phase. The unilamellar vesicles also present tend to be much smaller (>40 nm) than the multilamellar ones.

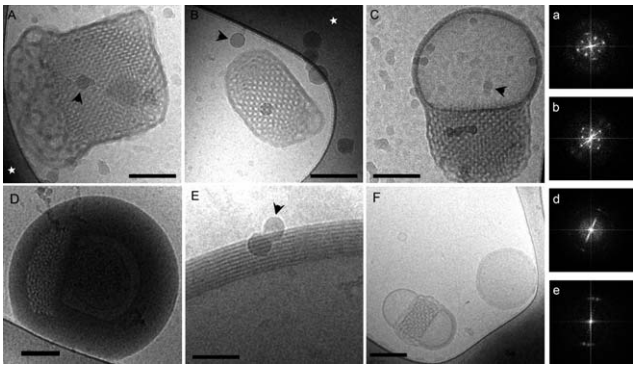

Fig. 1 Cryo-TEM images of cubosome-like particles (A–C and F) and multilamellar vesicles (D–F) present in the (DOPC/DOPE)/DOPURu/H_2O system at 60/40 and 50/50 phospholipids/Ru complex molar ratios. Selected area fast Fourier transform of A, B, D, and E can be seen in a, b, d, and e respectively. Scale bars in A, B, C, F are 100 nm, 150 nm in D and 50 nm in E. Note that the scale for a–e is not included. The spherical particles, indicated by black arrowheads, are frost, and the white stars indicate the lacey carbon support film, so these features are not part of the sample.

It is interesting to observe that, while for pure systems (*i.e.* (DOPC/DOPE)/H_2O and DOPURu/H_2O) only liposome aggregates are found, when the ratio of phospholipid/metal complex is around unity the presence of cubic phases occurs. In principle, this behavior could be rationalized in terms of the minimal surface exposed by the amphiphilic molecules toward the polar medium,[20] or more easily through a semi-qualitative analysis of the packing factor. As known, the formation of these structures is observed when the unimers have a critical packing parameter cpp ≥ 1.[21] Due to the synthesis, the DOPURu molecule is always coupled with the counter-cation displayed in Scheme 1 (step 3). This cation has a very similar structure to that of DOPURu and, because of the opposite charge, co-aggregates with this latter forming an ion pair complex. The resulting catanionic amphiphile presents a higher cpp parameter, because of the strict closeness of the two heads, and the subsequent partial overlap of the hydration cospheres. Modulation of the local curvature, achieved by the insertion of phospholipids, is then fundamental for the occurring of the bicontinuous phases.

In order to investigate the acyl chain micro-structuring in the bilayered structures, we incorporated 1% by weight of a phosphatidylcholine spin-labeled on the 5 C-atom of the *sn*-2 chain (5-PCSL) in the dispersions. The EPR spectra (see Table S3†) show the typical line shape of bilayered structures. Interestingly, no evident differences between samples in which only liposomes are formed and samples in which cubosomes are also present were found, suggesting a substantial similarity of the lipid bilayer forming both mesostructures.

The quantitative characterization of the structures has been obtained through SANS measurements. The scattering cross-sections $d\Sigma/d\Omega$ obtained as a function of the scattering vector modulus q are reported in Fig. S2† and 2. From the inspection of the figures it is quite straightforward to infer the presence of unilamellar vesicles for the system composed of DOPC/DOPE and (DOPC/DOPE)/DOPURu at a 70/30 molar ratio, where the power law $d\Sigma/d\Omega \propto q^{-2}$

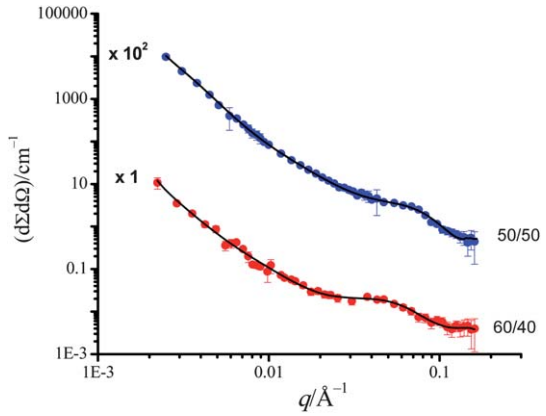

Fig. 2 Scattering cross-sections obtained at 25 °C for some aqueous dispersions (DOPC/DOPE)/DOPURu at different ratios between the total phosholipids and the metal complex, as indicated. Fitting curves to the experimental data through the models reported in the text are displayed. For a better comparison, data have been multiplied for a scale factor as displayed.

holds. Analysis of SANS data shows that (DOPC/DOPE) unilamellar vesicles have a thickness of (3.9 ± 0.2) nm.[19] Analysis of the other dispersions is more complex. From a qualitative point of view, analysis of Fig. 2 for mixed systems (DOPC/DOPE)/DOPURu shows the presence, in the low q region, of a power law $d\Sigma/d\Omega \propto q^{-\alpha}$ where the exponent α is gradually shifted from 2 (for the 100/0 and 70/30 ratios) to 3.7 (for the 50/50 ratio), indicating that the presence of unilamellar objects is progressively reduced.[22–24] At the same time, a correlation peak at $q \cong 0.05$ Å^{-1} appears in the middle q region, corresponding to a correlation distance among scattering regions of $\cong 2\pi/q = 130$ Å^{-1}, very close to the mean lattice parameter found through Cryo-TEM images.

To have a useful expression to be fitted to the experimental data for bicontinuous *Im3m* cubic structures, a very simple novel model has been developed and reported in the ESI†. Scattering cross-sections have been assumed arising from multilamellar vesicles and cubosomes with volume fraction of ϕ_{cub} and ϕ_{ves}, respectively; in the model the cubosome is represented as a cubic structure crossed inside by water channels. The geometrical quantities characterizing this structure are the number N of channels per side, the double layer thickness d and the length of a side $(D + d)$, as sketched in Fig. 3. This leads to the structure of a solid cube from which the regular lattice of cubic vacancies is subtracted. The solid cube in one dimension is described by

$$f_1(Q) = \frac{(D+d)\sin Q(D+d)/2}{Q(D+d)/2} \quad (1)$$

while the regular lattice of vacancies is described in one dimension by:

$$f_2(Q) = \frac{D-Nd}{N}\frac{\sin(Qd/2)}{\sin(Qd/2N)}\frac{\sin(Q(D-Nd)/2N)}{(Q(D-Nd)/2N)} \quad (2)$$

The overall dimension of the cubosome is $D + d$, while the bilayer thickness is d. The number of vacancies (or repetitions) is N. When the terms are multiplied to yield a three-dimensional structure, the result for the macroscopic cross-section can be obtained. Finally, a closed expression for $d\Sigma/d\Omega$ can be carried out, being more convenient for computational purposes:

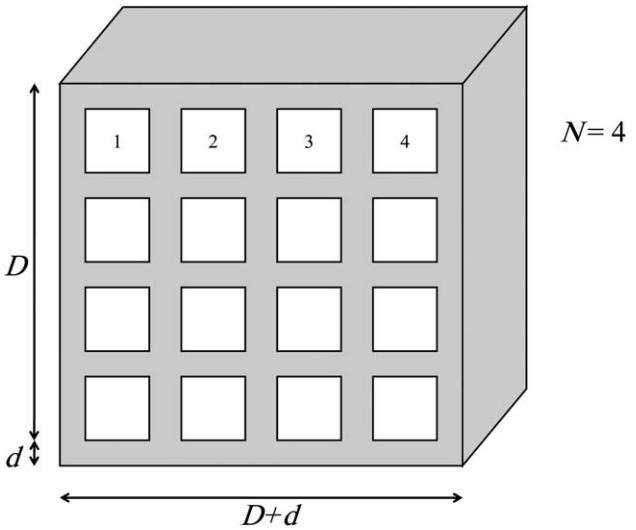

Fig. 3 Schematic illustration of a cubosome belonging to the primitive *Im3m* space group, as modeled in obtaining a small angle scattering form factor. The model is simplified by assuming a regular lattice of bilayers with the thickness d. The overall dimension of the cubosome is $D + d$. The number of vacancies in one dimension inside the cubosome is N. The ideal structure is three dimensional, and so the cubic vacancies are placed inside the volume. In this reported example $N = 4$.

$$\frac{d\Sigma}{d\Omega}(q) = \frac{\Delta^2\rho\phi}{v}\frac{\pi}{400}\sum_{j=0}^{9} \times \sum_{k=0}^{19} \sin t[\, f_1(q\cos y\sin t)f_1(q\sin y\sin t)f_1(q\cos t) - f_2(q\cos y\sin t)f_2(q\sin y\sin t)f_2(q\cos t)]^2 \quad (3)$$

where $t = \pi(k + 1/2)/40$ and $y = \pi(j + 1/2)/40$.

The experimental results (see Table S2†) show that the linear dimension $D + d$ of the bicontinuous structures is comparable with that estimable from CryoTEM images. The bilayer thickness d of cubosomes is very close to that found for the unilamellar vesicles of DOPC/DOPE, suggesting that the presence of DOPURu molecules does not considerably perturb the phospholipid double layer. The number of water channels per side N agrees quite well with the value estimable through the analysis of Cryo-TEM images, providing a very promising test for the reliability of the model developed, even if it is only an approximated sketch of the real situation.

In conclusion, this paper presents the first example of phospholipid-based bicontinuous phases with potential use for biomedical applications. Furthermore, such formulations open up new perspectives for the application of supramolecular systems in ruthenium anti-cancer therapy. At the same time a new and simple model developed for analyzing such complex structures by means of SANS data has been presented and its reliability has been shown by comparison with Cryo-TEM direct images.

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