Quatsomes: Highly Stable Nanovesicles formed by Sterols and Quaternary Amonium Surfactants

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ABSTRACT

There is a large interest in finding non-lipid building-blocks or tectons, which self-assemble into stable vesicles, and which satisfy the quality standards required in pharmaceutical formulations. Here we show the ability of quaternary ammonium surfactants and sterols to self-assemble forming stable amphiphilic bimolecular building-blocks with the appropriate structural characteristics to form, in aqueous phases, closed bilayers, which we named Quatsomes. When prepared by using compressed fluids, these colloidal structures are stable for periods as long as several years and their morphology do not change upon rising temperature or dilution. Many functionalities can be implemented simultaneously in Quatsomes, either by covalent attachment to sterol like molecules, by electrostatic interaction with the cationic head of surfactant units or by hydrophobic interaction with the bilayer. These possibilities open a broad range of applications in pharmacy, cosmetics and materials synthesis.

Keywords: vesicles, cationic surfactants, sterols, self-assembling, molecular dynamics simulations

1 INTRODUCTION

Liposomes made with phospholipids, are among the most studied self-assembled nanoobjects since their serendipitous discovery in 1964.[1] They are described as vesicles composed of one or more concentric lipidic bilayers, which separate a small enclosed liquid compartment from its surroundings. Their unique structure enables them to trap hydrophobic molecules within their bilayers and hydrophilic molecules within their lumen making liposomes excellent candidates to be used as nanocarriers for the protection and delivery of active ingredients in pharmaceutical and cosmetic formulations.[2] Despite their versatility, the translation to the clinic of liposomal formulations is often being limited by the tendency of these lipid self-assemblies to aggregate and by their low degree of structural homogeneity, which are critical quality attributes with a major impact on the in vivo pharmacokinetic and pharmacodynamic properties.

There is a large interest in finding non-lipid building-blocks or tectons, which self-assemble into stable vesicles, and which satisfy the quality standards required in pharmaceutical formulations.[3,4] Using CO_2-expanded solvents we have been able to prepare stable vesicular structures composed by quaternary amonium surfactants and sterols, which we named Quatsomes, with structural characteristcs not achievable by commonly used vesicle formation techniques, such as film-hydration.[5]

2 ONE-STEP PREPARATION OF QUATSOMES

Quatsomes, composed by sterols and quaternary ammonium surfactants, were prepared using compressed CO_2 following the DELOS-SUSP procedure schematically represented in Figure 1.[5] Briefly, the method consists in loading a solution of the desired sterol in an organic solvent (e.g. ethanol) into a high-pressure autoclave and pressurizing it with a large amount of compressed CO_2.

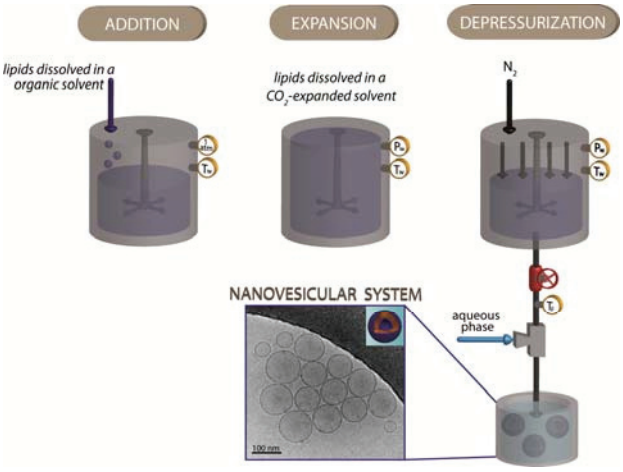

Figure 1. Scheme of the DELOS process for the preparation of vesicles.

Nanoscopic vesicular structures are straightforward formed, by depressurizing the resulting CO_2 expanded solution over an aqueous phase, which contain the water soluble surfactant. The CO_2 here acts as a co-solvent and its

evaporation from the organic expanded solution during the depressurization stage produces a fast, large and homogeneous cooling responsible of the high vesicle-to-vesicle structural homogeneity in comparison to the one reached by conventional thin-film hydration.[6] It should be pointed out, that lipids, such as sterols, have a great sensitivity to solvent media variations.[7] Therefore, homogeneous vesicle formation paths are required to guarantee a high degree of structural homogeneity. In Figure 2, are shown the cryo-TEM images of some representative Quatsomes prepared by this methodology.

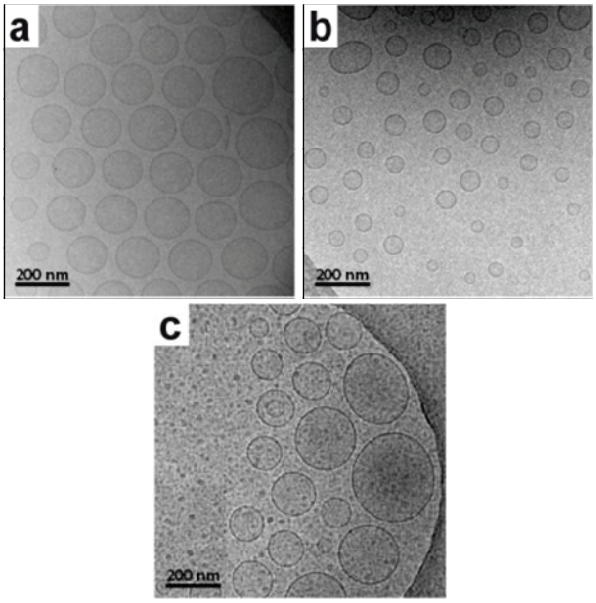

Figure 2. Representative cryo-TEM images of quatsomes prepared by DELOS-susp with composition: (a) Cholesterol/CTAB, (b) Cholesterol/MTAB and (c) β-sitosterol/CTAB. The scale bar represents 200 nm.

These vesicular systems are stable for periods as long as several years, their morphology do not change upon rising temperature up to 343 K or dilution and show outstanding vesicle to vesicle homogeneity regarding size, lamelarity and membrane supramolecular organization.

These quatsomes have a great potential in the development of new nanomedicines and they have already shown to be effective nanostructures to enhance specific bioactivity of proteins and to protect them against premature degradation in topical pharmaceutical formulations.[8] Worth to mention is that none of the individual components of a Quatsome spontaneously aggregate into vesicular structures since in water the quaternary ammonium surfactant forms micelles and the insoluble sterol species form crystals. Therefore, the self-assembly of sterol/quaternary ammonium surfactant mixtures into exceptionally homogeneous bilayer vesicles has to be attributed to a synergy between both molecular entities.

3 COOPERATIVE BEHAVIOR BETWEEN STEROLS AND QUATERNARY AMONIUM SURFACTANTS

To understand the molecular origin and driving force of the synergism between sterol species and quaternary ammonium surfactants, we have studied at molecular level the self-assembling of cholesterol and the cationic surfactant CTAB in aqueous medium, as model components of Quatsomes. For this purpose, sterol/surfactant mixtures at distinct molar ratios (Q) were prepared in aqueous media. Quasi-elastic light scattering (QELS), cryogenic transmission electron microscopy (cryo-TEM) and optical density (OD) were used to characterize the generated supramolecular phases. All-atomic molecular dynamics simulations were used to investigate with atomic resolution the nature of the interactions between the CTAB and cholesterol species in the process of the bilayer formation.[9] The analysis of each Chol/CTAB mixture by QELS and cryo-TEM evidenced the formation of distinct intermediate supramolecular phases. In the course of the micelle-to-vesicle-to-crystal transition induced by increasing the cholesterol content in the system, five distinct phase domains, each one governed by a predominant morphology, can be pictured:

- *First domain*, defined at Q=0. It is governed by CTAB micelles with their hydrocarbonated core sizing 1 nm.
- *Second domain*, at Q values between $1\cdot10^{-3}$ and 0.1. At $Q=1\cdot10^{-3}$ the micellar population coexists with a growing population of objects between 70 and 295 nm, identified as large micelles of elongated flexible shapes called wormlike micelles. The addition of cholesterol to the mixture until Q=0.1 provokes, according to cryo-TEM, the thickening, folding and partial self-closing of the worm membrane generating nascent bilayers. It is reported that above a critical worm size, worm-like micelles tend to curve and self-close in order to minimize their unfavorable edge energies giving rise to unilamellar vesicles.[10]
- *Third domain*, at Q=0.5. The priomary morphology consist on disk-like mixed micelles with a particle size mean of 115 nm. In this domain, disk-like micelles coexist with a considerable amount of closed vesicles . Upon addition of cholesterol to the mixture the destabilizing edge energies of the disk-like phase can be minimized either by growth of the floppy bilayers to reduce the overall edge length relative to the membrane area or by bending of the bilayer to form hemisphere cap-like structures with smaller peripheries, which eventually close up on themselves to form vesicles.
- *Fourth domain*, at Q=1. At the equimolar proportion the dispersed system contains a pure phase of spherical and unilamellar vesicles with mean diameter sizes around 40 and 160 nm that we have called Quatsomes. It is worth to say that these vesicular assemblies are stable for at least 36 months stored at 277 K and they do not change shape upon heating between 298 and 343 K.

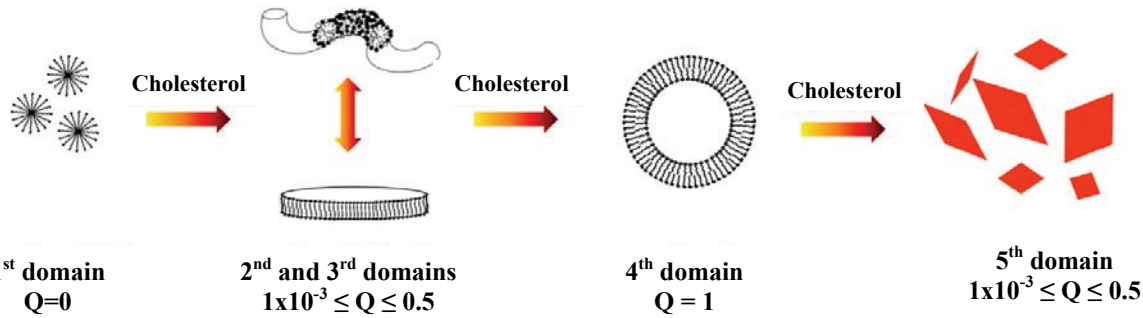

Figure 3. Schematic representation of the distict assemblies adopted by the cholesterol and CTAB molecules at increasing the cholesterol/CTAB molar ratio (Q).

- *Fifth domain*, defined at Q values between 1.5 and 3. At such cholesterol/CTAB molar ratio, the smaller number of CTAB molecules compared to the cholesterol ones promotes a macroscopic phase separation between self-assembled nanoobjects and plate-like cholesterol crystals. At Q=1.5, cholesterol crystals coexist with spherical vesicles but also with elongated and distorted vesicles with diameters ranging between 70 and 460 nm. At Q=3, the phase separation gets more pronounced and population of cholesterol crystals in the mixture dramatically increases.

To have a molecular picture of the process and the supramolecular structures underlying this transition we have performed all-atomic molecular dynamics (MD) simulations of mixtures of cholesterol and CTAB in water at distinct cholesterol/surfactant molar ratios. The outcome of our simulations suggest a thermodynamic preference of both CTAB and cholesterol molecules for the mixed environment of an equimolar Chol/CTAB bilayer rather than for a homogeneous environment of CTAB micelles and cholesterol crystals (Figure 4). The net free energy gain of 5 kcal/mol associated with the preference of a cholesterol molecule to be incorporated into a 1:1 cholesterol:CTAB bilayer (ΔG=-55 kcal/mol) rather than into a cholesterol nanocrystal (ΔG=-50 kcal/mol) is almost identical to the binding free energy (4.7 kcal/mol) calculated for the association of a single surfactant with a cholesterol molecule. Consequently the pair Chol/CTAB works as a unique supramolecular architecture for the formation of more complex colloidal phases such as vesicles. It is clear that the structure of the bilayer depends crucially on its equimolar composition. In order to understand the dependence with composition, we have performed further simulations of bilayers with different cholesterol/CTAB molar ratios. The atomic density profiles and snapshots of these simulations reveal that the bilayer becomes unstable as the cholesterol/surfactant ratio decreases and suggest the formation of distinct intermediate colloidal phases. We have also studied by MD, how a CTAB micelle is altered by the addition of a small quantity of cholesterol. To this end, we have performed a simulation in which a cholesterol molecule is added to a CTAB micelle in water. The cholesterol molecule is rapidly incorporated into the micelle and protected from water with surfactant molecules inducing certain degree of structuration in an inherently disordered micelle.

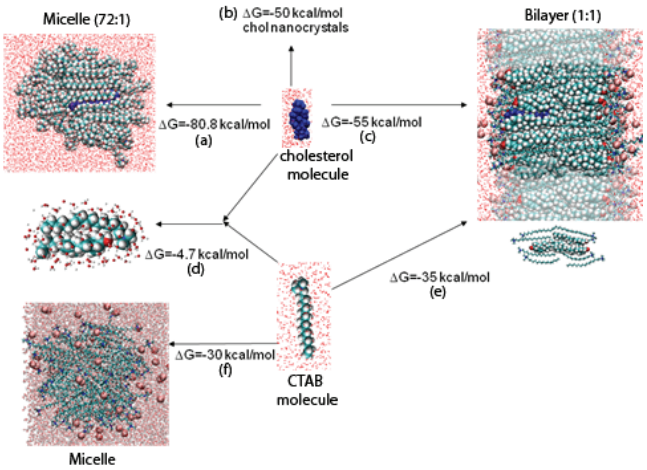

Figure 4. Diagram showing the free energies of interaction of the pairs (a) cholesterol molecule - CTAB micelle, (b) cholesterol molecule - cholesterol nanocrystal, (c) cholesterol molecule – Chol/CTAB bilayer (1:1), (d) cholesterol molecule - CTAB molecule, (e) CTAB molecule – Chol/CTAB bilayer (1:1) and (f) CTAB molecule - CTAB micelle.

Overall, all these remarkable structural and thermodynamic properties of a Chol/CTAB bilayer at 1:1 molar ratio predicted from MD simulations provide a theoretical support to justify the experimental high thermal stability and the exceptional morphological properties attributed to vesicles of such composition obtained following in-solution preparation routes in comparison to vesicles prepared by procedures involving a solvent-free stage.[11]

The transition pathway and transient morphologies induced by the addition of cholesterol to a micellar solution of CTAB observed here are similar to those found in previous studies of the micelle-to-vesicle transition that occur on mixing cationic and anionic surfactant.[12, 13]

However, the mechanism for which a mixture composed of a cationic surfactant, like CTAB, and a sterol, like cholesterol, self-assemble into a vesicle is rather different and does not have any precedent explanation. In water, cationic and anionic surfactants individually self-assemble in micelles but their mixtures are able to self-assemble in more complex structures (including vesicles at nearly equimolar concentrations) due to the electrostatic complexation between both molecules. In the case of the Chol/CTAB vesicle formation, the self-assembly driving force was not known from previous works. MD simulations confirm that, it is the synergy between the CTAB and the sterol entities that generates a bimolecular synthon that at certain proportions behaves as a single building block with adequate structural characteristics to self-assemble into vesicles. This behavior cannot be expected in a heterogeneous mixture of two dissimilar, noninteracting components.[2, 14]

In view of these structural results, it is worth emphasizing here the profound, conceptual differences with previous works on transitions from multicomponent membrane vesicles to micelles studied for mixed anionic-cationic surfactants and two-chain lipid systems. In such systems, both molecular types have head groups at the water-hydrocarbon interface.[15] In the mixed cationic-anionic surfactant systems, both polar groups electrostatically interact modifying the geometry of the system and causing the bilayer and vesicle formation. Instead, in the sterol/surfactant system, the sterol does not live at the surface but in the interior of the bilayer. Such particular arrangement of the sterol unit makes the volume of the hydrophobic region of the CTAB molecule apparently increase leading to the formation of a bimolecular synthon with a structural architecture similar to that of a double tailed amphiphile whose spontaneous aggregation geometry is that of a vesicle (Figure 5). Therefore, it is the synergy between one sterol and one single-tail quaternary ammonium surfactant that allows the self-assembling into multicomponent membrane vesicles in aqueous media only at the equimolar proportion leading to these fewly repeated structures that we have called quatsomes. These colloidal structures display the required properties to be established as a true thermodynamically stable phase.

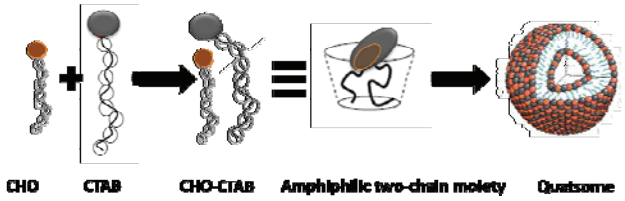

Figure 5. Cartoon showing the formation of a quatsome from a cholesterol and a CTAB molecule.

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