Preparation and Structural Characterization of Different Cholesterol-CTAB Supramolecular Assemblies Using CO_2-expanded Solvents

M. Cano,[†] A. Angelova,[‡] N. Ventosa,[†,*] S. Lesieur,[‡] J. Veciana[†,*]
[†]*Dep. Nanociencia Molecular y Materiales Orgánicos*
ICMAB-CSIC-CIBER-BBN
Campus de la UAB 08193 – Barcelona, Spain

[‡] *Equipe Physico-chimie de Systèmes Polyphasés,*
UMR CNRS 8612,
Université Paris-Sud, 92296 - Châtenay-Malabry, France

Contact e-mail: ventosa@icmab.es ; vecianaj@icmab.es

Nanoscopic and uniform unilamellar vesicles, rich in cholesterol, have been prepared using CO_2-expanded solvents. This procedure is based on the depressurization of a cholesterol solution in CO_2-expanded acetone into an aqueous solution containing a surfactant. The CO_2 is used here as a co-solvent, allowing the straightforward preparation of vesicular systems with controlled size distribution, uniform shape, and stability unachievable by conventional mixing technologies. The analysis of the different CTAB/cholesterol assemblies that can be formed by the tuning of CTAB/cholesterol molar ratios is crucial regarding the capacity of vesicles as intelligent drug biovectors. For this purpose, playing with operational parameters and surfactant/lipid ratios, we have been able to prepare different CTAB/cholesterol assemblies in a robust way, and characterize them by dynamic light scattering (DLS), ζ potential and cryogenic transmission electron microscopy (cryo-TEM).

INTRODUCTION

Vesicles are unilamellar or multilamellar spheroid structures composed of amphiphilic molecules assembled into bilayers. They are particularly attractive for drug delivery, since they may permit to incorporate larger molecules than micelles, and also provide unprecedented protection of the drug from biological degradation and denaturation before entry into the cell. In this context, uniform and small unilamellar vesicle (SUV) systems are attracting a great deal of interest as intelligent materials for drug-delivery since they can be used as nanocontainers sensitive to external stimuli -pressure, pH, temperature or concentration changes in the medium- triggering modifications in their supramolecular structures. The control of the size and the stability of vesicles are crucial for their application in material science and for drug delivery purposes, since their pharmacological features are related to its nanostructure.[1] Traditional vesicle preparation methods have many problems associated to process scaling up, numerous process steps, low reproducibility, large processing times, high energy consumption, and limited control of final particle sizes. Therefore, the development of reproducible, efficient, environmental friendly and easy to scale-up methodologies for the production of vesicular systems with controlled sizes and supramolecular organizations is of great industrial interest.[2] Herein we present a new method, which uses compressed CO_2 as co-solvent for the straightforward preparation of uniform unilamellar CTAB/cholesterol nanovesicles.[3] Particularly, in this work we show the

efficiency of this new methodology for preparing in a single-step and reproducible way different CTAB/cholesterol lipid phases. Playing with operational parameters and lipid-surfactant rates, we have been able to prepare, in a robust way, different CTAB/cholesterol supramolecular assemblies.

MATERIALS AND METHODS

Materials. 5-Cholesten-3β-ol (cholesterol, purity 95%) was obtained from Panreac (Barcelona, Spain), cetyltrimethylammonium bromide was purchased from Sigma-Aldrich (Steinheim, Germany), acetone (purity 99.5%) was obtained from Panreac (Barcelona, Spain) and carbon dioxide (purity 99.9%) was supplied by Carburos Metálicos S.A All chemicals were used without further purification. Water was deionized and purified by flowing it through a two cartridge Elix Water Purification System Millipore (conductivity lower than 0.2μS cm^{-1}).

CTAB/cholesterol mixture solution prepared by the DELOS-SUSP process. The equipment used for the preparation of CTAB/cholesterol mixtures using CO_2-expanded solvents is described elsewhere.[3] In a typical experiment, a known volume of a solution of cholesterol in acetone, with an initial supersaturation ratio β_I ($\beta_I = C/C^S$ where C is the initial cholesterol concentration in the non-pressurized acetone and C^S is saturation limit of the cholesterol in the acetone at a given working temperature, T_W) was loaded into a 300mL high-pressure autoclave at atmospheric pressure and at a given working temperature, T_W. The autoclave was then pressurized with compressed CO_2 producing a volumetric expanded liquid acetone solution with a given molar fraction of the compressed fluid, X_{CO2}, at a given working pressure, P_W. The concentration of cholesterol in the expanded mixture at this stage must remain below the saturation limit to avoid unwanted anti-solvent precipitation as solid. After leaving the system with mechanical agitation under the same conditions for 30-60 minutes, in order to achieve a complete homogenization and to attain a thermal equilibration, the liquid expanded solution was depressurized from the working pressure to the atmospheric one through a non-return valve with a flow rate of 5 $ml \cdot s^{-1}$ over a pumped aqueous solution of CTAB (0.01M) dissolved operating at 10-30 $ml \cdot s^{-1}$ flow rate. The aqueous solution flow rate is adjusted in order to fix a cholesterol/surfactant ratio in the final vesicular system. The temperature of the depressurized solution, T_F, was measured just before mixing with the aqueous solution which takes place in a T-mixer, in a cross-flow with turbulent conditions and with mixing times down to the millisecond range yielding the cholesterol/CTAB dispersed systems.

RESULTS AND DISCUSSION

In Table 1 is presented a selection of experiments for the preparation of CTAB/cholesterol dispersed systems, in which all process parameters were kept constant except the CTAB/cholesterol molar ratio. Previously, the solubility of cholesterol in CO_2-expanded acetone mixtures was studied by the vanishing point method, at different solvents compositions at 308K and 10MPa, in order to avoid undesired cholesterol precipitation at working conditions (P_w,T_w,X_w).[3] We have observed a broad range of solvent compositions where the CO_2 behaves as a co-solvent for cholesterol. The anti-solvent effect of the CO_2 over the "cholesterol/acetone/CO_2" system does not appear until a CO_2 molar fraction of 0.5. The particle size distributions of the different CTAB/cholesterol assemblies produced in each

experiment were measured by dynamic light scattering (DLS), the stability was studied by the ζ potential and the morphology by cryogenic transmission electron microscopy (cryo-TEM). Worth mentioning are the differences in the physicochemical characteristics observed in the different CTAB/cholesterol phases prepared by mixing different CTAB/cholesterol molar ratios (1/1, 0/1 and 1/4).

Table 1. Preparation of CTAB/cholesterol lipid phases using CO_2-expanded solvents: Operational parameters and structural characterization

	operational parameters				particle size distribution[a]		stability parameters[b]
sample	CTAB/chol[c] (mol/mol)	β_i	X_{CO2}	ΔT^d(ºC)	D(v,0.5) (nm)	U.I.	ζ (mV)
ES-1	1/1	0.8	0.58	-51	78	65.7	+74.5
ES-2	0/1	0.8	0.58	-57	3840	44.7	+27.6
ES-3	1/4	0.8	0.57	-63	2735	2.2	+54.4

[a] Volumetric particle size distributions measured by dynamic light scattering technique, are described by D(v,0.5), which is the volume median particle diameter (nm). The uniformity index (U.I.), is defined as U.I.=[D(v,0.1)/D(v,0.9)]·100 and describes the polydispersity of the system. [b] The dispersed system stability is described by the ζ potential. [c] CTAB/cholesterol molar ratio. [e] Temperature decrease, $\Delta T=T_F-T_w$, where T_w is the temperature of the expanded solution and T_F is temperature of the solution after the depressurization valve. [f] In all experiments CTAB concentration was kept constant at 0.01M. [f] T_w=308k and P_w=10MPA**.**

According to the DLS measurements and the cryo-TEM micrograph image (Figure 1A), the amphiphilic mixture produced at a CTAB/cholesterol molar ratio of 1/1 (sample ES-1) was a extremely uniform phase formed by small unilamellar spherical vesicles with a narrow particle size distribution and a mean particle size centered on 78 nm. Moreover, the ES-1 sample presents a large stability over time as it is indicated by the large ζ potential (+75.5mV). In contrast, the dispersed system prepared at CTAB/cholesterol=0/1 (sample ES-2), without CTAB, was an unstable suspension of crystalline cholesterol particles with a mean particle size centered on 3.8µm and a rectangular shape morphology, as shown Figure 1B. This fact remarks that the presence of a surfactant is a key point for the cholesterol self-assembling in an aqueous phase, since in its absence instead of forming vesicles, only cholesterol crystals are obtained. The dispersed system obtained using a CTAB/Cholesterol molar ratio of 1/4, presents similar characteristics to the one prepared in the absence of CTAB. As shown Figure 2, the DLS measurements reveal that in this case the system is constituted by two phases, vesicles and crystals.

CONCLUSIÓN

We conclude that our CO_2-expanded solvent based methodology permits to prepare in a robust way different CTAB/cholesterol assemblies from small unilamellar vesicles to uniform crystalline solid crystals. It is worth mentioning that this process overcomes some of the limitations related to traditional methods for preparing dispersed systems, offering alternative advantages for clean and non-toxic drug formulations. Moreover, it can be scaled up easily producing large amounts of the dispersed systems.

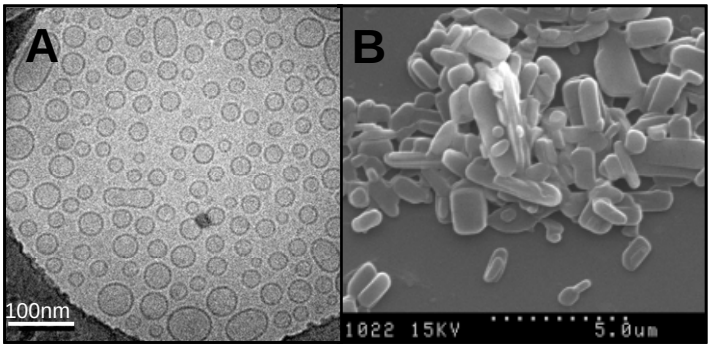

Figure 1: A) Cryo-TEM image of CTAB/cholesterol vesicular system prepared by using CO_2 at CTAB/cholesterol molar ratio=1/1; B) SEM image of cholesterol suspension prepared using CO_2 at CTAB/cholesterol molar ratio=0/1.

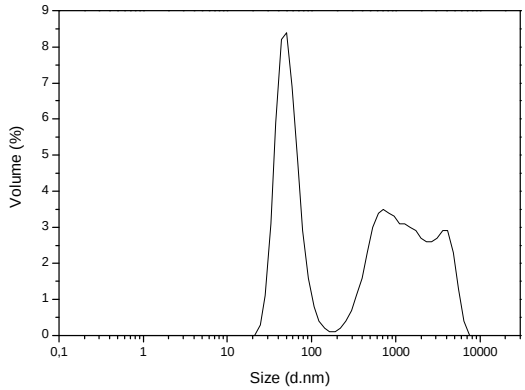

Figure 2: Particle size distribution curve measured by dynamic light scattering of CTAB/cholesterol dispersed system obtained through CO_2-expanded solvent based process at CTAB/cholesterol molar ratio=1/4

ACKNOWLEGMENTS

M.C.-S. thanks the MEC (Spain) for her Ph.D. grant. This work was supported by the "Plan Nacional de Investigación Científica y Desarrollo e Innovación Tecnológica", Iniciativa Ingenio 2010, Programa *Consolider*, under the projects NANOFAR(Grant NAN2004-09159-C04-01) and EMOCIONA (Grant CTQ2006-06333/BQU), and the Instituto de Salud Carlos III, through "Acciones CIBER". We also thank Raúl Solanas and Emili de la Serna for the equipment and practical assessment in the high-pressure experiments.

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