PREPARATION OF UNIFORM UNILAMELLAR NANOVESICLES USING CO_2-EXPANDED SOLVENTS

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Nanoscopic and uniform unilamellar vesicles, rich in cholesterol, have been prepared by a new procedure, named "DELOS-SUSP", which 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 cosolvent medium, allowing the straightforward preparation of vesicular systems with controlled size distribution, uniform shape, and stability unachievable by conventional mixing technologies. The resulting nanoscopic vesicular systems dispersed in water were characterized using dynamic light scattering, ζ potential, turbidity, and cryogenic transmission electron microscopy. The influence of operational parameters of this new methodology on the physicochemical characteristics of the vesicular systems is also reported.

INTRODUCTION

Nowadays, uniform and small unilamellar vesicular (SUV) systems are attracting a great deal of interest as intelligent materials since they can be used as containers sensitive to external stimuli- pressure, pH, temperature or concentration changes in the medium- triggering modifications in their supramolecular structures. The control of the supramolecular organization of these systems is of profound importance for applications in material science and for drug delivery purposes. Therefore, the development of reproducible, efficient, environmentally friendly and easy to scale-up methodologies for the production of vesicular systems with controlled sizes and supramolecular organizations is of great industrial interest.[1]

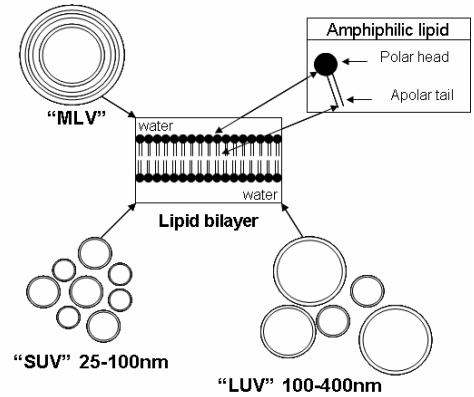

Figure 1. Classification of vesicular systems according to their size and membrane lamellarity: SUV: small unilamellar vesicles; LUV: large unilamellar vesicles; MLV: multilamellar vesicles

Lipid vesicle systems, named liposomes, are hollow spherical assemblies formed by a single lipid bilayer (unilamellar) or multiple, concentric bilayers (multilamellar) which entrap part of the aqueous medium in which they are suspended (see Figure 1). Because of the amphiphilic character of the lipids and their organization in closed structures, lipid vesicles can encapsulate hydrophobic molecules in the bilayer membrane or hydrophilic compounds in the aqueous internal cavity as well as amphiphilic

substances.[2] Lipid vesicle systems in aqueous phases are currently prepared by conventional mixing technologies producing multilamellar vesicles (MLVs) which must be sonicated and extruded down through filters of a defined pore size until uniform SUVs are obtained.[3] Such mixing methods have several drawbacks that can be summarized as follows: multiple steps of preparation, low reproducibility, long processing times, high consumption of energy, and limited control of particle sizes over the final particulate material. Such drawbacks are originated because these solvent-based processes are driven by temperature and/or composition changes (temperature decrease, solvent evaporation, addition of salts, etc.) on the processed systems which are always slowly and non-homogeneously transmitted in liquid media.[4] In contrast, processes based on compressed fluids (CFs) have gained increasing attention as alternative solvent media because they overcome some of the limitations related to the traditional methods, offering alternative advantages of the final product.[5] Thus, the solvation power of CFs in their liquid or supercritical state can be tuned by pressure changes, which propagate much more quickly and uniformly than temperature and solvent composition changes. Therefore, CFs allow a much greater control and tuning of the structural characteristics of the final material (size, size distribution, porosity, polymorphic nature, morphology, etc.) than with conventional organic solvents. Indeed, processing with CFs often leads to materials with unique physicochemical characteristics, unattainable by conventional processing methodologies.[6] Herein we present a novel method, called DELOS-SUSP,[7] for the preparation of aqueous dispersed vesicular systems of water non-soluble compounds, based on the DELOS precipitation technology, which uses compressed CO_2 as a cosolvent.[8] Particularly in this work, we show the efficiency of this new methodology for preparing in a single step, nano sized, stable, uniformly shaped, and unilamellar rich in cholesterol vesicles, unachievable by conventional procedures. The cholesterol is a functional lipid practically insoluble in water and somewhat soluble in supercritical CO_2, which is amply used as an active substance in cosmetic, nutraceutical, and pharmaceutical products.[9] The majority of liposomal systems are based on phospholipids; however, there has been a continuous interest in preparing liposomes from non-phospholipid amphiphiles, such as cholesterol.[10] This kind of vesicular formulation shows low passive leakage in comparison to liposomal systems based on phospholipid systems, and therefore, they have a higher ability to encapsulate materials, for example, therapeutically active molecules.

EXPERIMENTAL SECTION

Materials. 5-Cholesten-3β-ol (cholesterol; purity 95%) was obtained from Panreac (Barcelona, Spain), cetyltrimethylammonium bromide (CTAB), polyoxyethylammonium monooleate (Tween 80) and bis(2-ethylhexyl)sulfosuccinate sodium salt (AOT) were purchased from Sigma-Aldrich (Steinheim, Germany), acetone (purity 99.5%) was obtained from Panreac, and carbon dioxide (purity 99.9%) was supplied by Carburos Metálicos S.A. (Barcelona, Spain). All chemicals were used without further purification. Water was deionized and purified by being passed through a two-cartridge Elix water purification system (Millipore, conductivity lower than 0.2 μS cm-1).

Preparation of cholesterol/surfactant vesicular systems by the DELOS-SUSP process. The equipment used for the preparation of cholesterol vesicular systems is described in elsewhere.[7] In a typical experiment, a known volume of a solution of cholesterol in acetone, with an initial supersaturation ratio, β_I (β_I=C/C^S, where C is the initial cholesterol concentration in the nonpressurized acetone and C^S is the 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 antisolvent precipitation as a solid. After the system was left under mechanical agitation under the same conditions for 30-60 min, 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·s^{-1} over a pumped aqueous solution with 1 wt %surface-active compound operating at a 10-30 mL·s^{-1} flow rate. The aqueous solution flow rate is adjusted to fix the cholesterol/ surfactant ratio in the final vesicular system. The temperature of the depressurized solution, T_F, was measured just before it was mixed 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 vesicular dispersed systems.

Preparation of cholesterol/surfactant vesicular systems by the conventional mixing method. Conventional mixed experiments of cholesterol were performed in the same experimental setup used in the DELOS-SUSP process without the gas unit. The operational procedure used in these experiments is summarized as follows. A known volume of a solution of cholesterol in acetone, with an initial supersaturation ratio of β_I, was loaded into a 300 mL autoclave, R, at atmospheric pressure and at a given working temperature, T_W. A known volume of a surface-active compound solution in water was loaded into vessel D1. The mixing between the flows of the cholesterol solution and the surface-active compound aqueous phase in the T-mixer produce the precipitation of the cholesterol vesicles by the antisolvent effect produced by the aqueous phase over the cholesterol solution.[11]

RESULTS AND DISCUSSION

In Table 1 is presented a selection of experiments for the preparation of cholesterol vesicular systems, using the DELOS-SUSP procedure and a conventional mixing method. Previously, the solubility of cholesterol in CO_2-expanded acetone mixtures was studied by the vanishing point method, at different solvent compositions at 308 K and 10 MPa.[12] 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 is exceeded. Surfactants used in all DELOS-SUSP experiments where the cationic cetyltrimethylammonium bromide (CTAB), the anionic sodium bis(2-ethylhexyl)sulfosuccinate salt, known as AOT, and the neutral polyoxyethylene (20) sorbitan monooleate, known as Tween 80. The particle size characteristics of the vesicular system prepared was measured by dynamic light scattering (DLS), and the stability was studied by measuring the ζ potential and the turbidity of the dispersed system through its backscattering and transmission profiles. The corresponding data are given in Table 1.[13] Worth mentioning are the differences in the physicochemical characteristics of cholesterol vesicular systems obtained by the DELOS-SUSP (sample DS-1) and by a conventional mixing method (sample CM-1) operating at similar conditions.

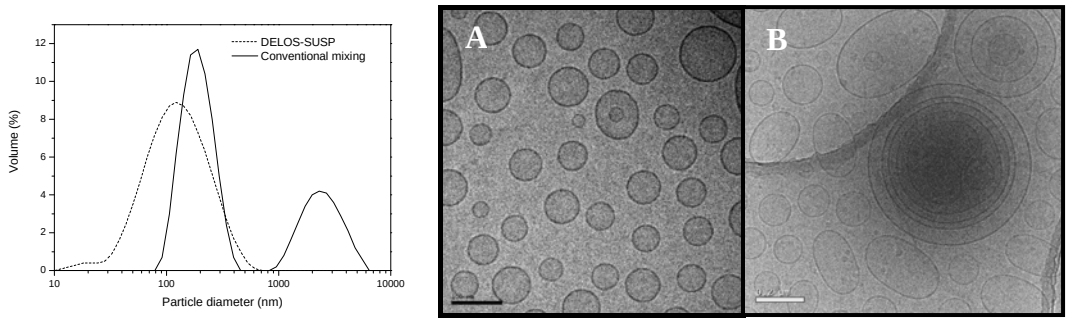

Figure 2. Particle size distribution curves measured by dynamic light scattering of disperse systems obtained through the DELOS-SUSP method (sample DS-1, dashed line) and the conventional mixing method (sample M-16, continuous line). Curves are represented in terms of particle volume percentage (left); Cryo-TEM micrograph images of cholesterol vesicular systems obtained through the DELOS-SUSP process (sample DS-1, right A) and the conventional mixing method (sample CM-1, right B).

Thus, the vesicles obtained through DELOS-SUSP (sample DS-1) have a mean particle size (167 nm) smaller than the dispersed system (sample CM-1) formed through the conventional mixing method

(301 nm) as well as a remarkable unimodal and narrow particle size distribution (Figure 2). Moreover, the DS-1 sample was more stable than the CM-1 sample, as the DELOS-SUSP vesicles have a ζ potential more positive (+88.8 mV) and a migration velocity 5 times lower (0.22 μ m/s) than those of the vesicles obtained through the conventional mixing method (+31.3 mV, 1.17 μ m/s). The cryo-TEM micrograph image of the dispersed system processed through DELOS-SUSP (sample DS-1) shows extremely uniform spherically shaped vesicles, which in all cases are unilamellar (Figure 2, right A). The observed vesicles have a mean size centred on 200 nm, in good agreement with DLS measurements. In contrast, with the conventional mixing method (sample CM-1), a multilamellar vesicular system, formed by multiple and concentric bilayers with a bigger and less homogeneous particle size distribution, is obtained (Figure 2, right B).

Table 1. Operational parameters and physicochemical characteristics of the dispersed "cholesterol/acetone/ H_20/surfactant" systems obtained with the DELOS-SUSP method at T w=308 K and $Pw = 10$ MPa.

	operational parameters					particle size distribution[a]			stability parameters[b]	
Sample	Surfactant[c]	[Chol/Surf][d]	X_{CO2}	β_I	ΔT[e]	Q_a[f]	$D[v,0.5]$	U.I	ζ	V_S
		mol/mol			ºC	mL·s^{-1}	nm		mV	µm/s
CM-1	CTAB	50/50	-	0.8	-	25	301	3.9	+31.3	1.17
DS-1	CTAB	50/50	0.6	0.8	-54	25	167	3.4	+88.8	0.22
DS-2	CTAB	50/50	0.7	0.8	-73	22	151	3.9	+91.1	0.3
DS-3	CTAB	50/50	0.8	0.8	-74	20	134	14.8	+92.3	1.17
DS-4	CTAB	50/50	0.9	0.8	-88	10	125	2.9	+101.1	0.30
DS-5	CTAB	50/50	0.6	0.6	-62	17	229	3.9	+76.6	0.57
DS-6	CTAB	50/50	0.6	0.7	-56	19	129	12.8	+74.4	-
DS-7	CTAB	50/50	0.6	0.9	-55	25	125	3.4	+92.3	-
DS-8	CTAB	50/50	0.6	1.1	-58	29	104	19.8	+84.1	-
DS -9	AOT	50/50	0.6	0.8	-65	20	2410	2.7	+23.2	-
DS-10	CTAB	50/50	0.6	0.8	-55	20	170	5.3	+87.4	0.57
DS-11	Tween 80	50/50	0.6	0.8	-61	20	3370	26.6	+11.3	5.25
DS-12[g]	-	100/0	0.6	0.8	-54	20	3840	44.78	+27.6	-

[a] Volumetric particle size distributions, measured by the 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. = $[D(v,0.1)]/D(v,0.9)]$x100 and describes the polydispersity of the system. [b] The dispersed vesicular stability is described by the ζ potential (ζ, mV), and the migration velocity of the dispersed vesicles (V_S, µm/s). [c]Surfactants: AOT, sodium bis(2-ethylhexyl)sulfosuccinate salt; CTAB, cetyltrimethylammonium bromide; Tween 80, polyoxyethylene (20) sorbitan monooleate. A 1 wt% wt concentration of surfactant in water was used in all experiments. [d] Cholesterol /surfactant 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 the temperature of the solution after the depressurization valve. [f]Aqueous solution flow rate. [g]This experiment has produced cholesterol crystalline particles instead of vesicular structures.

Figure 3. Image of cholesterol dispersed systems after one month of being prepared by the conventional mixing method (left and right samples) and by the DELOS-SUSP procedure (middle sample).

As shown in Figure 3, the properties of the vesicular system prepared by the CO_2-based method do not change noticeably over periods of several months while the dispersed systems obtained with the mixing methodology are unstable in a few weeks. Indeed, DLS measurements showed that vesicles prepared by DELOS-SUSP have a much higher mean particle size stability as a function of time than those obtained by conventional mixing. Thus, the as-prepared DS-1 sample shows a mean size of 167 nm which remains constant after 6 months (171 nm) while the as-prepared CM-1 sample shows a mean size of 301 nm which increases considerably after 6 months (26 μm). Cryo-TEM was used to characterize the morphology of the resulting dispersed systems.

We have also observed experimentally that the particle size distribution and dispersion stability of the cholesterol vesicular systems obtained with DELOS-SUSP do depend neither on the expanded solution formation step nor on the processing time. In contrast, both physico-chemical characteristics are dependent on the cholesterol concentration in the initial solution (i.e., initial supersaturation ratio β_I) and in the final aqueous phase, as well as on the CO_2 molar fraction (X_{CO2}), the nature of the surfactant and the molar ratio between such the surfactant and the cholesterol.[14] Table 1 shows the influence of these operational parameters over the physico-chemical characteristics of the resulting dispersed systems. The presence of a surfactant in the aqueous phase is a key point for the preparation of cholesterol vesicular systems by the DELOS-SUSP method, since in its absence instead of dispersed vesicles only suspensions of crystalline particles of cholesterol in a water phase are obtained (see experiment DS-12). Finally, we have also studied the influence of the surfactant nature on the preparation of cholesterol vesicles. We have studied three different surfactants: an anionic surfactant, such as AOT (sample DS-9), a cationic one, such as CTAB (sample DS-10), and a neutral one, such as Tween 80 (sample DS-11). According to the results of the particle size distribution and the dispersion stability, reported in Table 1, the cationic CTAB surfactant is a more suitable agent for producing nanoscopic spherically shaped unilamellar cholesterol vesicles dispersed in an aqueous phase. The vesicular systems achieved with the neutral and the anionic surfactants, have a much larger mean particle size and a much lower stability. Further work is ongoing to determine the supramolecular organization of cholesterol and CTAB molecules inside the vesicular membrane.

CONCLUSION

We conclude that stable and structurally well-defined uniform spherically shaped, unilamellar rich cholesterol nanovesicles dispersed in an aqueous phase are formed by the DELOS-SUSP process showing physicochemical characteristics unachievable by conventional mixing technologies. It is worth mentioning that this process overcomes some of the limitations related to traditional methods for preparing nanovesicles, offering alternative advantages for clean and nontoxic drug formulations. Moreover, it can be scaled up easily, producing large amounts of the dispersed vesicles. Application of this new technology for processing other water-nonsoluble polymeric and molecular bioactive compounds in the form of water-dispersed nanovesicles and the use of such systems as drug-delivery vehicles are currently in progress.

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