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Solid Lipid Nanoparticles (SLNs): Preparation and Properties of Calix[4]resorcinarene-Derived Systems

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Abstract

Solid Lipid Nanoparticles (SLNs) have been prepared from *c*-2,*c*-8,*c*-14,*c*-20-tetraundecyl-4,6,10,12,16,18,22,24-octahydroxyresorc[4]arene as colloidal suspensions. Photon Correlation Spectroscopy studies revealed a particle hydrodynamic diameter of 150 nm. Non-contact mode Atomic Force Microscopy allows observation of the particles as slightly flattened spherical objects of 236 (±40) nm diameter and 145 (±40) nm height. The study of the preparation parameters showed that shear force does not affect the hydrodynamic size of the SLNs. In contrast, the viscosity and the pH of the aqueous phase, the amphiphile concentration in the organic phase and the volume of organic phase used, all lead to variation in the size of the particles. In term of post-preparation parameters only the ionic strength has been shown to affect significantly the particle size; while the pH of the storing solution, microwave, ultrasonic and thermal treatments do not. Short- and long-term stability studies have been performed to measure the effect of the ionic strength on the stability of the particles. The use of carbohydrate cryoprotectants does not allow re-dispersion of the colloidal suspension after freeze-drying.

Introduction

The use of colloidal transport systems for bioactive molecules is a subject of wide interest. Transporters have been based on micelles [1], liposomes [2], polymeric micro- and nanoparticles [3] and protein derived colloids [4]. Recently solid lipid nanoparticles (SLNs) have been developed as an attractive alternative form of transport system [5, 6]. These colloidal systems are prepared from mixtures of solid lipids with, if needed, the presence of co-surfactants. The SLNs show, generally, long term stability, resistance to precipitation from ionic solutions, excellent encapsulation levels for guest molecules, and good protection of encapsulated products towards chemical and biological degradation. Their preparation is simple, avoids toxic organic solvents and is inexpensive [7, 8]. Tailoring of SLNs may allow targeting of biological receptors and also avoid bio-toxicity and immune reactions [9–13]. The preparation of SLNs has been extensively reviewed by Muller [5, 14, 15].

Recently, attention was turned to the use of supramolecular amphiphilic molecules to prepare SLNs. Initial research focused on cyclodextrin (CD)-derived SLNs, using amphiphiles derived obtained either by hydrophobic coupling at the secondary face [16] or by random esterification [17]. The large cavity of the cyclodextrins may allow double encapsulation of molecules, either in the host cavity or within the SLN matrix [18]. However, the known haemolytic properties of the parent CDs is a serious disadvantage to their use [19]. Furthermore, the chemical modifications used are generally costly and involve toxic solvents or reagents [20].

In the last year, we have focused on the use of amphiphilic calixarene-derived SLNs [21–27]. Interestingly the parent calixarenes, while derived from phenol/formaldehyde resins show neither toxicity nor provoke immune reactions [25, 28, 29]. The stability of calixarene-based SLNs is excellent, and no haemolytic effects have been observed [25]. Adsorption of serum albumins onto calixarene-based SLNs leads only to the deposition of mono- or bi-molecular surface layers, without precipitation [27].

In contrast to the amphiphilic calixarenes, which require three synthetic steps (all in good yields), the synthesis of amphiphilic calix[4]resorcinarenes is a one step procedure, with only ethanol as the solvent. In view of this ease of preparation, such molecules seem excellent candidates for the extension of supramolecular SLN systems and their molecular recognition properties with regard to bio-relevant molecules (amino-acids [30, 31], carbohydrate [32]) have been shown. The calix[4]resorcinarenes have been previously reported to form micellar [33] and liposomal [34] colloidal systems.

In this paper we describe the preparation, properties, stability and AFM imaging of SLNs derived from *c*-2,*c*-8,*c*-14,*c*-20-tetraundecyl-4,6,10,12,16,18,22,24-octahydroxyresorc[4]arene, **1a** (Figure 1a). The properties closely mirror those of the similar calixarene, [22], al-

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though **1a**-based SLNs show a lower resistance to high ionic strengths.

Experimental

General

1a was synthesized as previously described [36], physical and analytical properties are in accordance with the literature values.

Solvents (HPLC grade) and chemicals were purchased from Acros Organics (France) and used without further purification. Pluronic® F68 (Synperonic) was purchased from Fluka (France).

Photon correlation spectroscopy

The particle size and the polydispersity index were measured on a Malvern 4700 spectrometer and 7132 256-channel with a 40 mW He-Ne laser (633 nm). All values were measured at an angle of 90° in 10 mm diameter cells. The system was thermostated at 25 °C. All measurements were repeated five times, and the variance of the measurements was less than 5%. In the dynamic light scattering experiments, the intensity time autocorrelation function $G^2(t)$ is measured directly using the correlator and can be directly related to the normalized first-order electric field time correlation function $g^{(1)}(t, q)$ as

$$G^2(t) = \langle I(0)I(t)\rangle = B[(1 + \beta|g^{(1)}(t, q)|^2], \quad (1)$$

where q is the module of scattered intensity, B is the measured baseline, t is the delay time, and β is the parameter depending on the coherence of the detection.

In monomodal analysis, the cumulant method is used; here, the logarithmic correlation function is expanded in a series in terms of the delay time where Γ_1, Γ_2, and Γ_3 are the first, second, and third cumulants as given in Equation (2).

$$\ln g^{(1)}(t) = -\Gamma_1 t + (\Gamma_2/2!)t^2 - (\Gamma_3/3!)t^3 + \cdots. \quad (2)$$

The apparent hydrodynamic particle diameter $\langle d_h\rangle$ can be found using the Stokes–Einstein relation (Equation (3)),

$$\langle d_h\rangle = k_b T/3\pi\eta D, \quad (3)$$

where $q\Gamma 1 = Dq^2$, with D being the diffusion coefficient; k_b, the Boltzman constant; T, the temperature; and η, the viscosity of the solvent.

If the sample is polydisperse, the polydispersity index, PI, is defined as

$$\mathrm{PI} = 2\Gamma_2/\Gamma_1^2. \quad (4)$$

Atomic force microscopy

Imaging was carried out using a Thermomicroscope (Santa Clara, USA) Explorer AFM equipped with a 100 μm tripod scanner, in non-contact mode, using high-resonant-frequency (F_0 = 320 KHz) Si cantilevers at a scan frequency of 1 Hz. Images are processed with the SPMLab 5.01 software package and are presented unfiltered. Object dimensions were measured using the Line Analysis package in SPMLab 5.01.

Samples were prepared by depositing a volume of 10 μL of SLNs suspensions on either cleaned glass microscope slides or freshly cleaved mica. Imaging was carried out after drying the samples overnight at room temperature.

SLN preparation

A standard method for the preparation of the SLN suspensions is defined as follows: to a solution (1.5 ml) of **1a** in tetrahydrofuran (THF) (5 mg/ml), under magnetic stirring (500 rpm), is added 50 ml of pure distilled water (resistivity >18MΩ.cm). The emulsion, instantaneously formed, is stirred during an additional minute. The THF is removed under reduced pressure and the volume is adjusted with pure water to yield a final volume of 50 ml, giving a final concentration of 0.15 g/l.

Detailed study of the preparation parameters

Unless otherwise stated the standard preparation described above was used. All PCS measurements were carried out after leaving the SLN suspensions 24 hours at ambient temperature (20 °C).

Mixed resorcinarene-pluronic® F68 SLNs

Increasing quantities of pluronic® F68 were dissolved in THF (0.125, 0.25, 0.5 and 1 mg/ml) and were co-dissolved with **1a**. Water was added to the mixed solution under the standard conditions.

Viscosity of the aqueous phase

Increasing amounts of glycerol (2, 4, 6, 8 and 10%; vol:vol) were dissolved in the aqueous phase before SLNs preparation. Prior to measurement, the suspensions were diluted 10 times and the effect of viscosity was taken in account in the PCS measurements.

Influence of the pH of the aqueous phase

The SLN suspensions were prepared following the standard methodology, varying the pH of the aqueous phase from 2 to 13 with HCl and NaOH.

Stirring speed

The stirring speed values were set respectively as 300, 500, 700, 900 and 1100 rpm.

Concentration

Amphiphile concentration in the THF phase was varied between 5 and 25 mg/ml.

Proportion of organic phase

Increasing volumes of THF solutions, from 1 to 10 ml and keeping the final quantity of **1a** constant at 0.1g/l, were used to prepare the SLNs suspension.

Post preparation stability

All the following experiments were carried out on SLNs prepared following the standard methodology.

Storage in saline solutions and temporal stability

The influence of various sodium and potassium salts on SLN stability was measured by dilution in solutions of various salts (NaCl, NaH_2PO_4, Na_2SO_4, $NaCH_3CO_2$, Na_2CO_3, KCl, KH_2PO_4, K_2SO_4, KCH_3CO_2, K_2CO_3) at final concentrations of 10^{-3} and 10^{-1} M. The hydrodynamic diameter of the SLNs was then measured after 1 h, 1, 15, 50, 80 and 90 days.

Effect of pH

Suspensions of **1a**-based SLNs were diluted (vol:vol) in HCl and NaOH solutions in order to obtain pH values in the range 2 to 13. PCS measurements were carried out 24 h after the dilution.

UV irradiation

10 ml of the SLN suspension, in a beaker, was irradiated during 10, 20, 30, 40, 50 and 60 minutes with a 254 nm ultra-violet lamp.

Ultrasonic irradiation

100 ml of a SLN suspension were maintained in an ultrasonic bath during one hour. Aliquots of 1 ml were removed and analysed by PCS every 10 min.

Boiling

A volume of 100 ml of a suspension of SLNs was keep under reflux during one hour, 1 ml aliquots were removed every 10 min, cooled to room temperature and then analysed by PCS.

Cryoprotectant effects

To 1 ml of a SLN suspension was added an equal volume of an aqueous solution of the relevant carbohydrate (glucose, fructose, mannose, maltose, trehalose) in order to obtain a final carbohydrate concentration of 1, 2, 5, 10, 15, 20 and 25% in mass of sugar. The suspensions were frozen at −15 °C overnight in glass vials. Freeze-drying was carried out at −55 °C (±3 °C) at a pressure of 10^{-4} Torr during 24 h. Suspensions were reconstituted in 2 ml of pure water under vortex agitation. For the PCS experiments, viscosity differences at high sugar concentrations were taken in account in the Contin size calculation program.

Results and discussion

Both calix[4]arenes and calix[4]resorcinarenes are based on macrocycles having four aromatic rings bridged by methylene groups, two major structural differences exist, Figure 1; firstly for the *para*-acylcalix[4]arenes the hydrophobic substituents are directly bonded to the aromatic units *para* to the phenolic groups, whereas in the calix[4]resorcinarenes the hydrophobic substituents are bonded to the methylene bridges, and secondly for the calixarenes a single phenolic hydroxyl group is present whereas for the calix[4]resorcinarenes two phenolic hydroxyl groups are present per aromatic moiety. These differences can be expected to influence the hydrophobic-lipophilic balance (HLB) of the molecules.

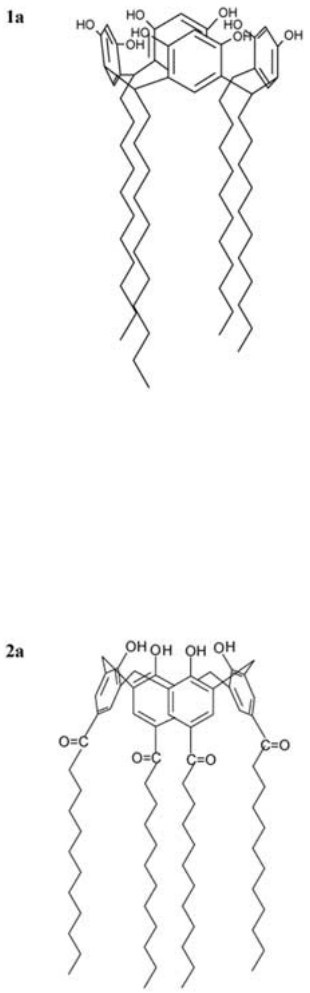

Figure 1. Formulae of *c*-2,*c*-8,*c*-14,*c*-20-tetraundecylclix[4]resorcinarene (a) and para-tetradodecanoylcalix[4]arene (b).

The physical and structural characterisation of Solid Lipid Nanoparticles (SLNs) prepared from **1a**, by the solvent diffusion method [22], was carried out by Photon Correlation Spectroscopy (PCS) and non-contact mode Atomic Force Microscopy (AFM).

PCS shows a hydrodynamic diameter for the calix[4]resorcinarene-based SLNs of 150 nm with a high degree of monodispersity, for SLNs prepared under the standard conditions, described in the experimental section.

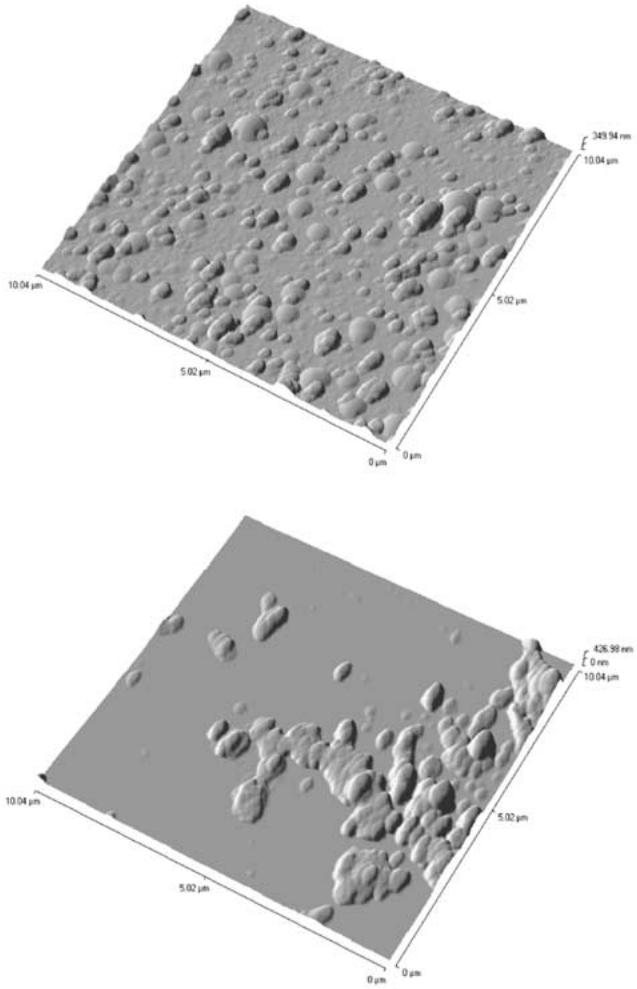

Figure 2. Non-contact mode AFM images of **1a**-based SLNs on mica (a) and on glass (b) at 10 μm scan ranges.

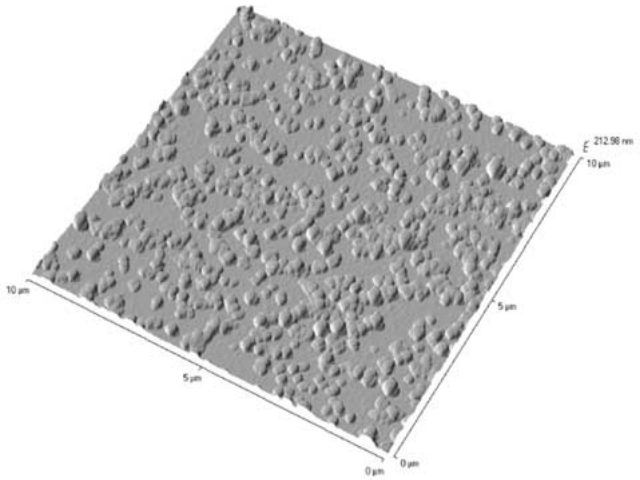

Figure 3. Non-contact mode AFM images of SLNs based on mixtures of **1a** and pluronic® F68 acid (10%) at 10 μm scan range.

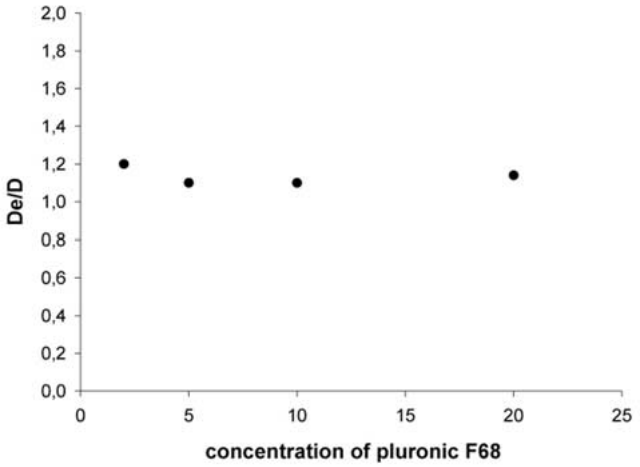

Figure 4. Effects of the presence of pluronic® F68 in the organic phase on the hydrodynamic diameter of **1a**-based SLNs; values are expressed as a ratio of the diameter of the SLNs prepared by the standard method.

In Figure 2 are given the non-contact mode AFM images of freshly prepared SLNs based on **1a** deposited on glass and mica and dried before imaging at a scan range of 10×10 μm. On the glass substrate (Figure 2a), the SLNs can clearly be seen as well dispersed round objects of 236 ($\pm$40) nm diameter and 145 ($\pm$40) nm height. While slightly flattened during the drying process [21], the observed volumes are consistent with those observed by PCS, implying a solid matrix for the colloidal objects. Liposomal systems collapse under such conditions to give heights equivalent to two bilayers for unilammellar vesicles [35], or often spread out to form Supported Lipid Bilayers [36].

On the mica substrate, SLNs formed from **1a** appear as flattened ovoid objects of 210 ($\pm$20) nm and 350 ($\pm$50) nm diameters, and 90 ($\pm$20) nm height. This effect may arise from the charges present on the surface of the mica (potassium alumino-silicate) which can interact with the polar function of **1a**. It can also be seen that the surface coverage is less important on the mica compared to the glass with a clear clustering of the SLNs. From the above, we can expect that the glass is a more appropriate substrate for the AFM imaging of SLNs, as they are apparently less deformed.

In Figure 3 are presented the AFM images of the SLNs prepared with mixture of **1a** and pluronic® F68 (10%), at a scan range of 10×10 μm, on a glass substrate. Less aggregation of the SLNs is observed, with observed diameter of 280 ($\pm$20) nm and observed height of 80 ($\pm$10) nm. Compared to the SLNs prepared from **1** only, the mixed SLNs tend to flatten more on the glass surface, implying slightly higher fluidity.

In view of the above AFM imaging results, we feel confident that the systems observed in this paper represent an extension to the family of supramolecular Solid Lipid Nanoparticles.

In terms of the parameters which may affect the formation and initial colloid size, we investigated: presence of a co-surfactant (Figure 4), effects of viscosity of the aqueous phase (Figure 5), aqueous phase pH (Figure 6), variation of the dispersion speed (Figure 7), final colloid concentration (Figure 8) and proportion of the organic phase (Figure 9).

In contrast to cyclodextrin-based SLNs [37, 38] and many other SLN systems but as with *para*-acylcalix[4]arene-

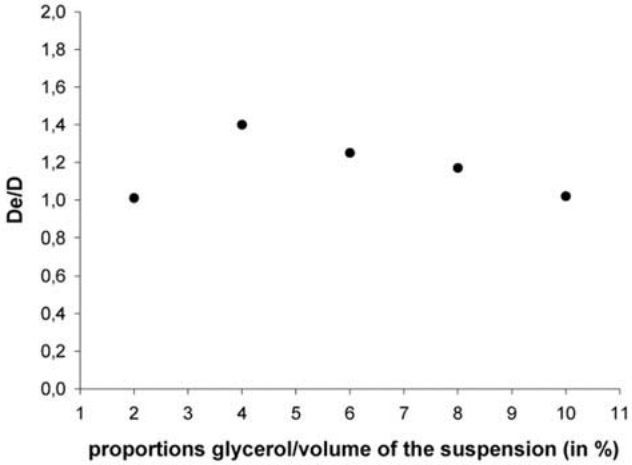

Figure 5. Effects of the presence of glycerol in the aqueous phase on the hydrodynamic diameter of **1a**-based SLNs; values are expressed as a ratio of the diameter of the SLNs prepared by the standard method.

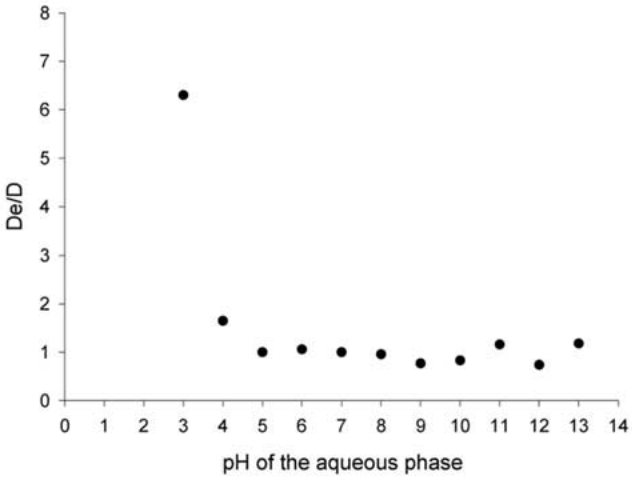

Figure 6. Influence of the pH of the aqueous phase used during the preparation process on the hydrodynamic diameter of **1a**; values are expressed as a ratio of the diameter of the SLNs prepared by the standard method.

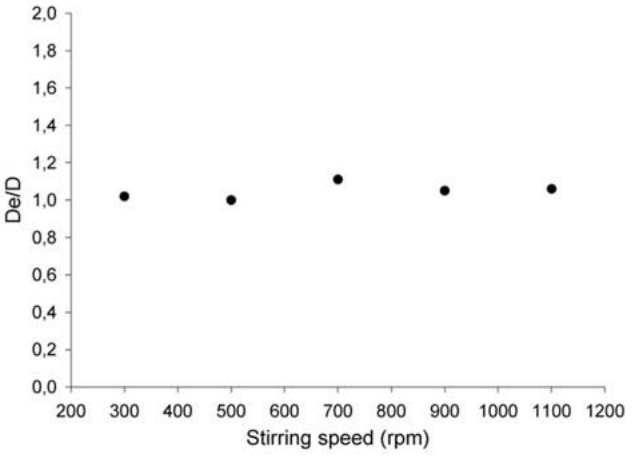

Figure 7. Effects of the stirring speed used during the preparation on **1a** based SLNs; values are expressed as a ratio of the diameter of the SLNs prepared following the standard method.

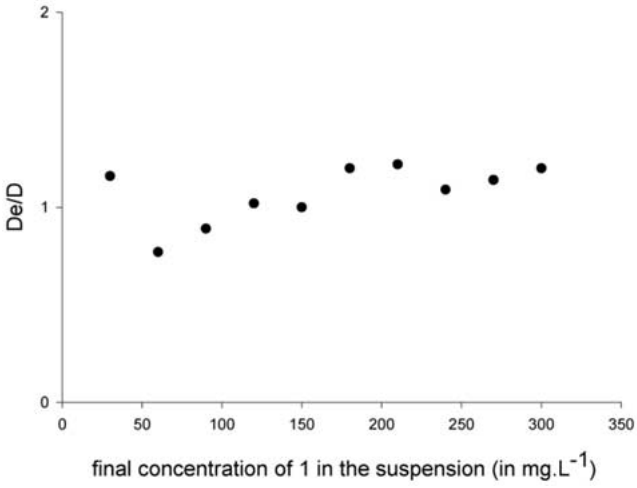

Figure 8. Effects of the concentration of **1a** in the organic phase in the SLNs production process; values are expressed as a ratio of the diameter of the SLNs prepared by the standard method.

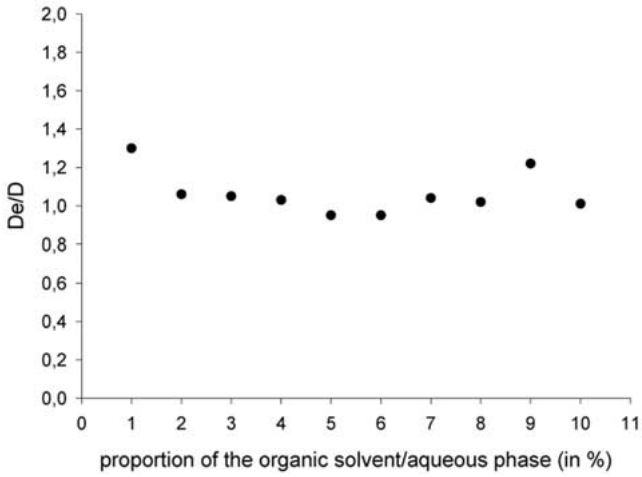

Figure 9. Effects of the volume of organic solvent in the **1a** based SLNs production process; values are expressed as a ratio of the diameter of the SLNs prepared by the standard method.

based SLNs, the calix[4]resorcinarene-based SLNs can be prepared in the absence of a co-surfactant.

The effects of a co-surfactant were measured by preparing colloidal suspensions of **1a** in the presence of pluronic® F68 in the organic phase (Figure 4). A small, but significant, increase in the hydrodynamic diameter is observed at 2% pluronic® F68 giving SLNs of 180 nm in diameter. This diameter is invariant with increasing co-surfactant concentration. These results accord the work of Shöler *et al.* [39], where it was demonstrated that the co-surfactant influences SLN size in the dynasan 114 system. However, the observed changes are in contrast to our previous work with *para*-dodecanoylcalix[4]arene, where a decrease in size with increasing co-surfactant concentration was observed [22].

The SLNs described here were prepared by the solvent diffusion method, for which it would be expected that variations in the viscosity of the preparation medium should influence the size of the SLNs by varying the kinetics of the solvent outflow. Such behaviour has been previously reported by Quintanar-Guerrero *et al.* [40] in the formation

of poly-lactic acid nanoparticles with increasing solvent viscosity diminishing the observed size of the carriers. The behaviour of **1a**-based SLNs is complex, Figure 5, increasing glycerol concentration to 4% with an increase in viscosity to 0.99 cp leads to a 40% increase in the observed size of the SLNs. At glycerol concentrations above 4%, the size decreases to reach a value of 150 nm, at a glycerol concentration of 10%, i.e., identical to that observed in pure water. Such behaviour is different from that observed for *para*-dodecanoylcalix[4]arene where the SLN size is effectively independent of viscosity changes.

The pH stability of SLNs is a key point in both the preparation and the application of such systems. In Figure 6 is given the size of the SLNs as a function of the pH of the aqueous phase used in the preparation. It can be seen that the SLNs are stable in the range of pH 4 to pH 8, i.e., throughout the range of physiological pH values. At acidic pHs between 2 and 4, the calix[4]resorcinarene based SLNs show lower stability than that observed for SLNs based on *para*-dodecanoylcalix[4]arene [22], however their stability with regard to basic conditions seems slightly improved.

In the solvent displacement method, the rate of dispersion of the lipid has been shown to be a key point, especially concerning the size of the particles formed. In Figure 7 are presented the size variations in function of the stirring speed. No significant variations occur when the stirring speed is varied from 300 to 1100 rpm. The SLN formation can also be influenced by the concentration of the amphiphile in the organic solvent. In Figure 8 are presented the variations in SLN size for varying concentrations of **1a** in the organic phase. At low final concentration, slight but significant decrease in the size of the particles is observed, the hydrodynamic diameter increases progressively for values above 100 mg/ml and reaches a value of 180 nm at a final concentration of **1a** equal to 0.3 g/l.

The final parameter tested was the proportion of organic solvent used during the preparation. Volumes of 1–10 ml of THF were used in the preparation, while holding the final concentration of **1a** constant; the results are presented in Figure 9. As for the *para*-tetradodecanoylcalix[4]arene, the observed behaviour is complex and may arise from the modification of the physical properties of water by THF clathration [41]. The apparent hydrodynamic radius shows two maxima, one at 1 mL of THF and the other at 9 mL of THF.

Post-preparation parameters have also been studied; in this section we treat those to which the SLNs might be subjected during formulation, storage and application. In terms of external post-preparation effects, 7 parameters were examined: (a) UV irradiation, (b) ultrasonic treatment, (c) boiling, (d) the pH of the suspension, (e) ionic strength of the suspension, (f) freeze-drying and cryo-protectant effects.

The effect of the ionic strength on the stability and the aggregation behaviour of **1a**-based SLNs is a key-point for their bio-medical and biological applications. The effect of the addition of various sodium and potassium salts on the hydrodynamic diameter of the SLNs are summarized in Table 1. It is clear that in the case of potassium carbonate, potassium sulphate and sodium acetate; no significant variations in the hydrodynamic diameter of the SLNs occur. In the case of sodium carbonate, sodium sulphate and potassium acetate, slight but significant increases in the hydrodynamic diameter of the SLNs are observed. Interestingly, these results show clearly that there is both an effect of the anion and the cation on the size and the stability of the SLNs; this is in contrast to the case of *para*-tetradodecanoylcalix[4]arene based SLNs where it has been shown that the effect of the cation was not significant [22]. In the case of sodium and potassium chloride, the SLNs are completely destabilized and precipitation/flocculation occurs.

Table 1. Effects of varying monovalent salts at a concentration of 0.1 M on the size of **1a**-based SLNs; values are expressed as a ratio of the diameter of the SLNs in pure water

Salt	De/D
NaCl	Precipitated
KCl	Precipitated
Na_2CO_3	1.32
K_2CO_3	1
NaH_2PO_4	Precipitated
KH_2PO_4	Precipitated
Na_2SO_4	1.11
K_2SO_4	1.02
CH_3COONa	0.96
CH_3COOK	1.30

The effect of modifying the pH of the suspension after preparation was observed; the results are presented in Figure 10. This result show that the destabilizing effect of the pH during the preparation is not observed when the pH of the suspension is modified after the preparation. The results concerning the effects on the stability of **1a**-based SLN of microwave irradiations, ultrasonic and thermal (boiling) treatment are presented in Figure 11. It is clear that these treatments have no effect on the stability of the SLNs.

The final post-preparation parameter tested is the possibility to freeze-dry and subsequently redisperse the SLNs. *para*-tetradodecanoylcalix[4]arene based SLNs can be freeze dried and redispersed only by the use of carbohydrate cryo-protectants. Here, for all the carbohydrates tested (glucose, fructose, mannose, maltose, trehalose), the polydispersity observed after redispersion was very high, showing the incomplete formation of the colloidal suspensions occurs. This fact can be explained by the high affinity of calix[4]resorcinarenes for carbohydrates [32], which does not allow the entire reconstitution of the SLN suspensions in aqueous media after the freeze-drying process, or may provoke restructuring of the colloids.

Finally, the temporal stability of the SLNs was studied. In Figure 9 are presented the stability curves for SLNs stored in suspension in pure water at two 25 and 40 °C.

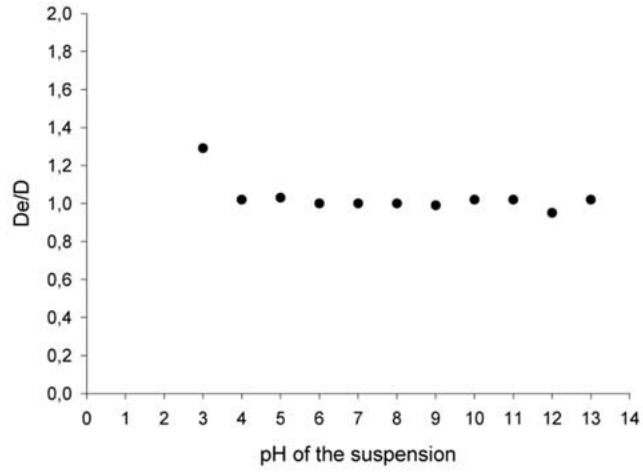

Figure 10. Influence of the pH in the stability of the suspension on **1** based SLNs; values are expressed as a ratio of the diameter of the SLNs maintained at pH 7.

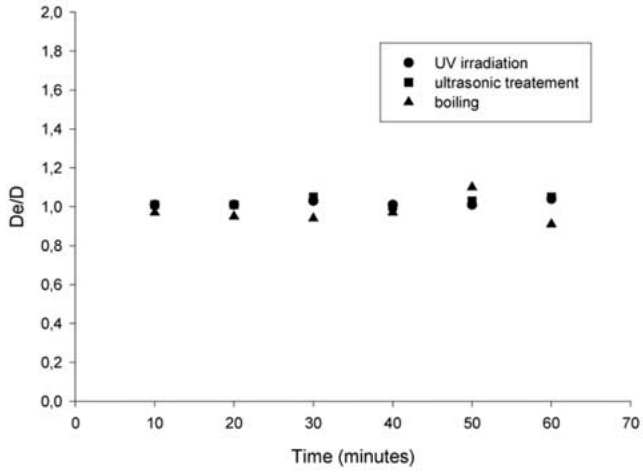

Figure 11. Effects of the UV irradiation, ultrasonic treatment and boiling on the stability of the suspension; values are expressed as a ratio of the diameter of the SLNs before treatment.

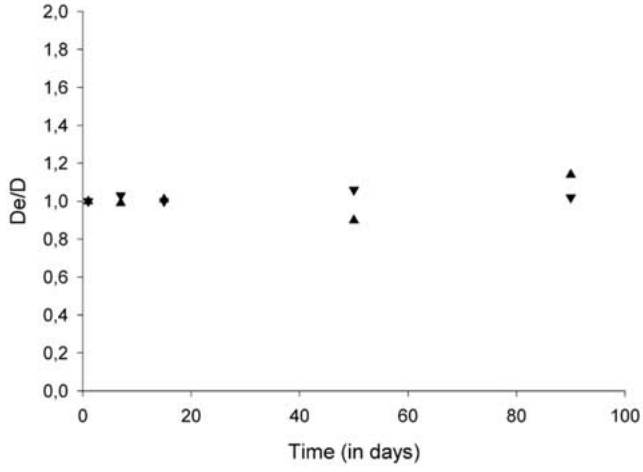

Figure 12. Temporal stability of the SLNs stored as suspensions in pure water at 25 and 40 °C; values are expressed as a ratio of the diameter of the SLNs at t_0.

Table 2. Effects of varying monovalent salts at a concentration of 0.1 M (a) and 10^{-3} M (b) on the temporal stability of **1a**-based SLNs for systems where instantaneous precipitation occurs

	Salt	De/D > 1.1	De/D > 1.5	Precipitation
(a)	NaCl	–	–	t_0
	KCl	–	–	t_0
	Na_2CO_3	t_0	24 h	5 days
	K_2CO_3	15 days	50 days	80 days
	NaH_2PO_4	–	–	t_0
	KH_2PO_4	–	–	t_0
	Na_2SO_4	–	–	24 h
	K_2SO_4	–	–	24 h
	CH_3COONa	–	–	–
	CH_3COOK	24 h	–	–
(b)				
	NaCl	80 days	–	–
	KCl	80 days	–	–
	Na_2CO_3	–	–	–
	K_2CO_3	–	–	–
	NaH_2PO_4	–	–	–
	KH_2PO_4	–	50 days	–
	Na_2SO_4	–	50 days	–
	K_2SO_4	–	–	–
	CH_3COONa	–	–	–
CH_3COOK	–	–	–	

Clearly there is no significant variation in the apparent hydrodynamic diameter of the SLNs stored during 90 days. In Table 2 are presented the temporal stability of **1a**-based SLNs during the storage in saline solutions. At 0.1 M (Table 2a), the long-term stability of **1a**-based SLNs is low. Only for sodium and potassium acetate, in spite of an increase in size, the SLNs show long-term stability. At 10^{-3} M (Table 2b), none of the salts cause precipitation, even for the four salts which provoke instantaneous precipitation at 0.1 M (NaCl, KCl, NaH_2PO_4, KH_2PO_4).

Conclusion

The systematic study of the preparation and post-preparation parameters of *c*-2,*c*-8,*c*-14,*c*-20-tetraundecyl-4,6,10,12,16,18,22,24-octahydroxiresorc[4]arene-based solid lipid nanoparticles has been undertaken. The results show that the behaviour of these SLNs closely mirror that of the *para*-tetradodecanoylcalix[4]arene based SLNs. The one-step synthesis of the calix[4]resorcinarenes may thus prove an advantage in their eventual applications.

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