Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 253 (2003) 23–38
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Para-acyl-calix-arene based solid lipid nanoparticles (SLNs): a detailed study of preparation and stability parameters

Patrick Shahgaldian [a], Eric Da Silva [a], Anthony W. Coleman [a,*], Beth Rather [b], Michael J. Zaworotko [b]

[a] *Institut de Biologie et Chimie des Protéines, CNRS UMR 5086, 7 Passage du Vercors, F-69367 Lyon Cedex 07, France*
[b] *Department of Chemistry, University of South Florida, 4202 East Fowler Avenue, Tampa, FL 33620-5250, USA*

Received 15 July 2002; received in revised form 6 November 2002; accepted 18 November 2002

Abstract

The preparation and stability parameters of *para*-acyl-calix[4]arene based solid lipid nanoparticles (SLNs) have been investigated. Atomic force microscopy (AFM) and photon correlation spectroscopy (PCS) show a mean particle size of 130 nm. In terms of preparation parameters, using the solvent displacement method, the nature and the volume of the organic solvent, the concentration of the amphiphile and the presence of a co-surfactant in the organic phase have been shown to affect significantly the size of the produced SLNs. In contrast, the stirring speed, the viscosity and the acidity of the aqueous phase and the amphiphile hydrophobic chain length have been shown to have no effect. In terms of stability parameters, the ionic strength has been shown to affect the short-time SLN stability depending on both the anion and the cation studied, with sodium sulphate causing precipitation. Ultrasonic, ultraviolet or microwave treatments of the SLN suspensions have no effect on the size of the SLNs. The study of the effects of short time thermal treatment revealed that the SLNs are not affected by one freezing–defreezing cycle and are stable at 100 °C in suspension. It is difficult to reconstitute the SLN suspensions after freeze-drying. Finally, the temporal stability of these suspensions in water has been shown to be superior to 1 year. The long-term temporal stability of suspensions stored in saline solution has been investigated. It has been demonstrated that the most destabilising effects arise from the presence in the storage suspension of sulphate ions.

^{1}H NMR, X-ray powder diffraction (XPD) and AFM have also been carried out on the calix-arene based SLNs and demonstrate the presence of a semi-organised matrix structure for the SLNs.

Keywords: Solid lipid nanoparticles (SLNs); Calix-arenes; Amphiphiles; Stability; Preparation; Atomic force microscopy

1. Introduction

In the recent years the solid lipid nanoparticles (SLNs^{TM}) (Mehnert and Mader, 2001; Müller et al., 2000; Müller and Runge, 1998), have emerged as an attractive colloidal transport system.

The SLNs, based on mixtures of solid lipids mixed, with if required, co-surfactants, may present all the advantages of other colloidal transporters, while possessing few or none of their problems. The advantages include high temporal stability, reduced effect of ionic strength on stability, possible targeting by suitable chemical modification, good protection of encapsulated bio-active molecules, high encapsulation loads, absence of carrier bio-toxicity, avoidance of toxic organic solvents, ease of scale-up procedures and low cost.

* Corresponding author. Tel.: +33-4-72-72-26-40; fax: +33-4-72-72-26-01.
E-mail address: aw.coleman@ibcp.fr (A.W. Coleman).

0378-5173/02/$ – see front matter © 2002 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0378-5173(02)00639-7

The production procedures for SLNs have recently been reviewed by Müller et al. (2000) and generally use natural solid lipids, emulsifiers, water and for certain preparation methods a co-solvent (which is generally removed). Six main procedures exist for the fabrication of SLNs: high shear homogenisation and ultrasound, high-pressure homogenisation, hot homogenisation, cold homogenisation, solvent emulsification/diffusion, micro-emulsion. Evidently, the nature of the lipid, the emulsifier, the production method and, if used, the co-solvent can influence the properties of the SLNs.

In the last decade, the range of solid lipids used to produce SLNs has been extended to include molecules based on cyclodextrins (Coleman and Kasselouri, 1993; Perrier et al., 2001; Skiba et al., 1993; Sommer et al., 1993; Duchêne et al., 1999a,b; Lemos-Senna et al., 1998). As one of the major classes of supramolecular systems (Schneider and Yatsimirsky, 2000; Steed and Atwood, 2000), the cyclodextrins present the added advantage of a molecular cavity capable of selectively including molecules (Szetjli, 1998) and thus may be of interest as carriers for two or more different molecules. However, certain problems, including some inherent toxicity, use of highly toxic chemicals in the synthesis, scale up problems and cost, appear to be limiting factors on their application. As a consequence we turned our attention to the calix-arenes (Gutsche, 1989), as another potential supramolecular skeleton (Fig. 1a) for the development of novel SLNs.

The calix-arenes are macrocyclic host compounds based on methylene bridged phenolic units. They are selectively available, in high yields under conditions suitable for industrial scale production. While they are purely synthetic, they show close structural analogy to natural polyphenols and indeed cyclobromoveratrylene has been found in red algae (Combaut et al., 1978). As yet no toxicity (Perret et al., 2000) or immunogenic properties (Gansey et al., 1999) have been found for simple derivatives. Importantly the chemistry for their conversion to amphiphilic (lipidic) derivatives is simple (Shahgaldian et al., 2001), and we have shown that in the solid-state they may be obtained in the absence of included solvent molecules (Shahgaldian et al., 2002).

We have previously communicated the preparation of SLNs based on several amphiphilic calix[4]arene

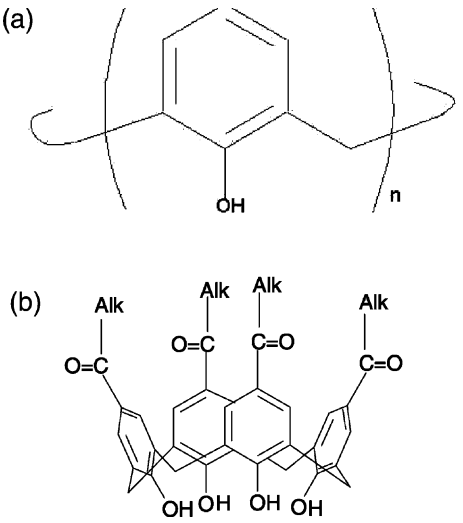

Fig. 1. General formulae of calix[*n*]arenes and *para*-acyl-calix[4]arene, where Alk = $CH_3(CH_2)_nCO$– (**1**: $n = 10$).

derivatives (Houel et al., 2002; Shahgaldian et al., 2002), and that these are both stable and may in fact, be produced by the solvent diffusion method, in the absence of co-surfactant and emulsifiers.

In this paper we present a detailed study of numerous parameters for the production of *para*-acyl-calix-[4]arene (Fig. 1b) based SLNs, including the nature and the volume of the organic solvent, the concentration of the amphiphile in this solvent, the kinetics of solvent–water dispersion, the pH and the viscosity of the aqueous phase, the presence of a co-surfactant and finally the hydrophobic chain length. The influence of ionic strength on the stability of the SLNs and cation or anion specific effects, and the effects of various post-production effects (pH, freeze drying, freezing, irradiations, freezing–unfreezing cycles) on the quality of the SLNs will be discussed. It will be demonstrated that the *para*-acyl-calix[4]arene based SLNs present an interesting alternative to more classical SLNs based on natural lipids.

2. Experimental

2.1. General

Para-acyl-calix[4]arenes were synthesised as previously described (Shahgaldian et al., 2001).

Chemicals (analytical grade) were purchased from Acros Organics (France) and used without further purification. Pluronic® F68 was purchased from Fluka (France).

2.2. Photon correlation spectroscopy (PCS)

PCS measurements were carried out using a Malvern 4700 spectrometer and a Malvern 71320, 256 channel correlator with a 40 mW He–Ne (633 nm) laser source. 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%. The analysis of the measurements was performed using the computer program Contin (Malvern). Prior to size determination by PCS, all suspensions were allowed to stand for 24 h.

2.3. Atomic force microscopy (AFM)

Imaging was carried out using a Thermomicroscope Explorer AFM equipped with a 100 μm tripod scanner, in non-contact mode, using high resonant frequency (F_0 = 320 kHz) pyramidal cantilevers with silicon probes at a scan frequency of 1 Hz. Images are processed with the SPMLab 5.01 software package and are presented unfiltered.

Samples were prepared by depositing a volume of 10 μl of SLNs suspensions on freshly cleaved mica plates, imaging was processed after drying the samples overnight at 37 °C.

2.4. X-ray powder diffraction (XPD)

XPD data were recorded on a Rikagu RU15 diffractometer at 30 kV, 15 mA for Cu Kα (λ = 1.5418 Å), with a scan speed of 1°/min and a step size of 0.02° in 2θ at room temperature.

2.5. ^{1}H NMR spectroscopy

NMR spectra were recorded on a Varian Unity 500 MHz spectrometer, on suspensions of SLNs prepared following the standard procedure described below, in H_2O/D_2O (95:5), 32 K scans were accumulated.

2.6. SLN preparation

A standard method for the preparation of SLNs is defined as follows.

The SLNs were prepared by the solvent displacement method (Perrier et al., 2001). *Para*-dodecanoyl-calix[4]arene was dissolved in tetrahydrofuran (THF) at a concentration of 5 mg/ml. Under magnetic stirring (500 rpm), in a conical flask, to a volume of 2 ml of a THF solution, was added 100 ml of pure water (resistivity > 18 MΩ) at a constant flow rate of 300 ml/min. The slightly milky suspension was stirred for an additional minute. After evaporation of the solvent at 40 °C under reduced pressure the final volume was adjusted at 100 ml, giving an SLN concentration of 0.1 g/l.

2.7. Systematic study of preparation parameters

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

2.7.1. Hydrophobic chain length effect

SLNs were prepared from *para*-hexanoyl-, *para*-octanoyl-, *para*-decanoyl-calix[4]arene were used at a concentration of 5 mg/ml in THF.

2.7.2. Organic solvent

Para-hexanoyl-calix[4]arene was dissolved in hot acetone, hot methanol and hot ethanol at a concentration of 5 mg/ml. Before the preparation of the SLNs, the glassware was heated at 50 °C to prevent the precipitation of the calix-arene. Otherwise the procedure was identical to that described above.

2.7.3. Mixed SLNs calix-arene/Pluronic® F68

Increasing quantities of Pluronic® F68 were dissolved (0.125, 0.25, 0.5 and 1 mg/ml) and were co-dissolved with *para*-dodecanoyl-calix[4]arene.

2.7.4. Viscosity of the aqueous phase

Increasing amounts of glycerol (2, 4, 6, 8 and 10%, v/v) were dissolved in the aqueous phase before SLNs preparation. Prior to measurement the suspensions were diluted 10 times to make the effect of viscosity negligible in the PCS measurements.

2.7.5. Influence of the acidity of the aqueous phase

The SLN suspensions were prepared following the standard methodology, varying the pH of the aqueous phase from 1 to 8 with HCl and NaOH.

2.7.6. Stirring speed

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

2.7.7. Concentration

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

2.7.8. Proportion of organic phase

Increasing volumes of THF solutions, from 2 to 10 ml and keeping the final quantity of calix-arene constant at 0.1 g/l, were used to prepare the SLNs suspension. All the organic solvent was removed under reduced pressure and the suspensions were stored at room temperature. Size measurements by PCS were carried out after 24 h.

2.8. Post-preparation stability

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

2.8.1. 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, NaI, NaH_2PO_4, Na_2SO_4, $NaCH_3CO_2$, $NaHCO_3$, KCl, KNO_3, KH_2PO_4, KI) at final concentrations of 10^{-4}, 10^{-3}, 10^{-2}, 5×10^{-2}, 10^{-1} and 2×10^{-1} M. The size of the SLNs was measured respectively after 1 h, 1, 7, 30, 60, 120 and 270 days.

2.8.2. Effect of pH

Suspensions of *para*-dodecanoyl-calix[4]arene (**1**) based SLNs were diluted (v/v) in HCl and NaOH solutions in order to obtain pH from 1 to 8. PCS measurements were carried out 24 h after the dilution.

2.8.3. UV irradiation

A volume of 10 ml of SLN suspension, in a beaker, was irradiated during 10, 20, 30, 40, 50 and 60 min with a 254 nm ultraviolet lamp.

2.8.4. Ultrasonic irradiation

One hundred milliliter of a SLN suspension were maintained in an ultrasonic bath during 1 h. Aliquots of 1 ml were removed and analysed by PCS every 10 min.

2.8.5. Microwave treatment

A volume of 100 ml of a suspension of SLNs was stored in a microwave oven during 1 h, 1 ml aliquots were analysed by PCS every 10 min.

2.8.6. Boiling

A suspension of SLN was allowed to stand at reflux during 60 min. One milliliter of the suspension was removed every 10 min, after cooling at room temperature, and allowing the system to stand during 1 h, PCS analysis was carried out.

2.8.7. Freezing–unfreezing

A volume of 100 ml of SLNs was stored during 12 h at $-15\,^{\circ}C$, unfrozen at room temperature. A 1-ml aliquot was analysed by PCS. The same procedure was repeated twice.

2.8.8. Freeze-drying

A volume of 100 ml of SLNs was concentrated to a volume of 10 ml under reduced pressure, the resulting suspension was dried at $-54\,^{\circ}C$ in vacuo overnight, the resulting white powder was re-suspended in water and analysed by PCS.

3. Results and discussion

The physical and structural characterisation of SLNs prepared from **1** (Shahgaldian et al., 2002) by the solvent diffusion method (Perrier et al., 2001) was carried out by PCS, non-contact mode AFM, XPD and 500 MHz ^{1}H NMR spectroscopy.

The results of the PCS experiments gave a hydrodynamic diameter for the SLNs of 147 nm with a high degree of monodispersity.

In Fig. 2 is given the non-contact mode AFM image of freshly prepared SLNs deposited on mica and dried before imaging; the scan range is $4\,\mu m \times 4\,\mu m$. The SLNs can clearly be seen as round objects of 250 nm diameter and 60 nm height. While slightly flattened during the drying process (Montasser et al., 2002),

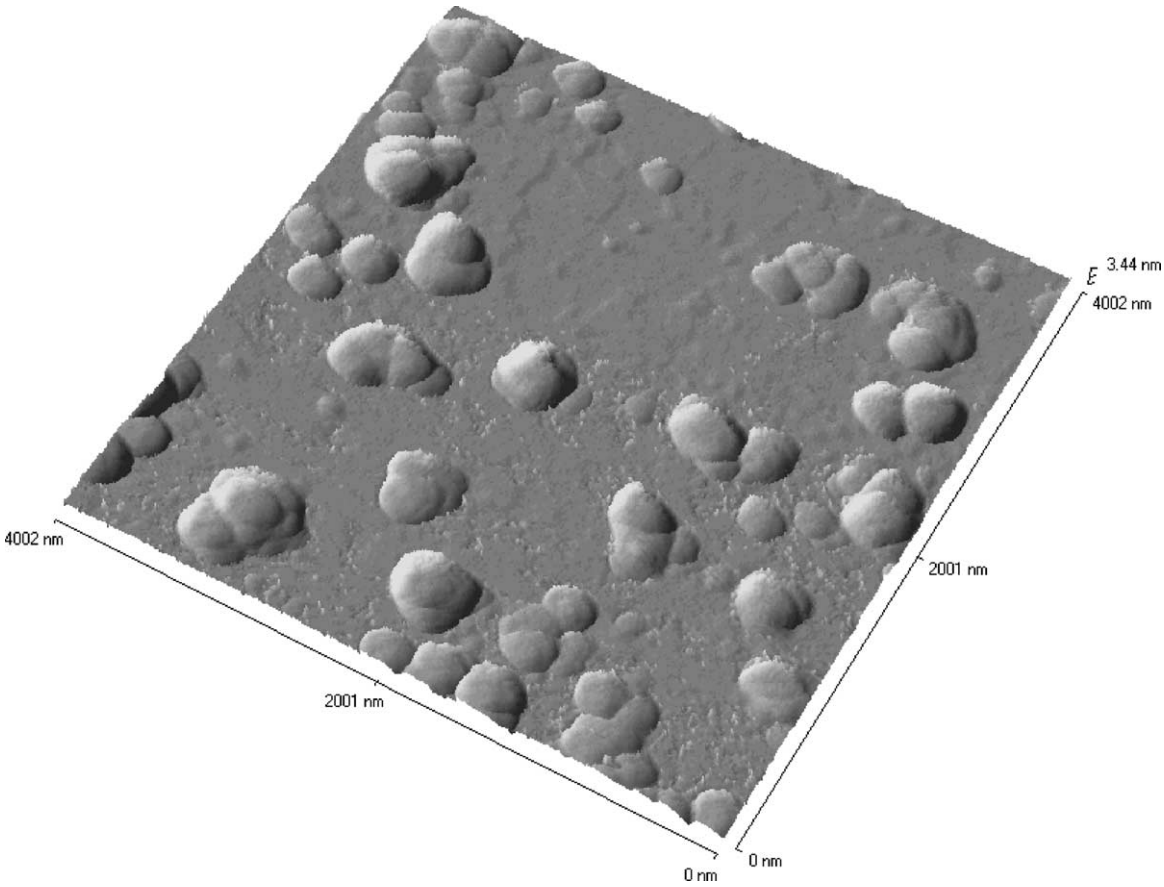

Fig. 2. Non-contact mode AFM image of freshly prepared **1** based SLNs.

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 (Paclet et al., 2000; Tanaka et al., 1999), or commonly spread out to form Supported Lipid Bilayer (Groves et al., 1997; Sackmann, 1996).

The XPD diagram is given in Fig. 3, two clear diffraction peaks are observed at $2\theta = 6.80$ and 7.68. Clearly, there is some degree of crystallinity present in the SLNs, other powder diffraction patterns have been observed for cetylpalmitate derived SLNs by Jenning et al. (2000b). The spacings in the diffraction pattern differ from those observed for single crystals of the parent system (Shahgaldian et al., 2002) implying a different crystalline arrangement.

The 500 MHz ^{1}H NMR spectrum of the SLNs was obtained in H_2O/D_2O (95:5) using a total of 32,000 scans. The extremely long collection time is consistent with a rigid crystalline matrix, and correlates well with the previously reported work of Jenning et al. (2000a) on the ^{1}H NMR study of glyceryl behenate based SLNs. As in that work the methylene protons are observed as broad peaks in the range 1.5–2.0 ppm. The terminal methyl protons are observed at 0.85 ppm as a slightly sharper signal, probably due to some degree of residual liberty at the chain ends. The aromatic protons are observed as one signal at 7.6 ppm, corresponding to the values of the parent molecule (Shahgaldian et al., 2001). The methylene bridge protons are not observed in this case.

In view of the above results, we feel confident that the systems observed in this paper represent a new

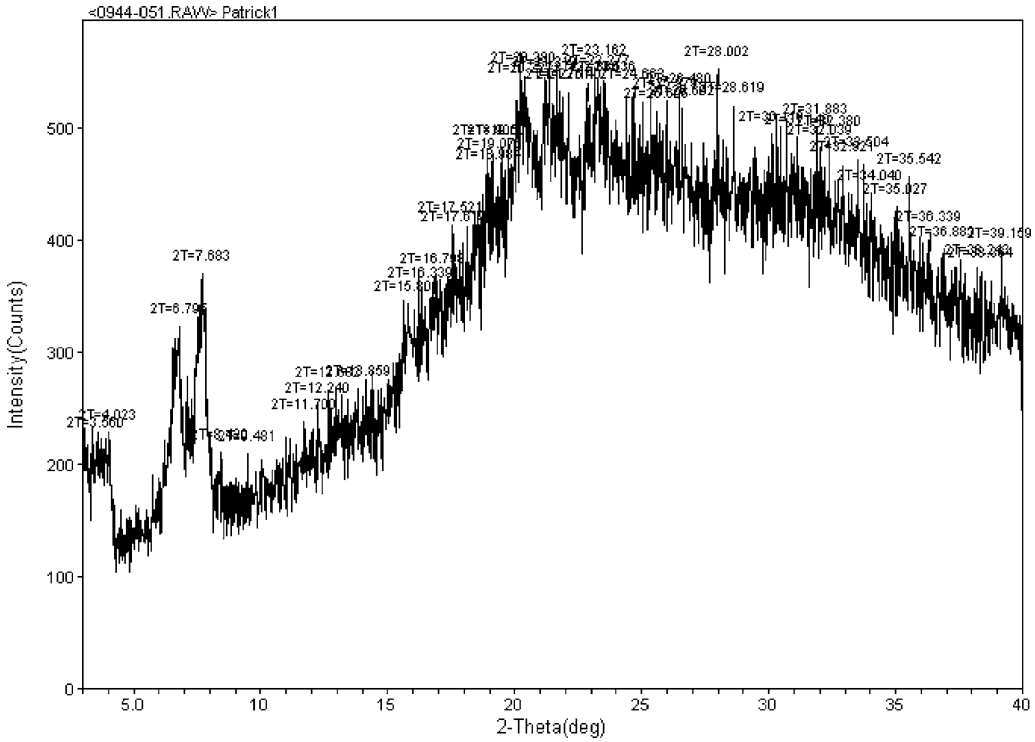

Fig. 3. X-ray powder diffraction pattern of freeze-dried *para*-octanoyl-calix[4]arene based SLNs.

group of SLNs based on solid lipid supramolecular systems.

In terms of the parameters which may affect the formation and initial colloid size, we investigated: (a) length of the hydrophobic chain at the *para*-position on the calix-arene, (b) nature of the organic solvent used in the preparation, (c) presence of a co-surfactant, (d) effects of viscosity of the aqueous phase, (e) aqueous phase pH, (f) variation of the dispersion speed, (g) final SLN concentration and (h) proportion of the organic phase.

In Fig. 4 are presented the observed hydrodynamic diameters of the *para*-acyl-calix[4]arene based SLNs, as a function of the acyl chain length, the values (D_e) are given as ratios the diameter of the *para*-dodecanoyl-calix[4]arene derived SLNs (D). The values are essentially constant. Given that the single crystal X-ray structures of *para*-octanoyl-calix[4]arene (Shahgaldian et al., 2002) and *para*-dodecanoyl-calix-[4]arene (unpublished results) show identical and interdigitated packing, such invariant size is not unexpected.

In Fig. 5 are presented, as ratios of the diameter for THF as the organic solvent diameters for SLNs prepared using acetone, methanol (MeOH) and ethanol (EtOH) as solvent phases for the amphiphile in the SLN preparation. While the SLNs produced using EtOH as the organic phase are the same size as those from THF, those using acetone or MeOH as the dispersant show larger, 1.33 and 1.44 times, respectively, diameters. This effect may arise from differences in the chain arrangements for *para*-acyl-calix-arenes when complexing certain of the co-solvents. These results are in good agreement with the size variations due to preparation solvent effects for poly(DL-lactide-co-glycolide) nanoparticles previously reported (Jeon et al., 2000; Jeong et al., 2001), and in contrast to those published by Debuigne et al. (2000) dealing with the preparation of organic nanoparticles using the water/oil emulsion technique, where no solvent dependant size variations were observed.

In contrast to cyclodextrin based SLNs (Skiba et al., 1993) and many other SLN systems, the *para*-acyl-calix-arene based SLNs can be prepared in

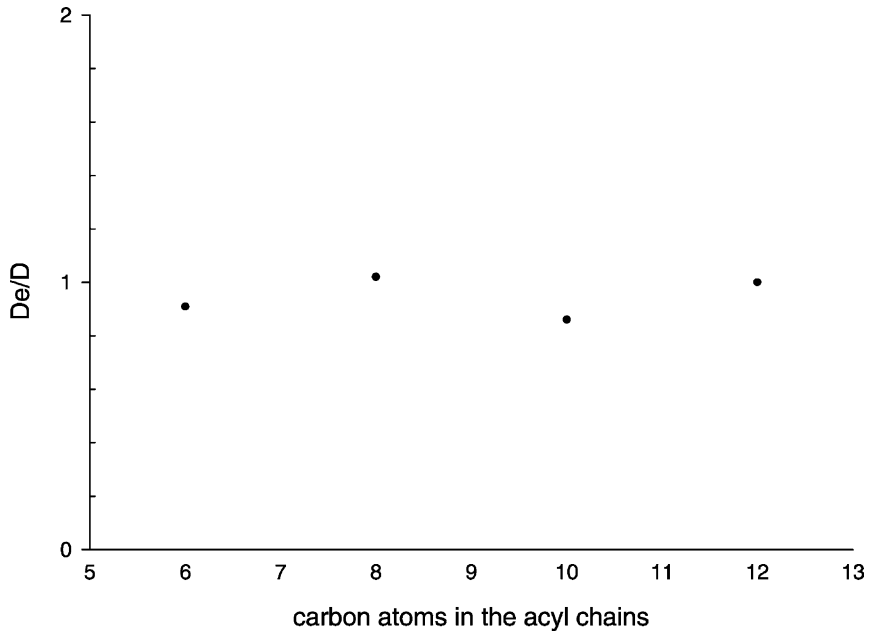

Fig. 4. Influence of the hydrophobic chain length of *para*-acyl-calix[4]arene on the hydrodynamic diameter measured by PCS, values are expressed as a ratio of the diameter of the SLNs prepared following the standard method.

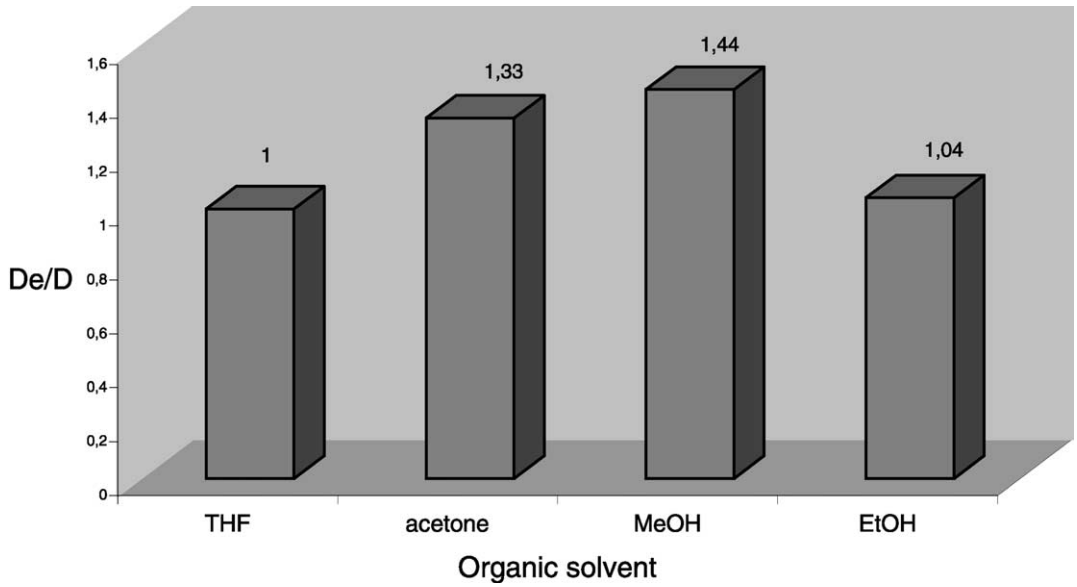

Fig. 5. Effects of the organic solvent used during the preparation on the hydrodynamic diameter of **1** based SLNs, values are expressed as a ratio of the diameter of the SLNs prepared following the standard method.

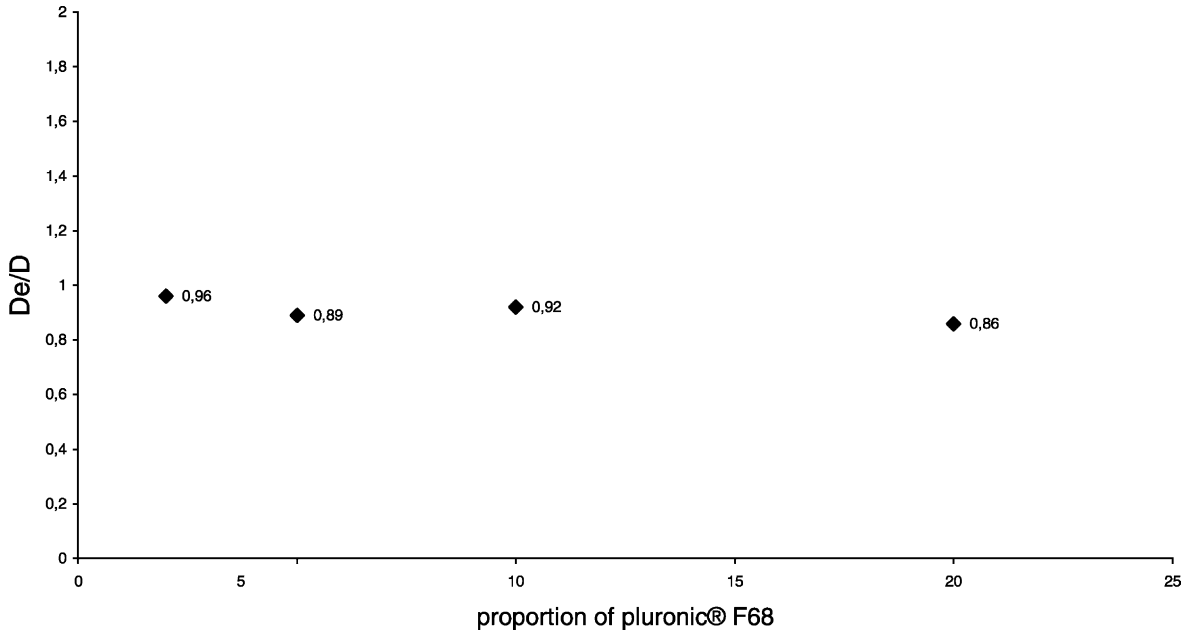

Fig. 6. Effects of the presence of Pluronic® F68 in the organic phase on the hydrodynamic diameter of **1** based SLNs, values are expressed as a ratio of the diameter of the SLNs prepared following the standard method.

the absence of a co-surfactant. In order to observe if a co-surfactant has any observable influence of the SLNs, the colloids were prepared with Pluronic® F68 present in the organic phase (Fig. 6). A small, but significant, diminution in the PCS derived hydrodynamic diameter is observed as a function of increasing Pluronic® F68 concentration. This effect may arise from the emulsifier role of the surfactant during the preparation, (Song et al., 1997) or as a stabilising agent for polymeric nanoparticles (Quintanar-Guerrero et al., 1996). These results are also in good agreement with those reported by Scholer et al. (2001), where it was shown that changing the nature of poloxamer used influences the size and the polydispersity index of dynasan 114 based SLNs.

In the solvent diffusion method, changes in the viscosity of the aqueous phase may affect the kinetics of the outward diffusion of the organic phase and hence the size of the SLNs. To test this, calix-arene based SLNs were prepared with aqueous solutions of up to 10% glycerol, i.e. with viscosity ranges of 0.89 cP for pure water to 1.15 cP in the latter case. As shown in Fig. 7, there is no significant variation in the observed hydrodynamic diameter. In the work of Quintanar-Guerrero et al. (1996) on the preparation of poly(DL-lactic acid) nanoparticles by the emulsification–diffusion technique, it was shown that an increase of viscosity of the external phases causes a decrease of the mean size of the nanoparticles produced.

The pH stability of SLNs is a key point in both the preparation and the application of such systems. In Fig. 8 is given the size of the SLNs observed 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 2–8, i.e. throughout the range of physiological pH values. The pH stability of other SLN has been studied by Zimmermann and Müller (2001), where it was shown that to produce stable SLNs, suitable systems for gastro-intestinal administration, the mixture of surfactant/lipid must be optimised.

The rate of dispersion of the amphiphile into the aqueous phase has been shown to affect the preparation of polymeric nanoparticles prepared by the emulsification–diffusion method (Quintanar-Guerrero et al., 1996) causing a decrease in nanoparticle size when the stirring speed is increased. In Fig. 9 is presented the size of the calix-arene based SLNs prepared

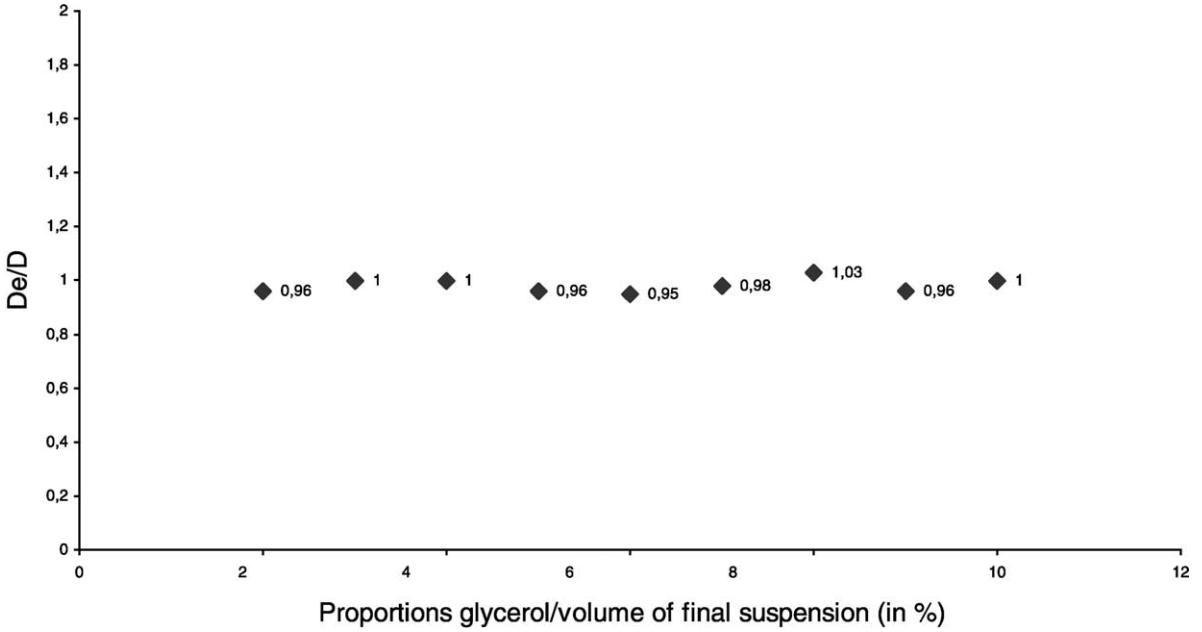

Fig. 7. Effects of the aqueous phase viscosity on the hydrodynamic diameter of **1** based SLNs, values are expressed as a ratio of the diameter of the SLNs prepared following the standard method.

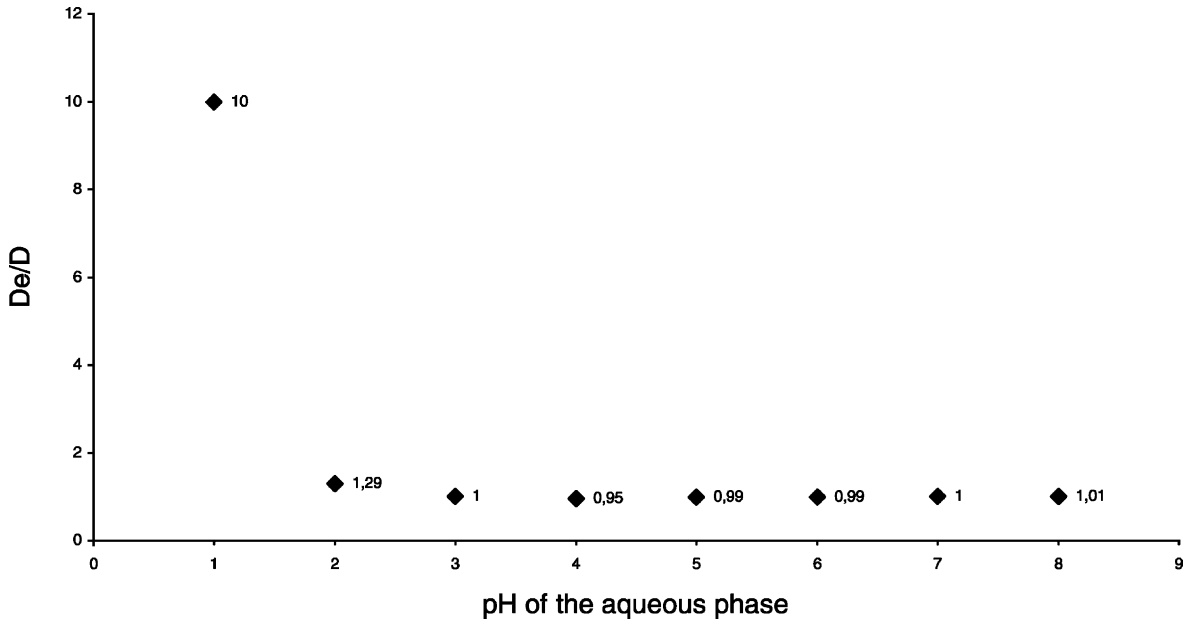

Fig. 8. Influence of the pH of the aqueous phase used during the preparation process on the hydrodynamic diameter of **1**, values are expressed as a ratio of the diameter of the SLNs prepared following the standard method.

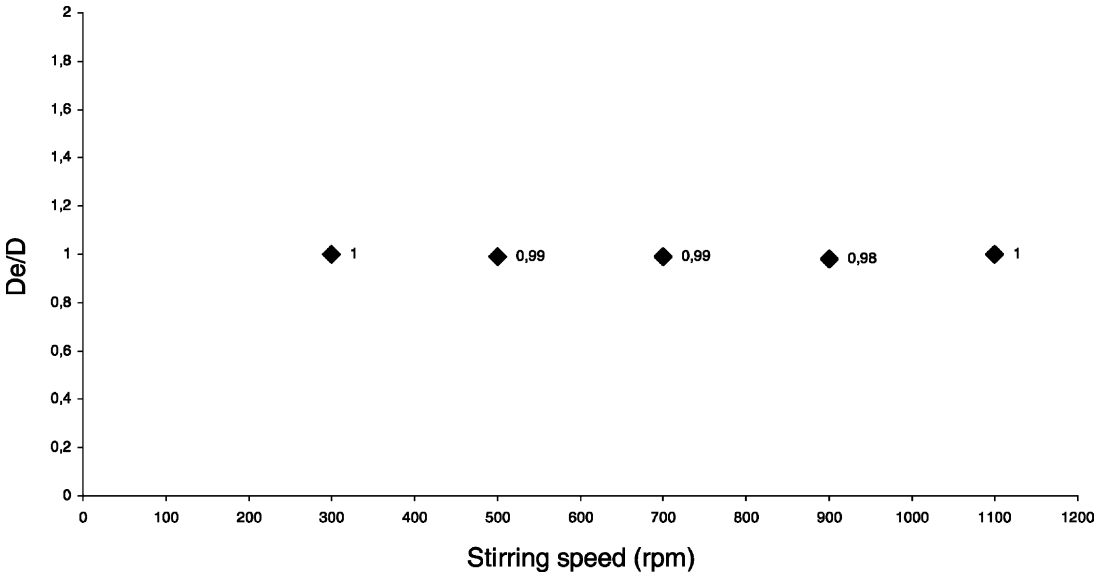

Fig. 9. Effects of the stirring speed used during the preparation on **1** based SLNs, values are expressed as a ratio of the diameter of the SLNs prepared following the standard method.

at stirring speeds between 300 and 11000 rpm. It can be seen that here the stirring speed and hence the rate of dispersion has no effect on the particle size.

In Fig. 10 are presented the variations in SLN size for final aqueous dispersion concentrations of between 100 and 600 mg/l. Taking the size at 100 mg/l as unity, an increase in size occurs up to 300 mg/l at which a plateau of about 1.8 relative size units is observed. This is in contrast to the work of Debuigne et al. (2000) where amphiphile concentration did not appear to affect drastically the size of the nanoparticles produced by the water/oil emulsion technique. In that case the invariant size was explained by thermodynamic stabilisation of the particles by the co-surfactant.

Given the lack of effects for the rate of diffusion due to change in stirring speeds, the above result can be explained by assuming that for low concentrations of **1** in the organic phase solvent droplets of the same size are dispersed into the aqueous phase, the solvent diffuses out into the aqueous phase and the size is determined by the residual quantity of **1** in the dispersion. However, the invariance in size above a concentration of 300 mg/l requires that the mass of the particle is the determinant factor in the final size of the colloid. Thus, here we have two different mechanisms of size control, at low concentration, i.e. below a critical mass droplet are of the same size and then above this value all droplets are dispersed with the same mass.

The final parameter tested involved the volume of organic solvent used in the preparation of the SLNs. Volume of 2–10 ml of THF were used, while holding the final calix-arene concentration constant, the results presented in Fig. 11. There is a bimodal effect with a maximum increase about 5 vol.% of THF. Such maxima are well known for physical properties of water with such solvents as THF (Franks, 1973) and even occur for cyclodextrin solubilities (Chatjikagis et al., 1992) and arise from clathration in solution. There is thus an increase related to solvent restructuring modifying the dispersion of the droplets during SLN formation.

Certain post-preparation parameters have also been studied; in this section we treat those to which the SLNs might be subjected during formulation, storage or application.

In terms of these external post-preparation effects, six parameters were examined: (a) ionic strength of the storage solution, (b) pH of this solution; (c) UV

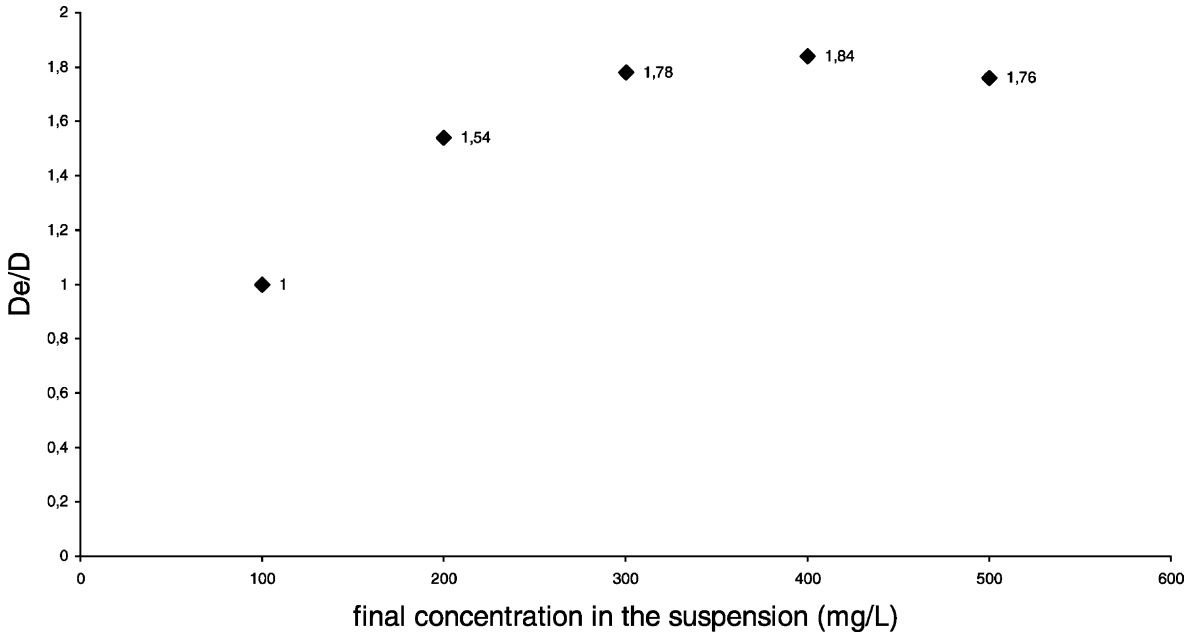

Fig. 10. Effects of the concentration of **1** in the organic phase in the SLNs production process, values are expressed as a ratio of the diameter of the SLNs prepared following the standard method.

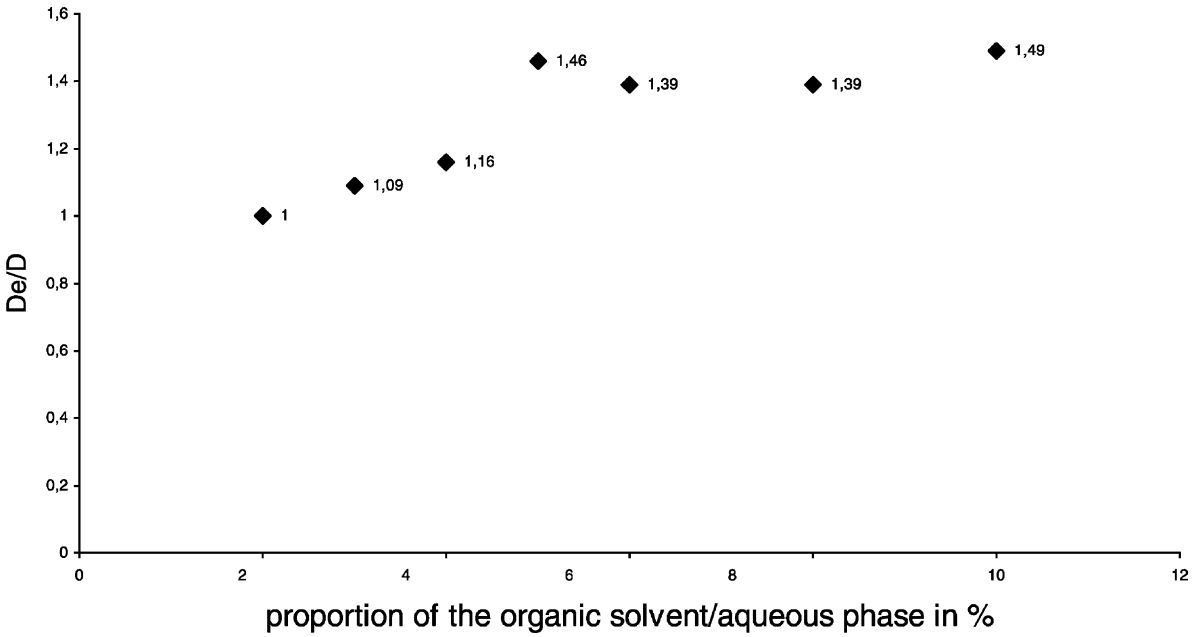

Fig. 11. Effects of the volume of organic solvent in the **1** based SLNs production process, values are expressed as a ratio of the diameter of the SLNs prepared following the standard method.

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

Salt	D_e/D
NaCl	1.01
NaI	1.08
NaH_2PO_4	1
Na_2SO_4	Precipitated
$NaCH_3CO_2$	1.12
$NaHCO_3$	1.01
KCl	0.98
KNO_3	0.99
KH_2PO_4	1.25

irradiation, (d) ultrasonic treatment, (e) short time temperature effects, (f) microwave irradiation, (g) centrifugation, (h) freeze-drying and (i) freezing–defreezing cycles.

The possibility to form SLNs, stable at near physiological ionic strengths is obviously a key element for the biomedical and biological applications of SLNs. The effects of various monovalent salts at an ionic strength of 0.1 M on the calix-arene based SLNs were studied. In Table 1 are summarised diameters of SLNs prepared in pure aqueous solutions and then added to the relevant sodium and potassium salt solutions, the values given are those observed immediately after mixing the SLNs and the salt solution, i.e. at time 0. For solution of sodium and potassium chloride, sodium phosphate, sodium carbonate, and potassium nitrate the size of the SLNs is the same as that observed in pure water. For the other salts, an increase in size of between 10 and 25% is observed revealing a certain instability. In the case of sodium sulphate SLNs are completely destabilised and precipitate.

Previous work on the effects of electrolytes on the stability of Compritol formulation has shown; for sodium, calcium and aluminium chloride, that a destabilising effect was observed with increasing electrolyte concentration and increasing valence, inducing gelation of the systems (Zimmermann and Müller, 2001).

Here there appear to be only slight differences between the effects of the monovalent cations Na^+ and K^+. This is in agreement with our previous work on the stabilisation of Langmuir monolayers of **1** (Shahgaldian and Coleman, 2001). However, as with the reported influence of anions on monolayer stabilisation, a stronger anion influence is observed. Treating the increase in size as a measure of the anion influence we find, for Na^+ as the cation:

$$SO_4^{2-} > CH_3CO_2^- > I^- > PO_4^{3-} \approx Cl^- \approx NO_3^- \approx CO_3^{2-},$$

this may be compared to the Langmuir film balance study on the film stability were

$$CO_3^{2-} > CH_3CO_2^- \gg I^- \approx Cl^- \approx NO_3^- \approx SO_4^{2-} > PO_4^{3-}.$$

We see, interestingly, little correlation between the effects of the anions on the stability of calix-arene based SLNs and monolayer stabilisation effects.

The SLNs are stable in the pH range 2–8 (Fig. 12). These values are essentially identical to those observed for the variation of pH of the preparation phase.

The results concerning the stability of calix-arene based SLNs, submitted to UV, ultrasonic and microwave irradiations as well as thermal treatment are given in Fig. 13, it can clearly be seen that for all post-preparation treatments no effect is observed. Thus, the SLNs prepared from calix-arenes will be stable for use in topical preparations, and may be sterilised by either heat or microwave treatment.

In the case of freezing–unfreezing cycles, the suspensions are stable at least for one cycle, after which the SLNs are destabilised and precipitate.

In the case of freeze-drying, the particle size is not apparently modified. They are, however, difficult to redisperse, requiring up to 1 h of ultrasonic treatment. Intravenous use of SLNs requires perfect redispersion, aggregates of more than 5 μm in diameter may cause embolism (Zimmermann et al., 2000). These above problems with freeze-drying and freezing–defreezing are the only clear weak points in the stability of the calix-arene based SLNs so far observed. However, preliminary results suggest that the use of cryoprotectant sugars may remove this disadvantage.

Finally, the temporal stability of the SLNs, both in the absence and presence of salts, and as a function of storage temperature were studied.

In Fig. 14 are shown the stability curves for aqueous dispersions of SLNs stored at 4, 20 and 40 °C, respectively over a 1-year period. During all the duration of this study, the calix-arene based SLNs remain

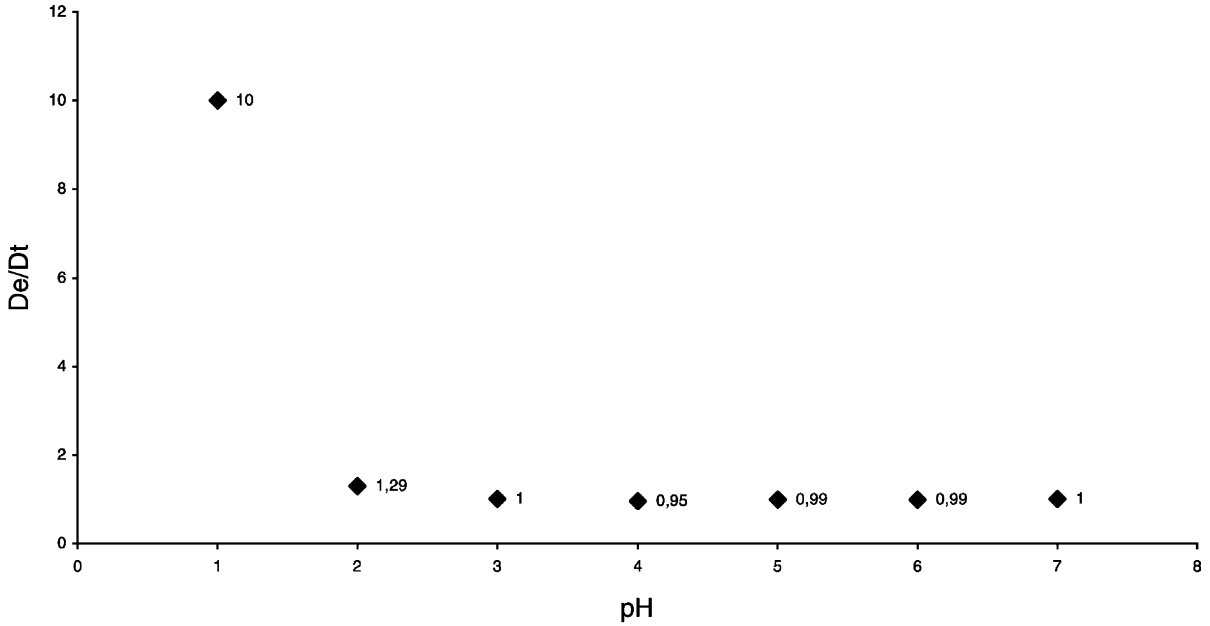

Fig. 12. Effects of the pH on the stability of **1** based SLNs, values are expressed as a ratio of the diameter of the SLNs in pure water.

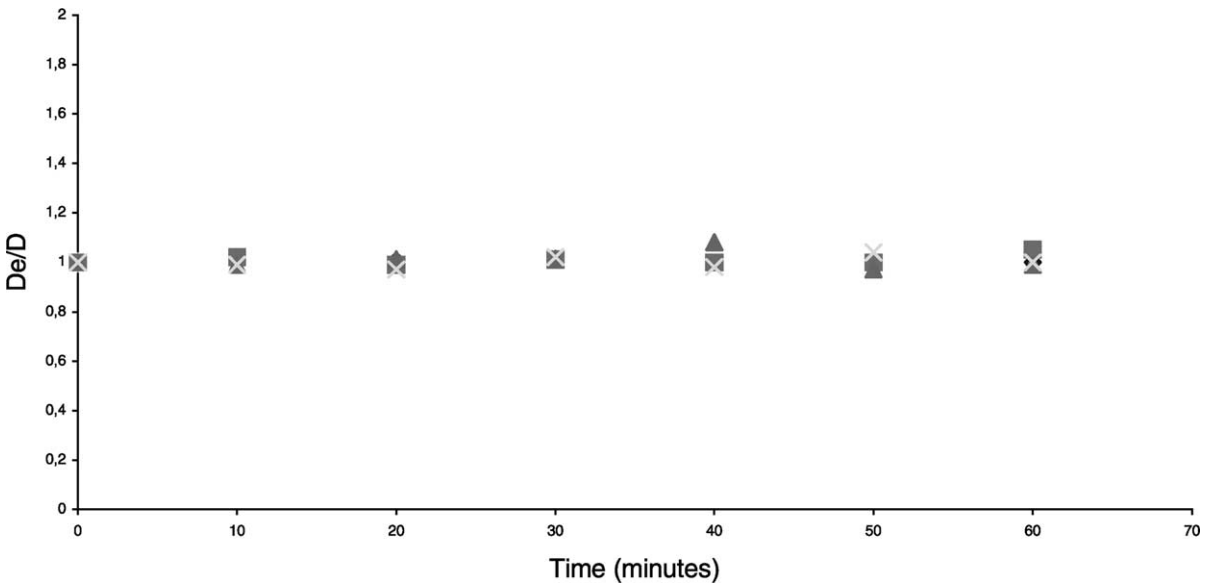

Fig. 13. Effect of post-preparations effects (■: boiling; ▲: UV irradiation; ◆: microwave treatment, ✕: ultrasonic treatment) on the stability of **1** based SLNs.

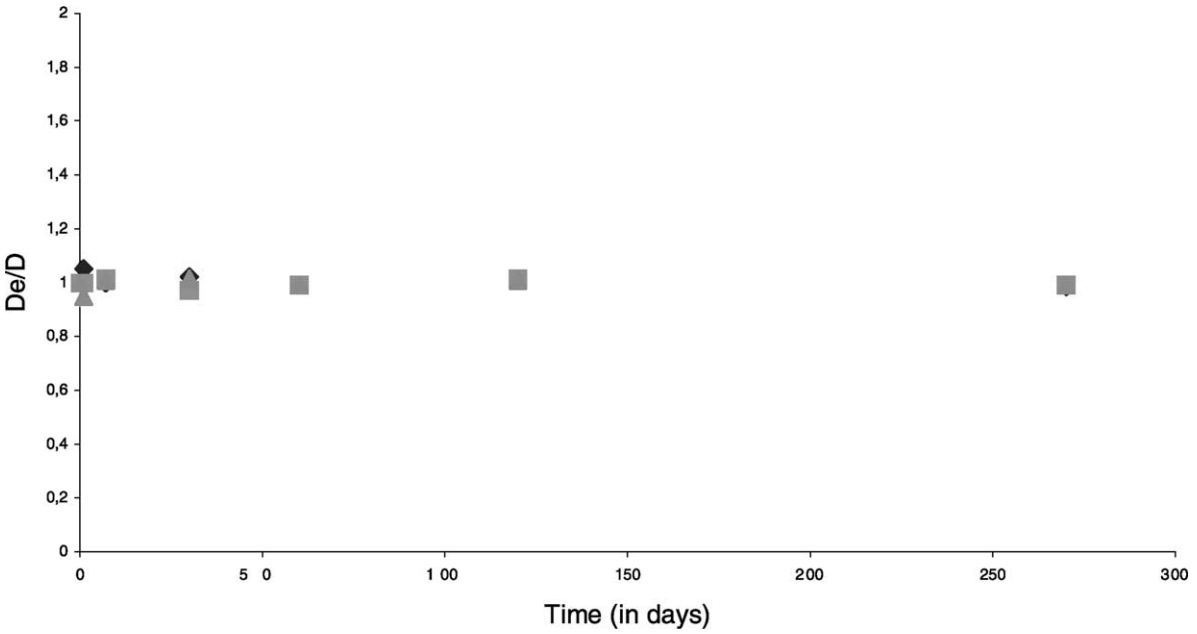

Fig. 14. Temporal stability of **1** based SLNs at 4 °C (■), 20 °C (▲) and 37 °C (◆), values are expressed as a ratio of the diameter of the SLNs prepared following the standard method.

as stable suspensions, and their size is unaffected under these typical storage conditions.

In Table 2 are presented the results for the temporal stability of SLNs stored in solutions containing various potassium and sodium salts at 0.1 (a) and 10^{-3} M (b). For salt concentrations of 10^{-3} M, only the sulphate and the dihydrogeno-phosphate have an effect on the size and the stability of calix-arene based SLNs, causing total destabilisation and precipitation in less than respectively 210 and 260 days. All other suspensions remain stable at least 260 days. For salt concentrations of 0.1 M, the most important destabilising effect is due to Na_2SO_4, SLNs precipitate immediately after mixing these solutions. In the case of KCl and NaH_2PO_4, the stability of SLNs is less than 24 h. For NaCl, KNO_3, KH_2PO_4, $NaCH_3CO_2$, the SLNs are destabilised in less than 7 days. Less important effects are observed for $NaHCO_3$ where precipitation occurs after about 7 months.

In Fig. 15 is presented a non-contact mode image of 1-year-old calix-arene based SLNs deposited from an aqueous suspension onto mica and then dried. Both size, height and surface dispersion are identical to those of freshly prepared SLNs (Fig. 1).

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 **1** based SLNs

Salt	$D_e/D > 1.1$	$D_e/D > 1.5$	Precipitation
(a)			
Na_2SO_4	–	–	t_0
KCl	–	–	24 h
NaH_2PO_4	–	–	24 h
NaCl	–	24 h	7 days
KNO_3	24 h	–	7 days
KH_2PO_4	t_0	–	7 days
$NaCH_3CO_2$	t_0	–	7 days
NaI	7 days	–	30 days
$NaHCO_3$	–	–	210 days
(b)			
Na_2SO_4	t_0	24 h	210 days
KCl	–	–	–
NaH_2PO_4	–	–	260 days
NaCl	–	–	–
KNO_3	–	–	–
KH_2PO_4	–	–	–
$NaCH_3CO_2$	–	–	–
NaI	–	–	–
$NaHCO_3$	–	–	–

Values are expressed in hours or days, t_0 means that the particles flocculate as soon as they are mixed with the salt solution.

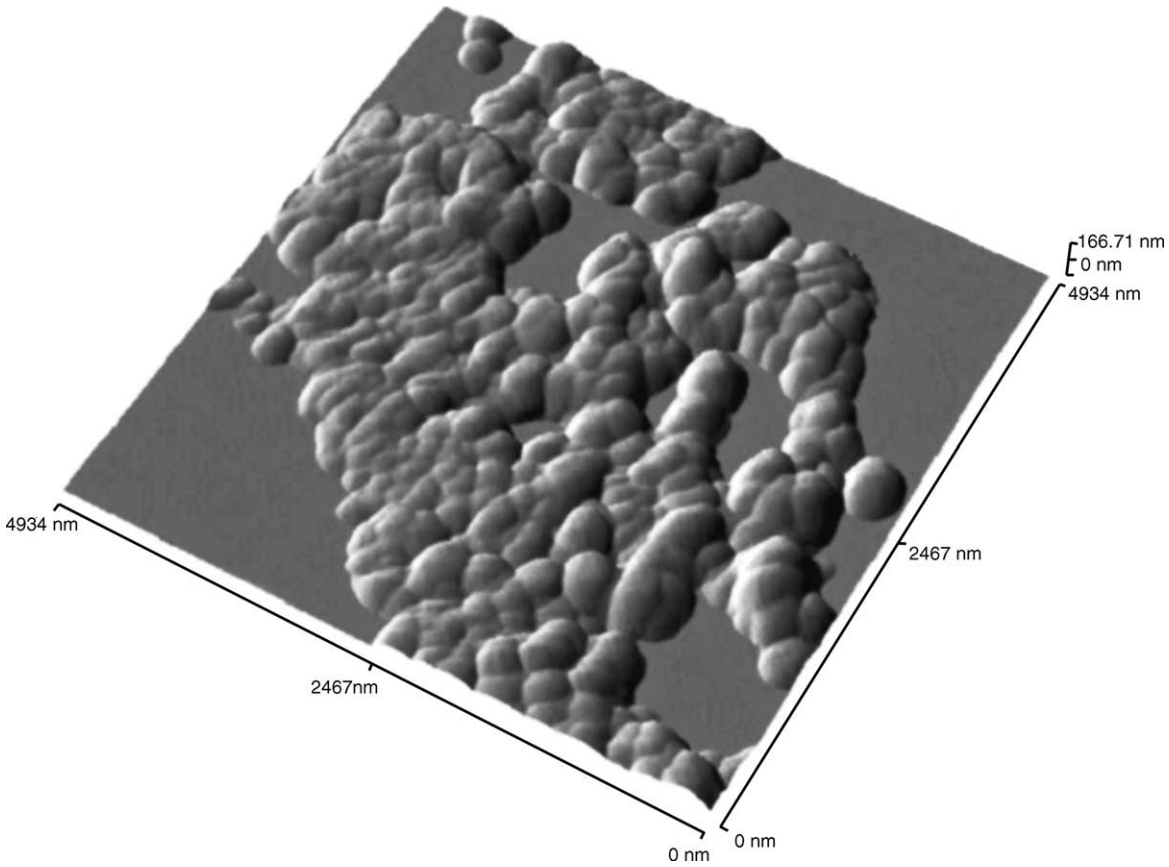

Fig. 15. Non-contact mode AFM image of 1-year-old **1** based SLNs.

4. Conclusion

We have systematically investigated a wide range of parameters on the stability of calix-arene based SLNs, these parameters concern effects which may occur during preparation, post-preparation formulation or treatment and long term storage of the SLNs. The calix-arene based SLNs are remarkably robust and stable systems, with destabilisation occurring only during freeze-drying/redispersion and in the presence of high ionic strengths of certain salts.

Acknowledgements

Authors gratefully acknowledge Central P bv and "La Fondation pour la Recherche Médicale" for their financial support.

References

Chatjikagis, A.K., Donze, C., Coleman, A.W., 1992. Solubility behavior of β-cyclodextrin in water/co-solvent mixtures. Anal. Chem. 64, 1632–1634.

Coleman, A.W., Kasselouri, A., 1993. Supramolecular assemblies based on amphiphilic cyclodextrins. Supramol. Chem. 1, 155–161.

Combaut, G., Chantraine, J.M., Teste, J., Glombitza, K.W., 1978. Bromophenols of red algae: cyclotribromoveratrylene, a new derivative obtained during extraction of *Halopytis pinastroides*. Phytochemistry 17, 1795–1797.

Debuigne, F., Jeunieau, L., Wiame, M., Nagy, J.B., 2000. Synthesis of organic nanoparticles in different W/O microemulsions. Langmuir 16, 7605–7611.

Duchêne, D., Ponchel, G., Wouessidjewe, D., 1999a. Cyclodextrins in targeting. Application to nanoparticles. Adv. Drug Deliv. Rev. 36, 29–40.

Duchêne, D., Wouessidjewe, D., Ponchel, G., 1999b. Cyclodextrins and carrier systems. J. Control. Release 62, 263–268.

Franks, F., 1973. Water, a Comprehensive Treatise, vol. 2. Plenum Press, London.

Gansey, M.H.B.G., De Haan, A.S., Bos, E.S., Verboom, W., Reinhoudt, D.N., 1999. Conjugation, immunoreactivity, and immunogenicity of calix[4]arenes; model study to potential calix[4]arene-based Ac^{3+} chelators. Bioconjugate Chem. 10, 613–623.

Groves, J.T., Ulman, N., Boxer, S.G., 1997. Micropatterning fluid lipid bilayers on solid supports. Science 275, 651–653.

Gutsche, C.D., 1989 Calixarenes Revisited: Monographs in Supramolecular Chemistry. The Royal Society of Chemistry, Cambridge.

Houel, E., Lazar, A., Da Silva, E., Coleman, A.W., Solovyov, A., Cherenok, S., Kalchenko, V.I., 2002. Interfacial interactions of cations with amphiphilic dihydroxyphosphonyl-calix-[4]-arene mesosystems. Langmuir 18, 1374–1379.

Jenning, V., Mader, K., Gohla, S.H., 2000a. Solid lipid nanoparticles (SLN) based on binary mixtures of liquid and solid lipids: a ^{1}H-NMR study. Int. J. Pharm. 205, 15–21.

Jenning, V., Thunemann, A.F., Gohla, S.H., 2000b. Characterisation of a novel solid lipid nanoparticle carrier system based on binary mixtures of liquid and solid lipids. Int. J. Pharm. 199, 167–177.

Jeon, H.-J., Jeong, Y.-I., Jang, M.-K., Park, Y.-H., Nah, J.-W., 2000. Effect of solvent on the preparation of surfactant-free poly(DL-lactide-co-glycolide) nanoparticles and norfloxacin release characteristics. Int. J. Pharm. 207, 99–108.

Jeong, Y.-I., Cho, C.-S., Kim, S.-H., Ko, K.-S., Kim, S.-I., Shim, Y.-H., Nah, J.-W., 2001. Preparation of poly(DL-lactide-co-glycolide) nanoparticles without surfactant. J. Appl. Polym. Sci. 80, 2228–2236.

Lemos-Senna, E., Wouessidjewe, D., Duchene, D., 1998. Amphiphilic cyclodextrin nanospheres: particle solubilization and reconstitution by the action of a nonionic detergent. Colloids Surf. B 10, 291–301.

Mehnert, W., Mader, K., 2001. Solid lipid nanoparticles. Production, characterization and applications. Adv. Drug Deliv. Rev. 47, 165–196.

Montasser, I., Fessi, H., Coleman, A.W., 2002. AFM imaging of novel type of polymeric colloidal nanostructures. Eur. J. Pharm. Biopharm. 54, 281–284.

Müller, R.H., Runge, S.A., 1998. Solid lipid nanoparticles (SLN) for controlled drug delivery. Drug Target. Deliv. 9, 219–234.

Müller, R.H., Mader, K., Gohla, S., 2000. Solid lipid nanoparticles (SLN) for controlled drug delivery—a review of the state of the art. Eur. J. Pharm. Biopharm. 50, 161–177.

Paclet, M.H., Coleman, A.W., Vergnaud, S., Morel, F., 2000. P67-phox-mediated NADPH oxidase assembly: imaging of cytochrome b558 liposomes by atomic force microscopy. Biochemistry 39, 9302–9310.

Perret, F., Shahgaldian, P., Mazzorana, M., Esposito, G., Morel-Desrosiers, N., Coleman, A.W., 2000. First steps in the study of the cellular toxicity of the calix-arenes. Book of Abstracts, International Symposium on Supramolecular Chemistry, Fukuoka, Japan.

Perrier, E., Terry, N., Rival, D., Coleman, A.W., 2001. Use of cyclodextrin derivatives for skin preparations, etc., their micelles or nanoparticles, and compositions containing the derivatives. French Patent 2000-6102.

Quintanar-Guerrero, D., Fessi, H., Allemann, E., Doelker, E., 1996. Influence of stabilizing agents and preparative variables on the formation of poly(D,L-lactic acid) nanoparticles by an emulsification–diffusion technique. Int. J. Pharm. 143, 133–141.

Sackmann, E., 1996. Supported membranes: scientific and practical applications. Science 271, 43–48.

Schneider, H.J., Yatsimirsky, A., 2000. Principles and Methods in Supramolecular Chemistry. Wiley, Chichester.

Scholer, N., Olbrich, C., Tabatt, K., Müller, R.H., Hahn, H., Liesenfeld, O., 2001. Surfactant, but not the size of solid lipid nanoparticles (SLN) influences viability and cytokine production of macrophages. Int. J. Pharm. 221, 57–67.

Shahgaldian, P., Coleman, A.W., 2001. Anion and cation interactions with *p*-dodecanoylcalix[4]arene monolayers at the air–water interface. Langmuir 17, 6851–6854.

Shahgaldian, P., Coleman, A.W., Kalchenko, V.I., 2001. Synthesis and properties of novel amphiphilic calix-[4]-arene derivatives. Tetrahedron Lett. 42, 577–579.

Shahgaldian, P., Cesario, M., Goreloff, P., Coleman, A.W., 2002. *Para*-acyl calix[4]arenes: amphiphilic self-assembly from the molecular to the mesoscopic level. J. Chem. Soc., Chem. Commun. 326–327.

Skiba, M., Wouessidjewe, D., Coleman, A., Fessi, H., Devissaguet, J.P., Duchene, D., Puisieux, F., 1993. Preparation and use of novel cyclodextrin-based dispersible colloidal systems in the form of nanospheres. PCT Int. Appl., WO, p. 23.

Sommer, F., Tran Minh, D., Coleman, A.W., Skiba, M., Wouessidjewe, D., 1993. Seeing is believing: imaging amphiphilic–cyclodextrin-derived liposomes by atomic force microscopy. Supramol. Chem. 3, 19–22.

Song, C.X., Labhasetwar, V., Murphy, H., Qu, X., Humphrey, W.R., Shebuski, R.J., Levy, R.J., 1997. Formulation and characterization of biodegradable nanoparticles for intravascular local drug delivery. J. Control. Release 43, 197–212.

Steed, J.W., Atwood, J.L., 2000. Supramolecular Chemistry. Wiley, Chichester.

Szetjli, J., 1998. Cyclodextrin Technology. Kluwer Academic Publishers, Dordrecht.

Tanaka, Y., Miyachi, M., Kobuke, Y., 1999. Selective vesicle formation from calixarenes by self-assembly. Angew. Chem. Int. Ed. 38, 504–506.

Zimmermann, E., Müller, R.H., 2001. Electrolyte- and pH-stabilities of aqueous solid lipid nanoparticle (SLN) dispersions in artificial gastrointestinal media. Eur. J. Pharm. Biopharm. 52, 203–210.

Zimmermann, E., Müller, R.H., Mader, K., 2000. Influence of different parameters on reconstitution of lyophilized SLN. Int. J. Pharm. 196, 211–213.