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The fractal hologram and elucidation of the structure of liposomal carriers in aqueous and biological media

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ABSTRACT

The present study deals with the physicochemical characterization (size, polydispersity, ζ-potential) of dipalmitoylphosphatidylcholine (DPPC) liposomes and DPPC:cholesterol (chol) (9:1 molar ratio) liposomes, and the determination of their fractal dimension (mass fractal (d_f) and surface fractal (d_s)), in an aqueous (HPLC grade water) and in a biological (fetal bovine serum – FBS) medium. Dynamic, static and electrophoretic light scattering and fluorescence spectroscopy are used as experimental techniques to elucidate the structure and physicochemical parameters of liposomes in an ageing study in two different media, as well as their structural response in changes in concentration and temperature. The extended DLVO theory would be the tool to explain the phenomenology of the colloidal behavior in these systems and of their aggregation process. The fractal dimensionality of DPPC liposomes was decreased while for DPPC:cholesterol (9:1) it remained unaffected in the two dispersion media. The structure of the liposomal systems, the process kinetics, and the fractal dimension are consistent with the diffusion-limited cluster aggregation (DLCA) and reaction-limited cluster aggregation (RLCA) models. On the contrary, hydrodynamic radius (R_h) was found to be stable during the variations of colloidal system conditions, especially due to concentration changes. Finally, we suggest that this study can be a rational road map to design advanced Drug Delivery nano Systems (aDDnSs) with improved pharmacokinetic profile which could be considered as crucial for their effectiveness.

1. Introduction

Pharmaceutical nanotechnology is considered to be an attractive area of drug delivery and targeted therapy, especially concerning colloidal nanocarriers (Hughes, 2005; Mishra et al., 2010; Souza et al., 2010). The classification of advanced Drug Delivery nanoSystems (aDDnS), which is mainly colloidal nanoparticles, has been achieved based on their surface functionality, surface morphology and composition (Demetzos, 2010a,b). Additionally, Chimeric aDDnS (chi-aDDnSs), the most attractive class of aDDnSs, combining dendritic and liposomal materials has only recently appeared in the research orientations of drug delivery (Gardikis et al., 2010, 2011; Kontogiannopoulos et al., 2012).

Liposomes represent one of the most thoroughly studied categories of colloidal nanoparticles with potential application as carriers of bioactive molecules (Bangham et al., 1965; Gregoriadis et al., 1974). The high biocompatibility of liposomes makes it possible to use them as models for the study of biological membranes. In the past decades, liposomes are finding a large number of biotechnology applications as drug delivery systems. Colloidal nanocarriers including liposomes, polymeric nanoparticles, etc., can be classified in a more general category of "soft matter", which include a wide range of materials, that cannot be classified as solid or liquid and that can deform easily because their molecules simply rearrange (Bonacucina et al., 2009).

The colloidal properties of nanocarriers (including liposomes) are of paramount importance for their applications. The *D*erjaguin-*L*andau-*V*erwey-*O*verbeek (DLVO) is the central theory for colloidal stability (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948). According to the literature, the hydration interaction energy can play an important role in the liposome aggregation and cluster morphology, though the classical DLVO theory considers only the Coulomb and van der Waals interactions (Roldán-Vargas et al., 2009). The determination of fractal dimension in order to investigate the aggregation mechanism of liposomes in the presence of calcium and its relation to the predictions of the classical DLVO theory on colloid stability has been proposed (Sabín et al., 2006, 2007a,b).

Furthermore, one of the simplest non-equilibrium growth processes that generates branched structures in colloidal systems

Abbreviations: FBS, fetal bovine serum; DPPC, dipalmitoylphosphatidylcholine; d_f, mass fractal; d_s, surface fractal; DLCA, diffusion-limited cluster aggregation; RLCA, reaction-limited cluster aggregation.

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characterized by a fractal dimension (d_f), which is synonymous to mass fractals, different from the Euclidean dimensionality (d), is the diffusion-controlled or limited aggregation process introduced by Witten and Sander (1981) and the diffusion-controlled deposition introduced by Racz and Vicsek (1983). It should be noted that in these models every collision between particles results in the formation of a permanent contact and the fractal dimensionality is insensitive to the sticking probability (Meakin, 1983a,b, 1986). On the other hand, several other aggregation mechanisms have been reported in the literature, for example, ballistic aggregation, chemically limited aggregation and aggregation with restructuring apart from diffusion-limited aggregation (Jullien, 1987). It should be emphasized that chemical aggregation leads to a more compact fractal compared to diffusion limited aggregation (Jullien, 1987).

Witten and Sander have investigated the colloidal aggregation from a theoretical point of view (Witten and Sander, 1981, 1983). Diffusion-limited aggregation (DLA) is a model where particles are added one at a time to a growing aggregate of particles via random walk trajectories. The determination of fractal dimension has been carried out with this model.

The diffusion-limited cluster aggregation was improved by Meakin (1983b) and is an extension of DLA. In this model, the cluster's growth is controlled by diffusion, the sticking probability is equal to one, all collisions between particles are effective and the aggregates are open and branched in their structure. The fractal dimension was estimated to be 1.8 in three dimensions from computer simulations. DLCA is a fast aggregation process, following a power law for the average radius of gyration, R_g:

$$R_g \sim t^{1/d_f} \tag{1}$$

where t is the time and d_f the fractal dimension (Forrest and Witten, 1979). On the other hand, reaction-limited cluster aggregation (RLCA) or Eden model is the slow aggregation process. The Eden model is a simple lattice model for the growth of the clusters, in which the particles are added one at a time at random to sites adjacent to occupied sites. The growth is limited by reaction kinetics due to the presence of an energy barrier to aggregation. The sticking probabilily is smaller than one because a large number of collisions are needed before the particles bind. The fractal dimension was estimated to be 2.1 in three dimensions from computer simulations and the aggregates are more compact and dense than DLCA clusters. The kinetics of RLCA are characterized by a power law for the average radius of gyration, R_g:

$$R_g \sim e^{at} \tag{2}$$

where a is a constant. The value of a depends on the sticking probability. The surface fractal dimension is d_s and the expression which describes the surface fractals is:

$$d_f = 6 - d_s \tag{3}$$

From an experimental point of view it is difficult to find aggregation phenomena described by the concept of surface fractals. Roldán-Vargas et al. reported the first experimental observation of a transition from surface fractals to mass fractal structures in a suspension of aggregating lipid vesicles (Roldán-Vargas et al., 2008) and presented a detailed description of structural and kinetic aspects of liposomal surface to mass fractal transition controlled by magnesium concentration (Roldán-Vargas et al., 2009). It must be noted that these two limiting regimes of irreversible growth of aqueous colloidal aggregates are universal. The "universality" of these aggregation phenomena was supported by Lin et al. (1989).

There are a large number of techniques available for the characterization of the structure of aggregates formed from suspensions and dispersions of particles in micro and nano scale and for the determination of their fractal dimension (Gregory, 2009). Light scattering provides the greatest potential for use as a tool for elucidation and characterization of the structure of nanoparticles and aggregates, alike. The physical theories, which were developed for aggregation phenomena, include a fractal formalism for elucidating the shape of the resulting aggregates. Recently, this formalism has been introduced to study the geometry of liposomal aggregates.

Additionally, the fractal analysis is used to determine the morphology of colloidal nanostructures other than liposomes. Fractal growth of poly(amidoamine) {PAMAM} dendrimers aggregates was observed in aqueous medium due to their self-assembling (Metulio et al., 2004; Jasmine and Prasad, 2010), while polymeric growth process and kinetics of gold nanoparticle aggregation were described by fractal geometry in order to better understand their physical behavior (Sotiriou et al., 2007; Ogasawara et al., 2000; Kim et al., 2008).

The goal of this study is to investigate the stability or the aggregation of liposomal carriers composed of DPPC with or without cholesterol, to determine their fractal dimension (mass fractal and surface fractal), specify the physicochemical characteristics in different media and assign the dependence of the fractal dimension on the concentration and temperature. Dynamic, static and electrophoretic light scattering and fluorescence spectroscopy were used to elucidate the structure and physicochemical parameters of liposomal carriers in an ageing study in two different media, as well as their structural response in changes of concentration and temperature. Results are discussed in the framework of available theories and physicochemical models.

2. Materials and methods

2.1. Materials

The phospholipids used for liposomal formulations were 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and cholesterol ((3β)-cholest-5-en-3-ol). They were purchased from Avanti Polar Lipids Inc., (Albaster, AL, USA) and used without further purification. Chloroform and all other reagents used were of analytical grade and purchased from Sigma–Aldrich Chemical Co. Fetal bovine serum (FBS) was purchased from Gibco® invitrogen™ and it was also used without further purification.

2.2. Liposome preparation

Two different liposomal formulations have been prepared using the thin-film hydration method, one composed of DPPC and one of DPPC:chol (9:1 molar ratio). Briefly, appropriate amounts of DPPC and DPPC/chol (9:1 molar ratio) were dissolved in chloroform and then transferred into a round flask connected to a rotary evaporator (Rotavapor R-114, Buchi, Switzerland). Vacuum was applied and the thin film was formed by slow removal of the solvent at 40 °C. The lipid film was maintained under vacuum for at least 12 h in a desiccator to remove solvent traces and subsequently it was hydrated in a sucrose solution (150 mM), by slowly stirring for 1 h, in a water bath above the phase transition of lipids (41° C). The resultant multilamellar vesicles (MLVs) were subjected to two, 5 min sonication cycles (amplitude 70, cycle 0, 7) interrupted by a 5 min resting period, in water bath, using a probe sonicator (UP 200S, dr. Hielsher GmbH, Berlin, Germany). The resultant small unilamellar vesicles (SUVs) were allowed to anneal for 30 min.

2.2.1. Freeze drying of liposomal suspensions

Liposomes were frozen at −80 °C overnight and were subjected to lyophilization in order to overcome stability issues concerning thermodynamic unstable liposomal suspensions. The lyophilization was achieved using a freeze drier (TELESTAR[Q7] Cryodos-50, Spain) under the following conditions: condenser temperature

from −50 °C, vacuum 8.2×10^{-2} mb (Wang et al., 2009). The lyophilized liposomal suspensions were stored at 4 °C.

2.2.2. Reconstitution of lyophilized liposomes

Freeze-dried liposomes were reconstituted by adding HPLC-grade water or fetal bovine serum to the original volume of the preparation under gentle agitation. Each sample was allowed to anneal for 30 min followed by vortexing, and a relaxation period of 15 min.

2.3. Dynamic and static light scattering

The hydrodynamic radius of liposomes and the polydispersity index (PD.I.) were measured by dynamic light scattering (DLS) and the fractal dimension was determined by static light scattering (SLS). For dynamic and static light scattering measurements, an AVL/CGS-3 Compact Goniometer System (ALV GmbH, Germany) was used, equipped with a cylindrical JDS Uniphase 22mV He—Ne laser, operating at 632.8 nm, and an Avalanche photodiode detector. The system was interfaced with an ALV/LSE-5003 electronics unit, for stepper motor drive and limit switch control, and an ALV-5000/EPP multi-tau digital correlator. Autocorrelation functions were analyzed by the cumulants method. For evaluating the temperature stability of the systems the cell temperature was varied from 25 °C (room temperature) to 45 °C (temperature higher than the phase transition of DPPC), using a temperature controlled circulating bath (model 9102 from Polyscience, USA). Heating and cooling cycles were performed, with equilibration of the systems at intermediate temperatures. Apparent hydrodynamic radii, R_h, at finite concentrations was calculated by aid of Stokes–Einstein equation:

$$R_h = \frac{k_B T}{6\pi n_0 D} \tag{4}$$

where k_B is the Boltzmann constant, η_0 is the viscosity of water at temperature T, and D is the diffusion coefficient at a fixed concentration. The polydispersity of the particle sizes was given as the μ_2/Γ^2 (PD.I.) from the cumulants method, where Γ is the average relaxation rate, and μ_2 is its second moment.

Light scattering has been used widely in the study of the fractal dimension of aggregates. In static light scattering, a beam of light is directed into a sample and the scattered intensity is measured as a function of the magnitude of the scattering vector q, with:

$$q = \frac{4\pi n_0}{\lambda_0} \sin\left(\frac{\theta}{2}\right) \tag{5}$$

where n_0 is the refractive index of the dispersion medium, θ is the scattering angle and λ_0 is the wavelength of the incident light. Measurements were made at the angular range of 30–150° and the range of wave vector was $0.01 < q < 0.03\ \mathrm{cm}^{-1}$.

The general relation for the angular dependence of the scattered intensity, $I(q)$ is:

$$I(q) \sim q^{-df} \tag{6}$$

where d_f is the fractal dimension of the liposomes or aggregates with $1 \le d_f \le 3$ ($d_f = 3$ corresponds to the limit of a completely compact Euclidean sphere). The above equation is the classical result used to determine the mass fractal dimension from the negative slope of the linear region of a log–log plot of I vs. q. This result is only strictly valid provided that the structures one is looking at are very much smaller than the overall size of the aggregate, and very much larger than the short range non-fractal structures induced by packing of the particles, i.e.: $(1/R_g) << q << (1/r_0)$. Evaluation of the behavior in the case of determination of aggregation kinetics by light scattering involves fractal analysis of aggregation process, which is valid for $qR_g \gg 1$, provided that R_g is much larger than r_0. The ratio of R_g/R_h, which is an important parameter to understand the conformation of nanoparticles in solution or in dispersion, was also determined. It has been reported that the ratio R_g/R_h takes the values of 0.775 for a hard uniform sphere and 1.0 for vesicles with thin walls, while values of 1.3–1.5 indicate a random coil (open/loose) conformation in the case of macromolecular chains (Burchard, 1983).

2.4. Electrophoretic mobility – microelectrophoresis

The zeta potential (ζ-potential) values play an important role in the colloidal stability. Zeta potential can be readily measured by the technique of microelectrophoresis (Delgado et al., 2007). The zeta potential of liposomes was measured using Zetasizer 3000HAS, Malvern Instruments, Malvern, UK. 50 μl of liposomes dispersion was 30-fold diluted in HPLC-grade water and ζ-potential was measured at room temperature at 633 nm. The zeta potentials were calculated from electrophoretic mobilities, μ_E, by using the Henry correction of the Smoluchowski equation:

$$\zeta = \frac{3\mu_E n}{2\varepsilon_0 \varepsilon_r} \frac{1}{f(\kappa a)} \tag{7}$$

where ε_0 is the permittivity of the vacuum, ε_r is the relative permittivity, α is the particle radius, κ is the Debye length, and n is the viscosity of water. The function $f(\kappa\alpha)$ depends on particle shape. While if $\kappa\alpha > 1$:

$$f(\kappa\alpha) = 1.5 + \frac{9}{2(\kappa\alpha)} + \frac{75}{2(\kappa\alpha)^2} \tag{8}$$

The above function refers to liposomal dispersions of the present study.

2.5. Fluorescence spectroscopy

Steady-state fluorescence spectra of pyrene probe in the solutions were recorded with a double-grating excitation and a single-grating emission spectrofluorometer (Fluorolog-3, model FL3-21, Jobin Yvon-Spex) at room temperature (25 °C). Excitation wavelength was λ = 335 nm for pyrene and emission spectra were recorded in the region 350–600 nm, with an increment of 1 nm, using an integration time of 0.5 s. Slit openings of 1 nm were used for both the excitation and the emitted beams. The dispersions containing the probe were left to equilibrate in the dark at 4 °C for 24 h before the measurements. Measurements were also made 7 days after the preparation of the dispersions. The pyrene monomer fluorescence has five predominant peaks. Peak 1 shows sufficiently enhanced intensity in polar environment, while peak 3 is strong and shows minimal intensity variation to changes in the polarity of the environment around the probe. Thus, the intensity ratio of peak 1 to peak 3 (I_1/I_3) serves as a measure of the micropolarity, i.e. larger I_1/I_3 ratio designate higher polarity of the surrounding medium. The microfluidity of liposomal bilayer membranes was estimated from the fluorescence intensity ratio I_E/I_M, where I_E and I_M are the fluorescence intensities of pyrene excimer and monomer, respectively. I_E and I_M were measured at emission wavelengths 480 and 372 nm, respectively. The fluorescence intensity ratio I_E/I_M offers an index of microfluidity, because the ratio I_E/I_M is proportional to the frequency of collision of pyrene molecules, namely the ability to form excimer. Typically, larger values of I_E/I_M imply a larger microfluidity.

Table 1
The physicochemical characteristics of liposomes before freeze-drying.

Sample	Diameter (nm)	PD.I.	ζ-Potential (mV)
DPPC liposomes	112.72 ± 12.02	0.519 ± 0.006	0.93 ± 2.74
DPPC:chol (9:1) liposomes	154.67 ± 16.8	0.487 ± 0.056	−9.93 ± 4.08

3. Results and discussion

3.1. Stability studies

3.1.1. Physicochemical characterization

Physicochemical characteristics of DPPC and DPPC:chol (9:1) liposomes before freeze-drying are presented in Table 1. Sucrose, which is used as lyoprotectant, influences the stability of the system, since sucrose molecules are located on the membrane surface and influence the hydration by replacing some part of bound water (Kiselev et al., 2003). The reconstitution was achieved by adding HPLC-grade water or FBS. The stability of liposomal systems was evaluated by measuring the hydrodynamic radius (R_h), polydispersity (PD.I.), ζ-potential and fractal dimension (d_f) in the two different media. The values of surface fractal of the systems under investigation were calculated from Eq. (3). It is important to characterize the physicochemical properties of liposomes, particularly in FBS because the proteins of the medium are expected to alter the properties of liposomes, thereby affecting the stability and clearance properties in the biological medium.

Aggregation of reconstituted DPPC liposomes was observed in HPLC grade water because R_h increased from 95 nm the day of reconstitution of liposomal cake to 680 nm after 20days (Fig. 1(a)). Generally, liposome dispersions are not thermodynamically stable, while the aggregation in this case is not ion-induced but mainly a result of the ageing of the system. The aggregation kinetics of DPPC liposomes in aqueous medium are represented by the equation:

$$R_h(t) = 101.7t + 55.196(r^2 = 0.7514) \quad (9)$$

which belongs to the first order kinetics. On the other hand, the R_h of reconstituted DPPC liposomes in FBS increased only by 10 nm during 20 days (Fig. 1(a)), that means that the liposomal dispersion is thermodynamically stable. The kinetics of the physical phenomenon is given by the expression:

$$R_h(t) = 1.9193t + 71.434(r^2 = 0.8061) \quad (10)$$

which also belongs to first order kinetics. For this reason, the studies on the fusion and aggregation processes of liposomes are necessary if efficient liposomes are to be designed as drug delivery systems. The size of reconstituted DPPC liposomes in FBS decreased by 25 nm, probably due to the membrane impermeability to some ions which generate osmotic forces, leading to water evacuation from the liposome interior (Sabín et al., 2006). The ζ-potential of reconstituted DPPC liposomes in HPLC grade water was found near zero (Fig. 1(c)), because of the absence of net charges on liposome surface. This results in the absence of electrostatic repulsion and the observed aggregation of DPPC liposomes in the aqueous medium. In FBS ζ-potential was +15 mV and remained stable for 20 days (Fig. 1(c)). The PD.I. measured on the first day was 0.45 and this value remained stable up to 20 days (Fig. 1(b)).

The use of liposomes as drug carriers may increase the therapeutic index of some toxic drugs or drugs with low bioavailability and high clearance rates. In targeted drug delivery, cholesterol is used to ameliorate the physicochemical properties and the biological properties of DDS. For example, the liposomal drug formulations in treatment of rheumatoid arthritis involve cholesterol to enhance the pharmacokinetic profile of antirheumatic bioactive molecules (van de Hoven et al., 2011). Cholesterol, a major constituent in

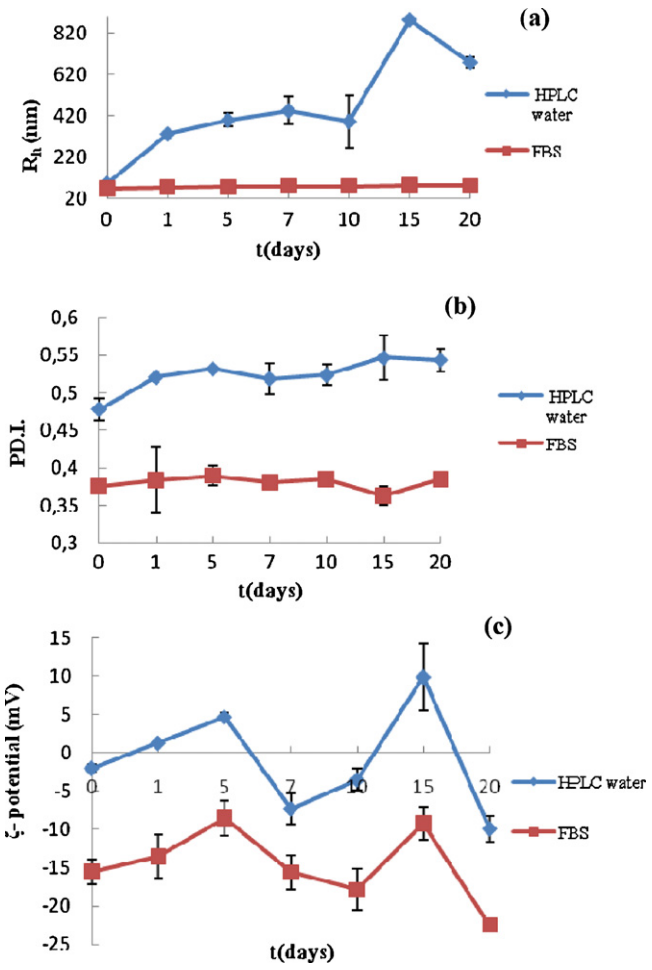

Fig. 1. Stability assessment of DPPC liposomes. (a) R_h, (b) PD.I., (c) ζ-potential of the liposomal dispersion. Mean of three independent experiments run in triplicate, SD < 10%.

many biological membranes, such as the plasma membrane of eukaryotic cells, on the other hand, is favored for incorporation into DPPC bilayers due to its easy-to-fit structure and has been assumed to provide stabilization. For this reason, we studied liposomal carriers incorporating cholesterol, which is an important compound to ameliorate the *A*bsorption, bio*D*istribution, *M*etabolism and *E*xcertion, ADME, profile of a candidate liposomal drug. The physicochemical stability of DPPC:chol (9:1) liposomes in HPLC water was observed. The stabilization effects of cholesterol on lipid bilayers emerge from the increase in the cohesion of lipids and the promotion of the liquid-ordered phase (Tierney et al., 2005). The aggregation kinetics of DPPC:chol (9:1) liposomes in biological medium are described by the equation:

$$R_h(t) = 4.362t + 135.96(r^2 = 0.6617) \quad (11)$$

A first order aggregation kinetics was observed for the liposomes incorporating cholesterol in FBS, while in HPLC water liposomes were physically stable (Fig. 2(a)). The liposomal stability indicates that another force should be responsible for keeping the liposomes far enough to avoid van der Waals attraction, which was predicted from classical DLVO theory. This force is probably the hydration force, which was predicted from modified or extended DLVO theory (Ohki and Ohshima, 1999; Ohki and Arnold, 2000; Sabín et al., 2005, 2006). The incorporation of cholesterol in DPPC liposomes caused a shift of ζ-potential to more negative values (Fig. 2(b)) An increase of the R_h of reconstituted DPPC:chol (9:1) liposomes

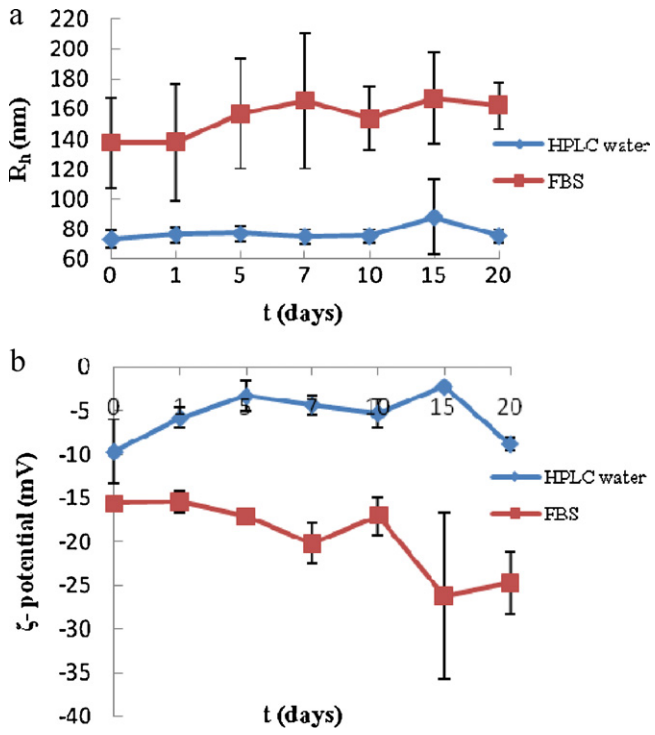

Fig. 2. Stability assessment of DPPC:chol (9:1) liposomes (a) R_h, (b) ζ-potential of the liposomal dispersion. Mean of three independent experiments run in triplicate, SD < 10%.

(60 nm) in FBS (Fig. 2(a)) and a change of ζ-potential (Fig. 2(b)) were observed, due to protein binding which can increase the nanoparticle's effective size and change its surface charge. Such changes can in turn influence uptake by the macrophages (Arnida Janát-Amsbury et al., 2011). After protein adsorption, the protein-covered liposomal surface may exhibit local positive or negative charge, depending on the orientation of the adsorbed proteins. The absorption of plasma proteins onto the surface of liposomes is known as opsonization, which is a crucial biological phenomenon for their recognition and clearance by cells of mononuclear phagocyte system (Semple et al., 1998; Dimitrova et al., 2000; Chen et al., 2010). It should be based on mind that the physical properties of a lipid bilayer could be transmitted to the membrane-associated proteins and likely affect their biological and pharmacological activity. Other serum components such as immunoglobulins, fibronectin and others can modify the physicochemical and the biological properties of liposomes by promoting interactions with reticuloendothelial cells and macrophages and activation of complement systems (Weissmann et al., 1974; Bonté and Juliano, 1986; Tsunoda et al., 2001; Sabín et al., 2009). The factors influencing plasma protein–liposome interactions are surface coatings, surface charge and lipid dose (Semple et al., 1998) and these are the strategic orientations to increase the bioavailability of liposomal drugs.

No significant difference in ζ-potential between the two kinds of liposomes was observed in FBS (Figs. 1(c) and 2(b)). This result suggests that the adsorption of albumin, the main protein of FBS, cannot be explained in terms of electrostatic interactions. The three-domain model of albumin, the hydrophobic hole in each domain and the configurational adaptability of bovine serum albumin are the key features that may explain the invariability in ζ-potential of different liposomal compositions in FBS. Taking these considerations into account, the hydrophobic interactions between bovine serum albumin and the overall zero charge of the membrane bilayer of liposomes play the leading role for the elucidation of the physical behavior of liposomes in FBS.

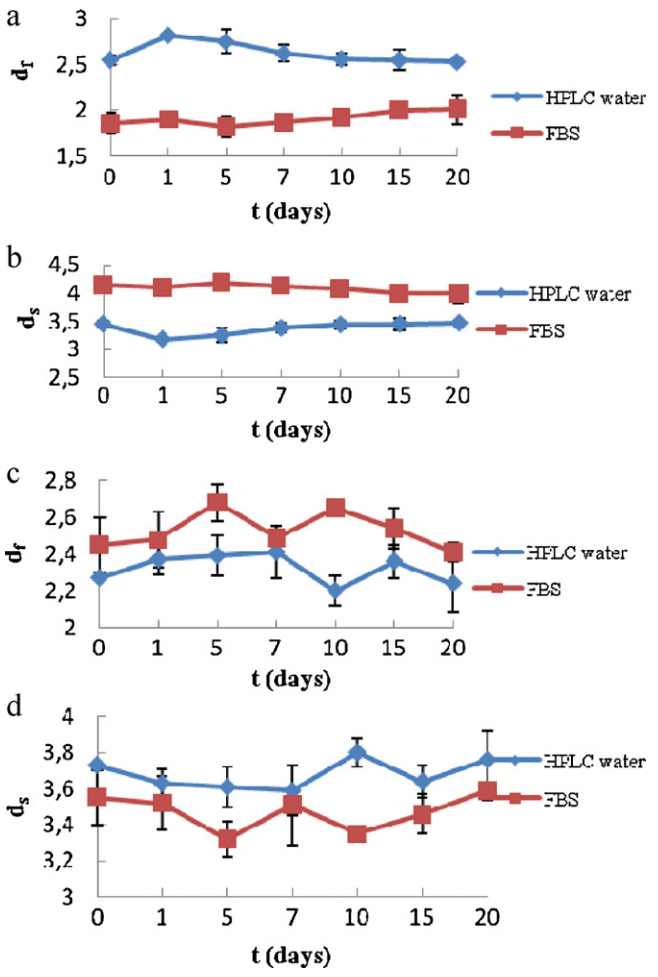

Fig. 3. Stability assessment of the liposomal formulations using the fractal approach (a) d_f of DPPC liposomes, (b) d_s of DPPC liposomes, (c) d_f of DPPC:chol (9:1) and (d) d_s of DPPC:chol (9:1) liposomes. Mean of three independent experiments run in triplicate, SD < 10%.

3.1.2. Fractal analysis

The d_f was found equal to 2.5 for reconstituted DPPC liposomes in HPLC water. The decrease of fractal dimension from 2.1 (in agreement with RLCA predictions) to 1.8 (in agreement with DLCA predictions) was observed for reconstituted DPPC liposomes in FBS (Fig. 3(a)). In the literature it has been reported that a decrease of the fractal dimension from $d_f = 2.1$ to 1.8 as the ionic strength increased (Sabín et al., 2007b). The value of d_f was not changed over the period of 20 days even though there was aggregation. The lateral cluster–cluster aggregation, which is a three-dimensional growth, could be an explanation for this observation for the morphology of DPPC liposomal dispersions in aqueous milieu (Grogan et al., 2011). Large liposomal aggregates possess fractal characteristics consistent with dimensional growth. The fractal dimension is a function of aggregate size and is correlated to the structural complexity of the system. The d_f values of DPPC:chol (9:1) did not present any significant differences in the two dispersion media (Fig. 3(c)). The incorporation of cholesterol into DPPC liposomes presumably resulted in a significantly decreased protein binding (Semple et al., 1996) and for this reason the morphological characteristics of the system in FBS is not quite different from those in HPLC water (while in general, the absorbed protein molecules change the particle surface characteristics). The extended or modified DLVO theory would be the physical formalism for the description of colloidal behavior of liposomes reconstituted in FBS because of the existence of

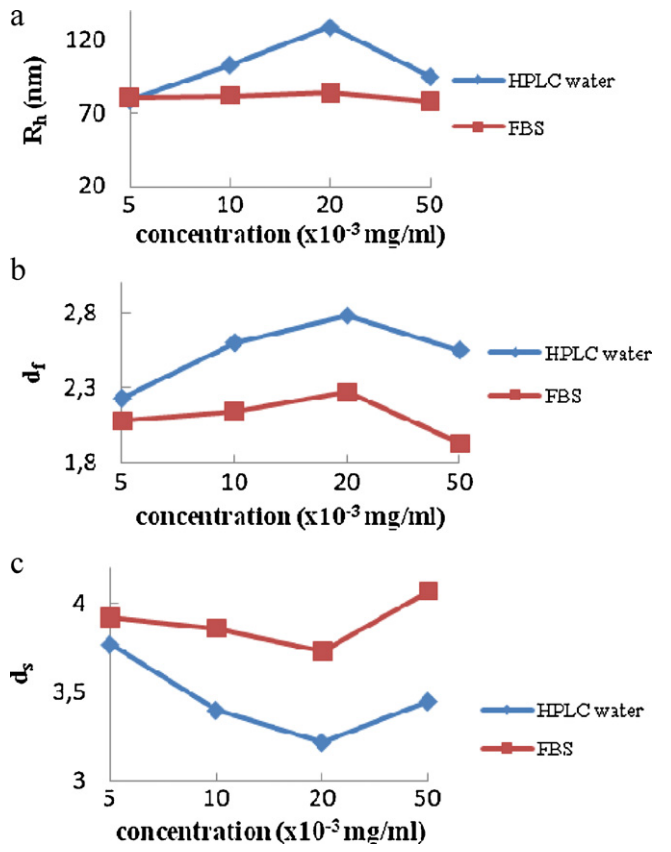

Fig. 4. (a) R_h, (b) d_f and (c) d_s vs. concentration of DPPC liposomes.

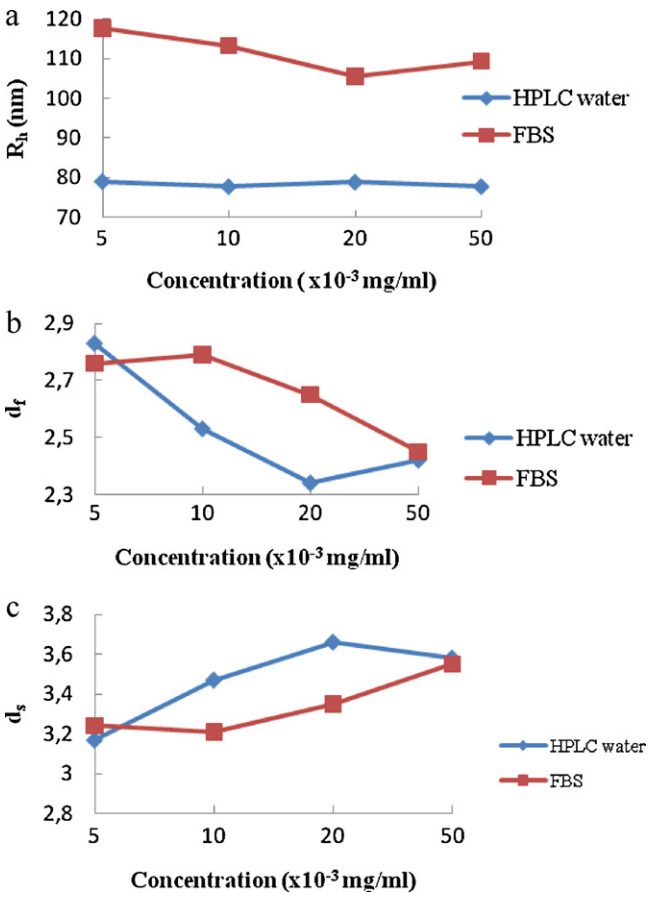

Fig. 5. (a) R_h, (b) d_f and (c) d_s vs. concentration of DPPC:chol (9:1) liposomes.

hydrophobic interactions. In particular, Ohki and Arnold define a hydrophobic index that represents the degree of hydrophobicity of the membrane surface (Ohki and Arnold, 2000). It should be pointed out that the adsorption of bovine serum albumin onto liposomes and the aggregation of colloidal particles covered with different amounts of bovine serum albumin have already been investigated (Law et al., 1986; Schmitt et al., 2000). To the best of the authors' knowledge this is the first report on fractal dimension of liposomal carriers reconstituted in FBS, leading to the determination of their structure in a biological milieu.

3.2. Concentration and temperature

R_h is independent of concentration changes for reconstituted DPPC and DPPC:chol (9:1) liposomes in the two different media (Figs. 4(a) and 5(a)). At the highest concentration the population of DPPC liposomes in HPLC grade water became more heterogeneous. On the other hand, d_f plays an important role for the elucidation of morphological characteristics, while R_h did not change by an increase of concentration. For DPPC liposomes d_f increased with the increase of concentration and for the DPPC:chol (9:1) the opposite was observed. It should be noted that the dispersion medium affects the changes in fractal dimension in the same manner for DPPC liposomes and in the opposite manner for liposomes incorporating cholesterol (Figs. 4(b), (c), 5(b) and (c)). The interactions between liposomes could play an important role for the morphological changes.

The rate of protein-induced liposome aggregation depends strongly on the ambient temperature (Dimitrova et al., 2000). We investigated the temperature dependence of the physicochemical parameters of liposomes in the process of cooling and heating. The R_h of reconstituted DPPC liposomes in HPLC water decreased in the process of heating up to 45 °C (Figs. 6(a) and 7(a)). At the lowest concentration, R_h attains the lowest value during cooling at 25 °C. The systems are not completely reversible. The irreversibility is most evident in physicochemical changes at the lower concentration (Fig. 7(a)). The d_f values also decreased during heating, especially at the higher concentration studied (Figs. 6(b) and 7(b)). These lower values are in agreement with the concept of a rough surface (to be expected for the presence of local domains in liposomes under non contrast-matching conditions (Vogtt et al., 2010)). Hence, this finding is a first indication of the presence of a heterogeneous microdomain structure of the liposomal system (Vogtt et al., 2010). The membrane surface became more hydrophobic at temperatures higher than the T_m of DPPC lipids (Shimanouchi et al., 2011). The curvature of the liposome membrane is expected to be larger also. The values of d_s demonstrate increased curvature at higher temperatures (Figs. 6(c) and 7(c)). The DPPC:chol (9:1) liposomes presented similar behavior with DPPC liposomes during the heating and the cooling cycles (Figs. 8 and 9). Cholesterol has been characterized as a "helper" lipid and "curvature loving" (Hirsch-Lerner et al., 2005), which is evidenced by the observed d_s values (Figs. 8(c) and 9(c)). The irreversibility could also describe the biophysical conduct of DPPC:chol (9:1) liposomes. Similar results were obtained in the study of liposomes with and without cholesterol in FBS at different temperatures. The irreversibility is pronounced probably due to the presence of proteins.

3.3. Microfluidity – micropolarity

The microfluidity of the hydrocarbon region of DPPC and DPPC:chol liposomal bilayers membranes decreased by the

Table 2
Pyrene fluorescence intensity ratios I_1/I_3 (indicating micropolarity) and I_E/I_M (indicating microfluidity) of liposomal bilayers.

Reconstituted in	HPLC water		FBS		FBS		HPLC[a] water		FBS[a]	
Diluted in	HPLC water		HPLC water		FBS		HPLC water		HPLC water	
Liposomal composition	I_1/I_3	I_E/I_M	I_1/I_3	I_E/I_M	I_1/I_3	I_E/I_M	I_1/I_3	I_E/I_M	I_1/I_3	I_E/I_M
DPPC	1.36	0.76	1.37	0.27	1.23	No excimer	1.34	0.54	1.31	0.26
DPPC:chol (9:1)	1.29	0.59	1.29	0.29	1.22	No excimer	1.27	0.28	1.29	0.18

[a] Measured 7 days after preparation.

absorption of proteins, as shown in Table 2. In the literature, it was reported that bovine serum albumin is likely to interact with lipidic bilayer membrane with higher hydrophobicity and make the microenvironment of liposomes polar. In this case, the permeability could be increased by the formation of a temporary gap and the existence of phase separation in the lipid membranes (Yokouchi et al., 2001; Tsunoda et al., 2001; Hioki et al., 2010). This means that the absorbed protein molecules allow one to change the particle surface characteristics quite drastically and thus, the question of to what extent the particle surface characteristics alter the aggregation behavior of colloidal liposomal suspensions should be answered. It is suggested that the absorption of proteins, especially albumin, on liposome based drug carriers brings about the change of membrane characteristics and probably the leakage of encapsulated drug molecules during the circulation of liposomes in the blood stream (Hioki et al., 2010). The microviscosity was increased in FBS for the two liposomal compositions (I_E/I_M values in Table 2), as a result of the limiting phase transition from gel phase to liquid crystalline phase (Tsunoda et al., 2001). The cooperativity is also loosened (Hashizaki et al., 2006). The micropolarity of liposomal membranes is smaller in FBS and further decreases after 7 days as I_1/I_3 values indicate (Table 2).

3.4. The R_g/R_h ratio

The calculated values of R_g/R_h are shown in Table 3. This ratio is sensitive to the shape of particles in solution and can be used as a rough estimate of the internal morphology of the particle. This is based on the notion that R_g is a measure of the mass density distribution around the center of the structure, while R_h defines the outer dimensions of the particle. It has been reported that the ratio R_g/R_h takes the values of 0.775 for a hard uniform sphere and 1.0 for vesicles with thin walls, while values of 1.3 to 1.5 indicate a ran-

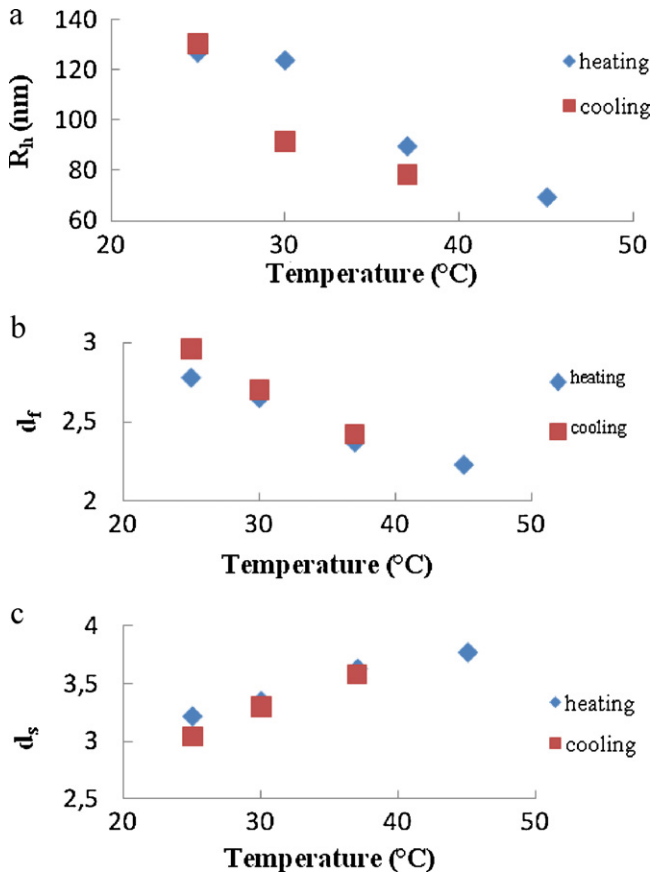

Fig. 6. (a) R_h, (b) d_f and (c) d_s vs. temperature for DPPC liposomes reconstituted in HPLC water (the liposomal concentration is constant at 10×10^{-3} mg/ml).

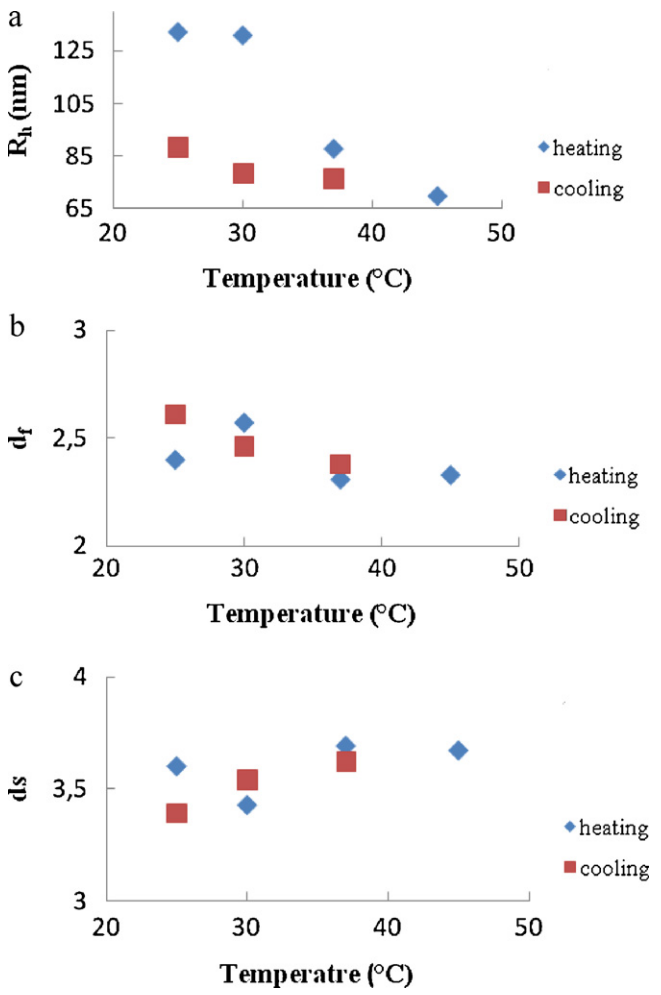

Fig. 7. (a) R_h, (b) d_f and (c) d_s vs. temperature for DPPC liposomes reconstituted in HPLC water (the liposomal concentration is constant at 5×10^{-3} mg/ml).

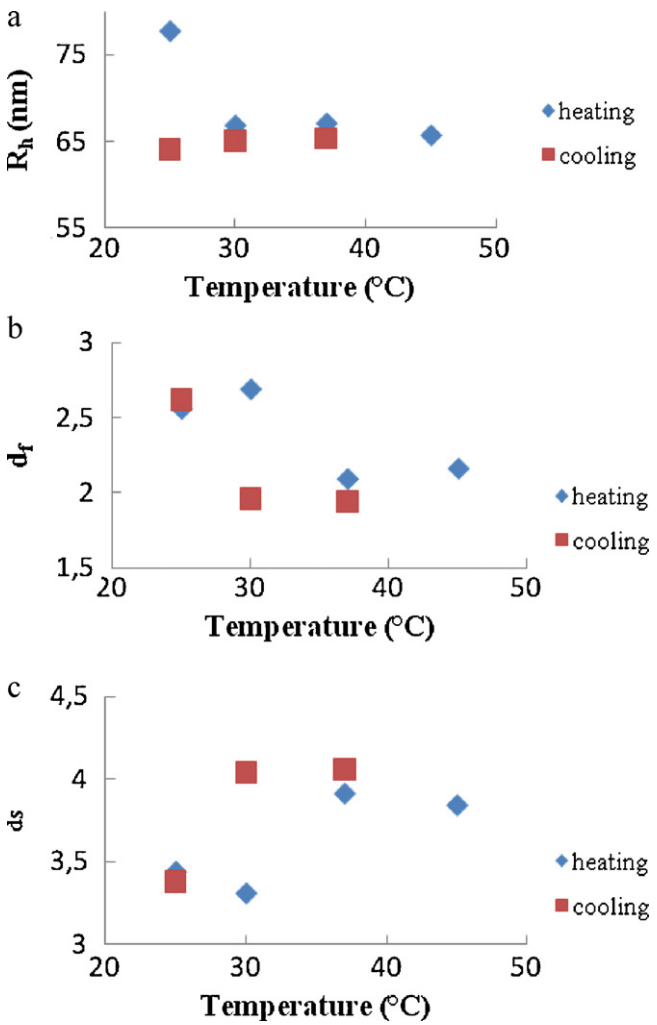

Fig. 8. (a) R_h, (b) d_f and (c) d_s vs. temperature for DPPC:chol (9:1) liposomes reconstituted in HPLC water (the liposomal concentration is constant at 10×10^{-3} mg/ml).

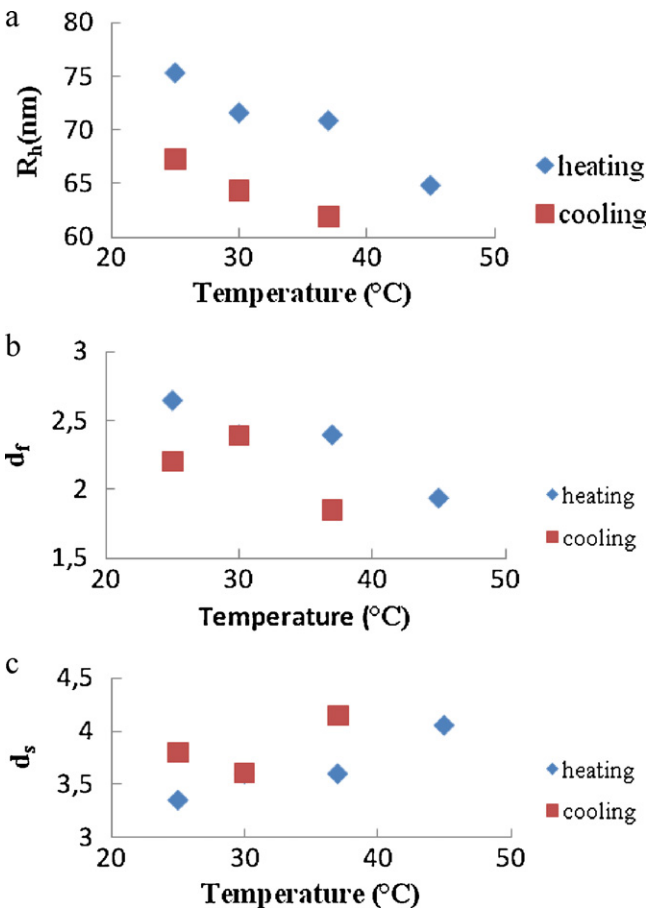

Fig. 9. (a) R_h, (b) d_f and (c) d_s vs. temperature for DPPC:chol (9:1) liposomes reconstituted in HPLC water (the liposomal concentration is constant at 5×10^{-3} mg/ml).

dom coil (loose) conformation in the case of macromolecular chains (Burchard, 1983). In the present case R_g/R_h changes from 1.37 in HPLC water to 0.98 in FBS and from 1.43 in HPLC water to 1 in FBS for DPPC and DPPC:chol (9:1) liposomes, respectively. These observations indicate a change in the apparent structure of liposomes from close to an open/loose structure towards a more well-defined hollow sphere (vesicle like) structure in FBS. The absorption of proteins, especially of albumin, would be a possible explanation for this change in morphology, besides the larger interactions in the biological medium. Protein adsorption increases the mass density on the periphery of the particles, with possible increase in the thickness of the membrane and possibly a parallel change of the size (decrease of the outermost dimensions) of the particle.

Table 3
The calculated R_g/R_h ratio for DPPC and DPPC: cholesterol (9:1) liposomes in HPLC water and FBS.

Sample	R_g/R_h	
Reconstituted in	HPLC water	FBS
DPPC	1.37	0.98
DPPC:chol (9:1)	1.43	1

4. Conclusions

Aggregation of DPPC liposomes in aqueous medium was observed, while d_f remained unchanged. The existence of lateral cluster–cluster aggregation could be a possible explanation to the observed behavior. Physicochemical stability was observed for the DPPC:cholesterol (9:1) liposomes in the two dispersion media. The structural properties of DPPC liposomes in aqueous medium are quite different from those in FBS, as demonstrated from fractal analysis, especially for liposomes without cholesterol. Cholesterol plays a major role on the fluidity of membranes by regulating their functions, as shown by the slight variation of mass and surface fractal dimension in the two media. To corroborate the experimental results, we have used the extended or modified DLVO theory to describe the colloidal stability of liposomes with or without cholesterol in FBS. The hydrophobic and steric interactions play a significant role for the preservation of morphological characteristics of liposomes over time. The physical theories, which were developed for aggregation phenomena, include the fractal formalism for elucidating the shape of the resulting liposomal aggregates and structures. In addition, the microfluidity of the hydrocarbon region of DPPC and DPPC:chol liposomal bilayers membranes decreased by the absorption of proteins, while the micropolarity did not present significant changes. Finally, determination of fractal dimension is very important for colloidal systems, since fractal geometry allows scientists from different fields to formulate alternative hypotheses for experimental observations, which lead to more realistic explanations compared to the traditional approaches, especially for drug delivery colloidal nanosystems.

These research orientations could be a road map for designing aDDnSs with complete knowledge of their morphological characteristics, their fractal hologram, which modifies the ADME profile of the candidate drug and defines their efficacy, effectiveness and safety.

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