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RESEARCH PAPER

The Shape/Morphology Balance: A Study of Stealth Liposomes via Fractal Analysis and Drug Encapsulation

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ABSTRACT

Purpose Fractal analysis was used as a tool in order to study the morphological characteristics of PEGylated liposomes. We report on the morphological characteristics of stealth liposomes composed of DPPC and DPPE-PEG 3000 in two dispersion media using fractal analysis.

Methods Light scattering techniques were used in order to elucidate the size, the morphology and the surface charge of PEGylated liposomes as a function of PEGylated lipid concentration and temperature. Fluorescence spectroscopy studies revealed a microenvironment of low polarity inside the liposomal membranes.

Results All formulations were found to retain their physicochemical characteristics for at least 3 weeks. The hydrodynamic radii (R_h) of stealth liposomes were stable in the process of heating up to 50°C; while the fractal dimension values (d_f) which correspond to their morphology, have been changed during heating. Hence, these results are a first indication of the presence of a heterogeneous microdomain structure of the stealth liposomal system. The amphiphilic drug indomethacin (IND) was successfully encapsulated within the liposomes and led to an increased size of stealth liposomes, while the morphology of liposomal vectors changed significantly at the highest molar ratio of PEGylated lipid.

Conclusions We can state that this approach can promote a new analytical concept based on the morphological characteristics and quantify the shape of drug carriers complementary to that of the conventional analytical techniques.

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KEY WORDS fractal dimension · indomethacin · morphology · pegylated nanoparticles · shape

ABBREVIATIONS

d_f	mass fractal
DLCA	diffusion-limited cluster aggregation
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DPPE-PEG 3000	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-3000]
d_s	surface fractal
IND	indomethacin
NSAIDs	nonsteroidal anti-inflammatory drugs
PBS	phosphate buffer saline
RLCA	reaction-limited cluster aggregation

INTRODUCTION

Nanotechnology is a challenging scientific field and its application in Pharmaceutics will bring significant advantages in the development of innovative drugs (1–3). The role of the nanocarrier on the pharmacological activity of the therapeutic agent is of great importance, since it drastically affects its pharmacokinetic profile, as well as its effectiveness, efficacy and toxicity, and has, therefore, created great expectation in the field of targeted drug delivery (4). Liposomes and other colloidal drug delivery systems modify the kinetics, body distribution and drug release of an associated bioactive compound. Stealth liposome technology is one of the most often used in liposome-based systems for delivery of bioactive molecules. Stealth liposomes belong to "second-generation liposomes", while conventional liposomal vectors are characterized as the "first-generation liposomes" (5,6). Stealth liposome technology strategy was

achieved by modifying the surface of liposomal membrane by engineering hydrophilic polymer conjugates, such as polyethylene glycol (PEG), which is the most widely used polymer conjugate, while reducing mononuclear phagocyte system uptake (7). These liposomal nanocarriers are characterized by high biocompatibility, nontoxicity, low immunogenicity and antigenicity and have been shown to extend blood-circulation time. The colloidal properties of high-stability, long-circulating liposomes are of paramount importance for their clinical, medicinal and pharmaceutical applications (8,9).

The studies of colloidal properties using liposomes have already been evaluated, including the fractal approach (10–15). Furthermore, the extended **D**erjaguin-**L**andau-**V**erwey-**O**verbeek (**DLVO**) theory is used as the biophysical formalism for the description of the phenomenological liposomal vectors' behavior (10,11,13,16–19). Diffusion-limited cluster aggregation (DLCA) and reaction-limited cluster aggregation (RLCA) are the two limited regimes which describe the aggregation mechanism, using fractal geometry. The fractal dimension was estimated to be 1.8 and 2.1 for diffusion-limited cluster aggregation (DLCA) and reaction-limited cluster aggregation (RLCA) aggreagates, respectively (10,11,13,18,19). It is worthy to note that the Euclidean and Newtonian approaches which can efficiently describe the shape and the physicochemical characteristics of nanocarriers are not effective to quantify their morphology. Fractal analysis has recently been used to describe the morphology of liposomal nanoparticles and it has been considered as a complementary analytical tool. The quantification of the morphological picture of nanoparticles could be achieved via fractal analysis. The "universality" of these fractal aggregation phenomena and the two limiting regimes were supported by Lin and co-workers (20). There is a large number of techniques available for the characterization of the structure and the morphology of aggregates formed from suspensions and dispersions of microparticles and nanoparticles and for the determination of their fractal dimension. Additionally, light scattering, especially static light scattering, provides the greatest potential for use as an *in situ* convenient tool for elucidation and characterization of the morphology of liposomal nanoparticles in aqueous dispersions (21). The physical theories, which were developed for aggregation phenomena, include a fractal formalism for elucidating the shape and the morphology of the resulting aggregates.

Indomethacin (IND) belongs to the class of nonsteroidal anti-inflammatory drugs (NSAIDs), which are highly effective in the treatment of a wide variety of illnesses and diseases, including inflammation, cancers, cardiovascular disease and degenerative diseases of central and peripheral nervous system such as Alzheimer's and Parkinson's disease, respectively (22–24). Several epidemiological, clinical and experimental studies established NSAIDs as promising cancer chemopreventive agents and in the prevention of human cancer because growing knowledge in this scientific area has brought about innovative approaches using combine actions of NSAIDs with other agents that have different modes of actions (22–24). The long term use of NSAIDs results in gastrointestinal toxicity in many cases, like ulceration leading to severe bleeding, perforation and obstruction (25–27). In view of the required decreased adverse drug reactions of IND formulations, the encapsulation within nanostructures, including micelles and liposomes, seems to be an attractive approach from the pharmaceutical nanotechnology orientation (28–32). Alternative formulations are being investigated to obtained pharmaceutical products with lower toxicity and higher stability. It should be noted that the application of lipid-based nanocarriers, including micelles, liposomes, solid lipid nanoparticles, nanoemulsions and nanosuspensions, in drug formulation is one approach to improve drug safety (33). Due to indomethacin's insolubility in aqueous media, it has been incorporated into liposomal nanoparticles resulting in an increase of solubility, which affected their activity against cancer cell lines followed by reduction of its toxicity, as mentioned above. Furthermore, it has being shown that its incorporation into liposomal carriers modified its cellular uptake by favoring the selective accumulation in cell membranes from where it had demonstrated prolonged release (29). The concept of fractal geometry can be applied to describe the complexity of the heterogeneous nature of drug processes in the human body and the dissolution of bioactive compounds (34–37).

Dynamic, static and electrophoretic light scattering and fluorescence spectroscopy were used as experimental techniques to elucidate the morphology, the internal structure and microenvironment and the physicochemical parameters of the stealth liposomal carriers in different aqueous media, their ageing characteristics, as well as their structural response in changes of temperature. This research is a continuation of the authors' efforts in order to elucidate the structure and morphology of stealth liposomal carriers aiming at the rational design of advanced Drug Delivery nano System (aDDnSs) based on their fractal morphology, which can effectively elucidate the structural characteristics of these nanoassemblies (14,15).

The goal of this study is to investigate the stability of stealth liposomal carriers composed of DPPC:DPPE-PEG 3000 (9:0.1, 9:0.5 and 9:1 molar ratios), to determine their fractal dimension (mass fractal and surface fractal), specify the physicochemical characteristics in different media (HPLC grade water) and PBS (phosphate buffer saline), assign the dependence of the fractal dimension on temperature and to detect the morphological variations of liposomal vectors induced by the incorporation of a NSAID, such as indomethacin. Even thought, PEGylated liposomes have

been studied widely, this is the first report, which contributes to the quantification of their morphological characteristics via fractal analysis. This approach can be considered as novel and contributes to a deeper understand of analytical way, based on the temperature dependent morphological quantification of the liposomal carriers incorporating indomethacin (IND). This research is a continuation of the authors' contribution in order to elucidate the structure and morphology of stealth liposomal carriers aiming at the rational design of advanced Drug Delivery nano System (aDDnSs) based on their fractal morphology, which can effectively elucidate the structural characteristics of these nanoassemblies

MATERIALS AND METHODS

Materials

The phospholipids used for liposomal formulations were 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[Methoxy (Polyethylene glycol)-3000] (Sodium salt) (DPPE-PEG 3000). 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. Indomethacin was supplied by Fluka and was used as received.

Preparation of Stealth Liposomes

Different liposomal formulations have been prepared using the thin-film hydration method, composed of DPPC:DPPE-PEG (9:0.1, 9:0.5, and 9:1 molar ratios). Briefly, appropriate amounts of DPPC:DPPE-PEG 3000 phospholipid mixtures (9:0.1, 9:0.5, and 9:1 molar ratios) were dissolved in chloroform/methanol (9:1 v/v) and then transferred into a round flask connected to a rotary evaporator (Rotavapor R-114, Buchi, Switzerland). Vacuum was applied and the phospholipid thin film was formed by slow removal of the solvent at 40°C. The phospholipid film was maintained under vacuum for at least 24 h in a desiccator to remove traces of solvent and subsequently it was hydrated in HPLC-grade water and Phosphate Buffer Saline (PBS), respectively, by slowly stirring for 1 h in a water bath above the phase transition of lipids (41°C for DPPC). The resultant multilamellar vesicles (MLVs) were subjected to two, 3 min and 2 min sonication cycles (amplitude 70, cycle 0.7) interrupted by a 3 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. The liposomal formulation with drug were prepared by dissolving IND in the initial lipid mixture resulting in the following molar ratios: DPPC:DPPE-PEG 3000:IND 9:0.1:1, DPPC:DPPE-PEG 3000:IND 9:0.5:1 and DPPC:DPPE-PEG 3000:IND 9:1:1. The effect of IND incorporation in liposomal preparations was evaluated by measuring the size distribution and ζ-potential of the liposomes. The mean hydrodynamic diameter, polydispersity index (PD.I.) and ζ-potential of the particles were used for the characterization of the liposomal dispersion immediately after preparation (t=0d), as well as for the monitoring of their physical stability over time (t=20d).

Dynamic and Static Light Scattering

The hydrodynamic radius (R_h) 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). Mean values and standard deviations were calculated from three independent samples. 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 22 mV 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 50°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 were calculated by aid of Stokes—Einstein equation:

$$R_h = \frac{k_B T}{6\pi n_o D} \tag{1}$$

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 dimensions 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:

$$q = \frac{4\pi n_o}{\lambda_o} \sin\left[\left(\theta/_2\right)\right] \tag{2}$$

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° to 150° (i.e. the range of the wave vector was $0.01 < q < 0.03\ cm^{-1}$).

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

$$I(q) \sim q^{-df} \tag{3}$$

where d_f is the fractal dimension of the liposomes or aggregates with $1 \leq d_f \leq 3$ ($d_f = 3$ 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. $1/Rg << q << 1/r0$. Evaluation of the behavior in the case of determining aggregation kinetics by light scattering involves fractal analysis of aggregation process, which is valid for $qR_g >> 1$, provided that R_g (radius of gyration) is much larger than r_0. The relative error of the measurement of fractal dimension is less than 10%.

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. 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:

$$\zeta = \frac{3\mu_E n}{2\varepsilon_0\ \varepsilon_r} \frac{1}{f(\kappa a)} \tag{4}$$

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(κα) depends on particle shape. While if κα > 1:

$$f(\kappa\alpha) = 1.5 + \frac{9}{2(\kappa\alpha)} + \frac{75}{2(\kappa\alpha)^2} \tag{5}$$

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

Fluorescence Spectroscopy

Steady-state fluorescence spectra of pyrene probe in the liposomal 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 $\lambda = 335$ 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. 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 designates 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 collisions of pyrene molecules, namely the ability to form excimer. Typically, larger values of I_E/I_M imply a larger microfluidity.

Incorporation Efficiency

The percentage of IND incorporated into stealth liposomes was estimated by spectrophotometer (Stat Fax® 4200, Microplate Reader, NEOGEN® Corporation). The absorbance was measured at 492 nm. The incorporation efficiency of IND into liposomes was calculated after quantification. Non incorporated IND was separated from liposomal formulations on a Sephadex G75column. Incorporation efficiency (IE) was calculated by using the following equation:

$$\%IE = \frac{IND\ (after\ column)}{IND\ (initial)} \times 100 \tag{6}$$

IND concentration was measured by spectrophotometer at λ = 492 nm, with the aid of the following IND calibration curve:

$$\text{IND concentration } \left({}^{mg}/_{ml}\right) = \frac{\text{absorbance} + 0.0567}{0}.0667 \quad \left(R^2 = 0.9985\right) \tag{7}$$

In Vitro IND Release Studies

The release profile of IND from DPPC:DPPE-PEG 3000 (9:0.1:1, 9:0.5:1 and 9:1:1 molar ratio) stealth liposomes was studied in PBS at 37°C. Stealth liposomes incorporating IND (1 ml of each sample) were placed in dialysis sacks (molecular weight cut off 12,000; Sigma-Aldrich). Dialysis sacks were inserted in 10 mL (PBS) in shaking water bath set at 37°C. Aliquots of samples were taken from the external solution at specific time intervals and that volume was replaced with fresh release medium in order to maintain sink conditions. The amount of IND released at various times, up to 10 h, was determined using spectrophotometry (Stat Fax® 4200, Microplate Reader, NEOGEN® Corporation) at λmax = 492 nm with the aid of the calibration curve of the Eq. (7).

Statistical Analysis

Results are shown as mean value ± standard deviation (S.D.) of three independent measurements. Statistical analysis was performed using Student's *t*-test and multiple comparisons were done using one-way ANOVA. *P*-values <0.05 were considered statistically significant. All statistical analyses were performed using "EXCELL".

RESULTS AND DISCUSSION

Physicochemical and Morphological Characterization of Stealth Liposomes

Physicochemical and morphological characteristics of DPPC and DPPC:DPPE-PEG 3000 (9:0.1, 9:0.5 and 9:1 molar ratios) liposomes in two aqueous media are presented in Table I. It should be noted that the pH and the ionic strength of PBS resembles the conditions met within the human body. The PD.I. values indicate quite monodisperse liposomal formulations only for the stealth vectors. The size distribution is also increased in HPLC-grade water (Table I). According to the literature, well-formulated liposomal systems should display a narrow nanoparticle size distribution (38). Liposomes incorporating PEGylated lipid are smaller than the conventional DPPC liposomes in the two dispersion media. The incorporation of 10% molar ratio PEGylated lipid leads to liposomes of smaller size. It should be pointed out that the DPPC:DPPE-PEG 3000 (9:1molar ratio) in PBS exhibit smaller R_h than all formulations (Table I). Taking into consideration the grater ionic strength of PBS one can assume that the above observation may be due to differences in the solvation state of PEG polymeric chains when ions are present in the solution. Another explanation can be based on the changes in the hydration power of water, due to the presence of ions in PBS, as well as the, at least partially, coordination of these ions to the PEG polymeric chains, something that would make them more hydrophilic, improving the hydration characteristics of the PEG chains (39). Stealth liposomal formulations exhibit negative ζ-potential values attributed to the incorporation of the negatively charged DPPE-PEG lipid (Table I). The PEGylated lipid used in this work was commercial phosphoethanolamine lipid with covalently attached PEG chain. This modification gives the lipid a net negative charge (dissociated phosphate group). On the other hand, the ζ-potential of DPPC liposomes in two dispersion media was found near zero, because of the absence of net charge on the liposome surface (Table I). The reconstitution in PBS caused a shift of ζ-potential to less negative values for stealth liposomes, probably because of screening effects as a result of the higher ionic strength of PBS. According to the literature, this results in the absence of electrostatic repulsion and the aggregation of conventional DPPC liposomal carriers in the aqueous media (14,15,38).

The fractal dimension (d_f) was found equal to 2.5 for conventional DPPC liposomal carriers in HPLC-grade water. A decrease of d_f values was observed for stealth

Table I The Physicochemical (R_h, PD.I. and ζ-potential) and Morphological (d_f, R_g/R_h) Characteristics of Stealth Liposomal Formulations in Different Dispersion Media

Sample	Dispersion Medium	R_h (nm)	PD.I.	ζ-pot (mV)	d_f	R_g/R_h
DPPC	HPLC water	62.50 ± 2.5	0.605 ± 0.016	+1.0 ± 0.4	2.51	0.99
DPPC:DPPE-PEG 3000 (9:0.1 molar ratio)	HPLC water	40.45 ± 0.4	0.354 ± 0.002	−19.9 ± 3.1	1.99	1.25
DPPC:DPPE-PEG 3000 (9:0.5 molar ratio)	HPLC water	39.75 ± 0.4	0.327 ± 0.008	−35.6 ± 7.6	1.98	1.16
DPPC:DPPE-PEG 3000 (9:1 molar ratio)	HPLC water	36.40 ± 2.4	0.392 ± 0.010	−28.7 ± 2.4	1.98	1.07
DPPC	PBS	95.85 ± 6.8	0.699 ± 0.007	+0.7 ± 0.2	2.73	1.24
DPPC:DPPE-PEG 3000 (9:0.1 molar ratio)	PBS	42.90 ± 1.5	0.290 ± 0.012	−12.1 ± 1.5	2.13	1.45
DPPC:DPPE-PEG 3000 (9:0.5 molar ratio)	PBS	40.35 ± 0.3	0.279 ± 0.020	−10.2 ± 5.7	1.95	1.25
DPPC:DPPE-PEG 3000 (9:1 molar ratio)	PBS	29.85 ± 1.3	0.238 ± 0.083	−19.7 ± 3.4	2.29	0.93

liposomes in aqueous medium (Table I). However, the mixing of different biomaterials (different in nature phospholipids) in order to produce stealth liposomes should be taken into consideration for controlling the surface morphological characteristics, which can be quantified by using fractal analysis.

The morphology of stealth liposomal systems in HPLC-grade water did not present statistically significant difference as the ratio of PEGylated lipid increased (Table I). The same results were observed for conventional and stealth vectors in PBS. In the literature, it has been reported that a decrease of d_f values for conventional liposomes can take place as ionic strength of the dispersion medium increases due to osmotic forces (11). This phenomenology was not observed for the stealth liposomes of this study in PBS (Table I). The changes in the d_f values indicate differences in the internal morphology of the stealth liposomes due to the anchoring of the PEG chains (i.e. an increase in the width of the mixed liposome membrane should take place as a result of the presence of solvated PEG chains).

Furthermore, the ratio of R_g/R_h, which is an important parameter to understand the conformation of liposomal nanoparticles 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 to 1.5 indicate a random coil (open/loose) conformation in the case of macromolecular chains (14,40). The calculated values of R_g/R_h are shown in Table I. This ratio is sensitive to the shape of particles in solution or in dispersion and can be used as a rough estimate of the internal morphology of the nanoparticles. The R_g/R_h values are close to unity for DPPC liposomes, indicating a vesicle like morphology, as expected. The R_g/R_h ratio increased with the increase of PEGylated lipid. These observations should be attributed to the "thickening" of the liposome membrane in stealth liposomes containing DPPE-PEG 3000, indicating a change in the apparent structure of liposomes from an open/loose to a more dense overall nanostructure.

Colloidal temporal stability of the initially formed stealth liposomal nanostructures was investigated. Size distribution control is of a great importance in preparing liposomal dispersions, because liposomal size can also modulate the biological stability and the capture mechanism by macrophages. The physical stability over time of all liposomal formulations was assessed by measuring liposome size and size distribution for a period of 20 days. Stealth liposomes in two dispersion media were found to retain their original physicochemical characteristics (size and size distribution) at least for the time period that they were studied (Fig. 1). The liposomal stability indicates that electrostatic and steric repulsions should be responsible for keeping the liposomes far enough to avoid aggregation or fusion and avoid van der

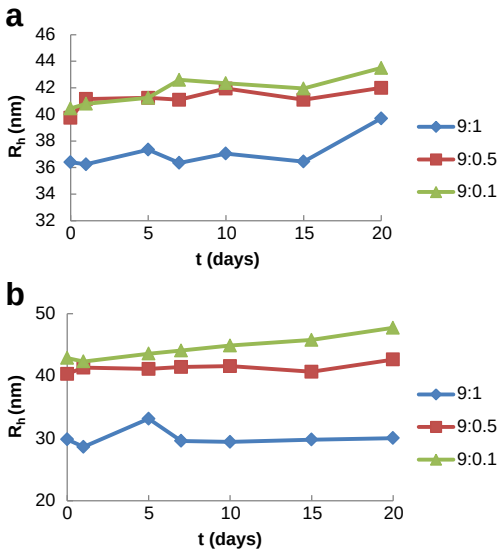

Fig. 1 Stability assessment of stealth liposomal formulations in (**a**) HPLC-grade water and (**b**) PBS.

Waals attraction, which was predicted from classical DLVO theory (16,17). High ζ-potential values demonstrated the existence of electrostatic repulsion. Furthermore, the protective PEG layer is thought to act as a steric barrier inhibiting liposomal vesicle fusion, because the addition of PEG polymeric chains increase the packing order in the stealth liposomal bilayer (41,42). It has been well established that the biodistribution of any colloidal drug delivery system depends on the size and the size distribution of the nanoparticles (43). On the other hand, the nanoparticles' *in vivo* circulation properties and the rate of liposome clearance from blood by the reticulo-endothelial system are dependent on the surface properties and on liposomal composition (44).

Effects of Temperature on Liposomal Morphology

The major advantage of liposomal vectors is the possibility of controlling the morphology and the size distribution, and therefore system's physicochemical properties, with the aid of a wide variety of physicochemical parameters. Maybe the most effective of these is temperature, especially for applications in pharmaceutical industry on stability studies.

In our previous work we have shown that the fractal dimension plays a significant role for the elucidation of morphological characteristics of conventional liposomes during changes of physicochemical parameters of the liposomal solution, such as concentration and temperature (14,15). Here, we investigated the temperature dependence of the physicochemical parameters of stealth liposomes in the process of heating in the two dispersion media (Figs. 2 and 3). These types of experiments may simulate the response of stealth liposomal formulation after injection to a fever situation or inflammatory conditions. The

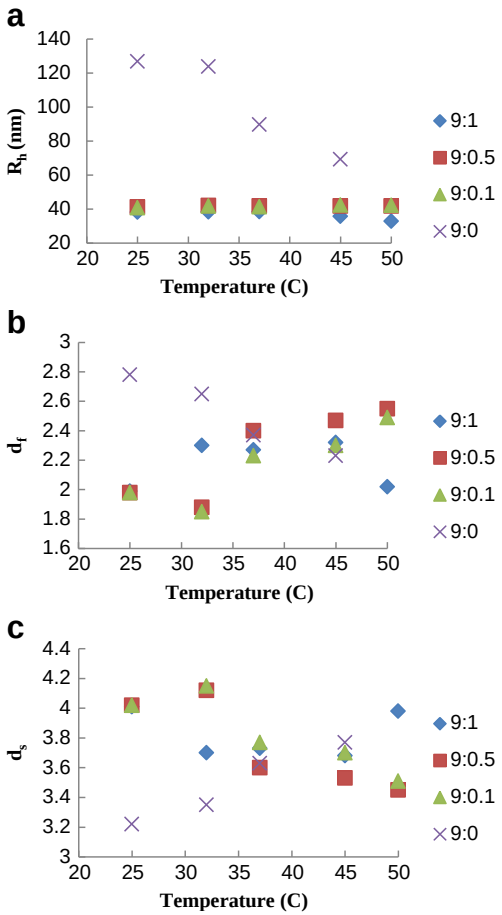

Fig. 2 (**a**) R_h, (**b**) d_f, (**c**) d_s *vs.* temperature for stealth liposomes in HPLC grade water (the liposomal concentration is constant at 5×10^{-3} mg/ml).

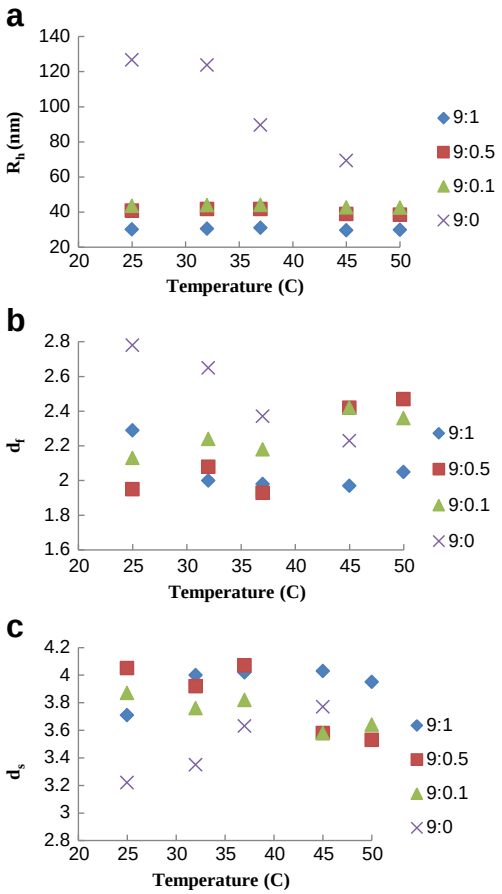

Fig. 3 (**a**) R_h, (**b**) d_f, (**c**) d_s *vs.* temperature for stealth liposomes in PBS (the liposomal concentration is constant at 5×10^{-3} mg/ml).

R_h values of stealth liposomes were stable in the process of heating up to 50°C (Figs. 2a and 3a); while the d_f values increased during heating (Figs. 2b and 3b), especially for stealth vectors in HPLC-grade water. At the highest temperature the population of liposomes became more homogeneous. On the other hand, R_h of conventional DPPC liposomes decreased in the process of heating up to 50°C (Figs. 2a and 3a) in the two dispersion media, while the d_f values decreased. At the highest temperature the population of stealth liposomal nanocarriers became more homogeneous also in PBS. It should be noted that while the average size of different chimeric nanocarriers did not change considerably in PBS, the fractal dimensions determined show some difference related most probably to changes in stealth nanocarrier morphology, due to the incorporation of PEG chains. The values of surface fractal for systems under investigation were calculated from equation:

$$d_f = 6 - d_s \tag{8}$$

where d_s is the surface fractal dimension (12,13). The d_s values demostrate increased curvature at temperatures higher than the main transision temperature of DPPC lipids (Tm=41°C for DPPC lipids) (Figs. 2c and 3c). The membrane surface became also more hydrophobic at temperatures higher than the T_m of DPPC lipids (45). Hence, these results are a first indication of the presence of a heterogeneous microdomain structure in the stealth liposomal system and that the liposomal membrane became more hydrophobic (45,46). Additionally, there is an increase in the headgroup mobility when the liposomal membrane is in the liquid crystalline phase and this affects the dynamics of the lipid membrane. It should be noted that while the average size of different stealth liposomes did not change considerably, the fractal dimensions determined show some difference, related to changes in liposomal morphology in the two dispersion media. The fractal dimension and the surface fractal offer a quantification of the changes on stealth liposomal morphology under different temperature conditions, detecting differences that the R_h values alone do not reveal.

Micropolarity and Microfluidity of Stealth Liposomal Membranes

Fluorescence spectroscopy has been utilized in an attempt to extract information on the internal nanostructure and

microenvironment of stealth liposomal nanostructures in aqueous media. Changes occurring in the stealth liposomal bilayer structure were monitored by fluorescence spectroscopy measurements of incorporated pyrene in the two media. The ratio I_1/I_3 in the fluorescence emission spectrum of pyrene is used as a measure of polarity of the environment surrounding the pyrene probe. In aqueous or similarly polar environment this ratio is found between 1.6 and 1.9. The micropolarity of the hydrocarbon region of DPPC and DPPC:DPPE PEG 3000 liposomal bilayers membranes did not change significantly in HPLC grade water and PBS, as shown in Table II. Micropolarity changes may be expected due to incorporation of the more polar PEGylated lipid into the hydrocarbon tails region of the lipid bilayer, as well as to possible induced changes in the structure of the bilayer, but something like that is not observed. Micropolarity of the tail region is unchanged and a bit higher than in the case of single tail surfactant micelles. On the other hand, the microfluidity of the hydrocarbon region of stealth liposomal bilayer membranes decreased with the change in the medium ionic strength (from HPLC water to PBS) and by increasing incorporation of PEGylated lipid (I_E/I_M values in Table II). It can be concluded that the microviscosity was increased for stealth liposomes in PBS, presumably due to the increase in ionic strength (lower I_E/I_M values in PBS). Alternatively, the increase in excimer formation in HPLC grade water may be due to an enhanced encapsulation of pyrene in the stealth liposomes, leading to an excessive aggregation of the probe within the bilayer. Pyrene aggregation seems to be less in PBS dispersions apparently due to the changes in the structure of the bilayer, as a result of changes (increase) in the ionic strength of the medium.

Physical Characteristics and Incorporation Efficiency of IND into Stealth Liposomes

IND is an amphiphilic drug and like all NSAIDs interacts strongly with lipidic membranes (47–52). The effects of liposome preparation method, lipid composition, charge and cholesterol content on encapsulation of indomethacin in liposomal vectors, as well as the drug release profile and *in vivo* experiments have been already investigated (31,53–55). In this study IND was used as a model compound for monitoring the changes of stealth liposomal morphology due to incorporation of an amphiphilic therapeutic agent.

IND was incorporated into stealth liposomes composed of DPPC:DPPE-PEG 300:IND (9:0.1:1, 9:0.5:1 and 9:1:1 molar ratios). Liposome size and size distribution, ζ-potential values and d_f are presented in Table III. The incorporation of indomethacin led to a slightly increased size of stealth liposomes. The ζ-potential values presented a shift to less negative values after the incorporation of IND (Table III). On the other hand, the morphological characteristics of stealth liposomes did not present any significant differences after the incorporation of IND. The d_f of DPPC:DPPE-PEG 3000 liposomes slightly decreased after the incorporation of the drug (especially for the stealth liposomes with the higher content in DPPE-PEG). It should be pointed out that these small changes in the microstructure of the lipid bilayer, as the fractal analysis indicated, are due to the IND incorporation. Generally, IND alters the spatiotemporal liposomal phase behavior, induces membrane fusion and changes proteins' organization on plasma membranes (56). It should be noted that the effect of NSAIDs on the physical properties of lipid bilayers has been already investigated (51,52). These research orientations are a biophysical approach providing detailed information of the membrane/drug interactions (51,52). According to the literature, indomethacin is located in the chain region closer to polar head groups at the pH conditions of the HPLC grade water (52). For this reason, the morphology of stealth liposomal vectors did not change significantly after the incorporation of the drug. To best of the authors' knowledge this is the first report on the determination of fractal dimension of stealth liposomal carriers incorporating a bioactive molecule, leading to the determination of their structural and morphological changes after the encapsulation of the drug.

The stability study over time of all formulations incorporating IND was performed by measuring the liposome size distribution for a period up to 3 weeks. All stealth liposomal formulations incorporating the amphiphilic drug were found to retain their physicochemical characteristics at least for 10 days (Fig. 4). More importantly, the incorporation efficiency of stealth vectors for IND was increased by the

Table II Pyrene Fluorescence Intensity Ratios I_1/I_3 (Indicating Micropolarity) and I_E/I_M (Indicating Microfluidity) of Stealth Liposomal Bilayers

Composition	HPLC water		PBS	
	I_1/I_3	I_{ex}/I_m	I_1/I_3	I_{ex}/I_m
DPPC	1.36	0.76	1.35	0.36
DPPC:DPPE-PEG 3000 (9:0.1 molar ratio)	1.30	0.51	1.27	0.07
DPPC:DPPE-PEG 3000 (9:0.5 molar ratio)	1.34	0.08	1.35	0.12
DPPC:DPPE-PEG 3000 (9:1 molar ratio)	1.40	0.38	1.36	0.16

Table III The Physicochemical (R_h, PD.I. and ζ-potential) and Morphological (d_f) Characteristics and Incorporation Efficiency (%IE) of Stealth Liposomal Formulations Incorporating IND in HPLC Grade Water

Composition	R_h (nm)	PD.I.	d_f	ζ-pot (mV)	% IE
DPPC:DPPE-PEG 3000:IND (9:0.1:1 molar ratio)	38.0 ± 0.9	0.279 ± 0.014	1.93	−7.8 ± 9.1	12.40
DPPC:DPPE-PEG 3000:IND (9:0.5:1 molar ratio)	42.4 ± 0.2	0.314 ± 0.032	2.04	−11.8 ± 1.6	13.70
DPPC:DPPE-PEG 3000:IND (9:1:1 molar ratio)	43.9 ± 0.5	0.311 ± 0.002	1.83	−23.6 ± 3.4	14.80

increase of PEGylated lipid, probably because the membrane became more polar in the presence of PEG-lipid, as also the fluorescence spectroscopy results indicate (Tables II and III). There is a direct correlation between the decrease of determined d_f values and the increase in the incorporation efficiency of the drug.

The *in vitro* release of the IND from the stealth liposomes at 37°C is presented in Fig. 5. It is observed that the *in vitro* release of the drug from the prepared stealth liposomes is quite fast only for nanovectors prepared with the lowest ratio of PEG-ylated lipid (DPPC:DPPE-PEG 3000:IND 9:0.1:1) (Fig. 5) at 37°C. The combination of PEG-ylated lipids with phospholipids for the development of stealth nanovectors appears very promising, mostly due to the fact that the presence of PEG-ylated lipid alters the fractal morphology of the mixed nanocarriers and affects the release rate of the drug. Actually drug release is slower as more DPPE-PEG 3000 is present in the nanocarriers. Our results show the interrelationship between fractal dimension, drug incorporation and release profile. This phenomenology could serve as a control factor for the preparation and development of stealth liposomal formulations with the desired release profile, modulating the release rate of the IND, improving its therapeutic index and finally decreasing any unwanted side effects.

CONCLUSIONS

The liposomal carriers composed of DPPC:DPPE PEG 3000 (9:0.1, 9:0.5 and 9:1 molar ratios) were studied in two dispersion media and were found to retain their size distribution at least for the time period that they were studied (ca. 20 days). Their stability indicates that electrostatic and steric repulsion should be responsible for keeping the liposomes far enough to avoid aggregation. High ζ-potential values demonstrated the existence of electrostatic repulsion, especially in HPLC-grade water. The dispersion in PBS caused a shift of ζ-potential to less negative values for stealth liposomes, probably due to screening effects. The morphology and the structural properties of the three liposomal formulations are not significantly different in the two dispersion media, as demonstrated by fractal analysis. The morphological characteristics (d_f and d_s) changed in different temperatures, detecting differences that the size measurements did not reveal. The membrane micropolarity remains unaltered by the increase of PEGylated lipid content in the two dispersion media. Pyrene aggregation, probed via I_E/I_M values, indicates changes in the membrane structure and microfluidity. IND was incorporated into different stealth liposomal formulations and primary results on the incorporation of IND showed that the size of liposomes remained unaffected contrary to the values of ζ-potential at the lowest PEGylated lipid concentration. d_f values were slightly decreased indicating changes in the morphology of stealth liposomes. To best of the authors' knowledge this is the first report on the encapsulation of indomethacin in stealth liposomes and on the quantification of the morphological characteristics of stealth liposomes incorporating this Non Steroid Anti-Inflammatory Drug (NSAIDs).

In conclusion, the experimentally determined fractal dimension illustrates in a comprehensive way the self-assembly and the morphological complexity of stealth liposomal formulations. This in turn could be a useful tool for the development and characterization of innovative nanocarriers for

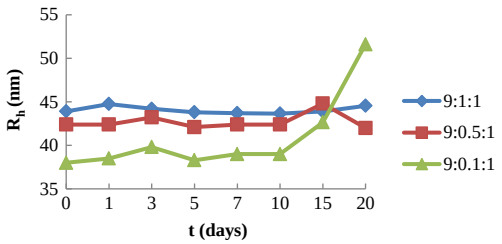

Fig. 4 Stability assessment of stealth liposomal formulations incorporating IND in HPLC-grade water.

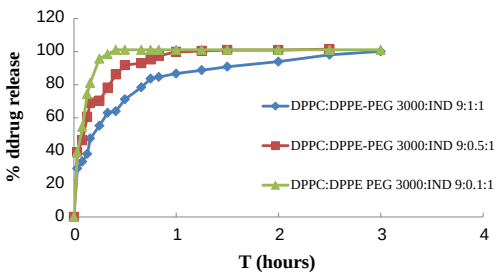

Fig. 5 Cumulative drug release from DPPC: DPPE-PEG 3000: IND 9:0.1:1, 9:0.5:1, 9:1:1 molar ratio at 37°C. Mean of three independent experiments run in triplicate, SD < 10%.

drugs with complete knowledge of their structural characteristics. We can conclude that fractal analysis can promote a new analytical concept based on the morphological characteristics of liposomal drug carriers complementary to that of the conventional techniques. Finally, this approach can disclose the pharmacokinetic reality of behavior of NSAID and can improve their therapeutic value and smoothes their side effects because the interdependence of particle size, shape and morphology effect on biological and pharmacokinetic processes, too. These research orientations could formulate a mathematical model for prediction of the physical status of liposomal nanocarriers.

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