Rapid report

Lipid membrane with low proton permeability ☆

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Abstract

This work reports the production of a liposomal formulation, having a lipidic membrane with known chemical composition and a low proton permeability, as confirmed by physicochemical characterization of the maintenance of a transmembranic pH gradient. These liposomes consist of DSPC, DSPE-PEG, DSPG and cholesterol, with low internal pH. To verify the low proton permeability of these liposomal bilayers, a study of proton migration according to the fluorescence quenching of 9-aminoacridine (9AA), as well as CPT-11 encapsulation, were used to monitor the acidification of the intravesicular space. Both experiments showed that this liposomal formulation is able to maintain a transmembranic proton gradient.
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Many reports have demonstrated that the encapsulation of compounds that are susceptible to degradation in physiological pH in liposomes containing a high internal proton concentration can avoid this degradation by using a transmembranic pH gradient [1–3]. However, these reports did not show experimentally the maintenance of the pH gradient across the lipidic bilayers.

Some researchers have speculated that substances such as sterols (cholesterol, ergosterol, hopanoid) [4], lipids with branched chains [4], isoprenes (carotene, dolichol, ubiquinone) [4], polyethyleneglycol [5], cardiolipin [6], phosphatidylglycerol [7] and sugars [8], such as sucrose and trehalose, could dehydrate the lipid bilayer and therefore maintain the proton gradient for long periods of time across the bilayer of cellular membranes and liposomes. However, no practical and conclusive results of this gradient maintenance have been reported. In 1994, Díaz and Monreal [9] published an excellent paper about liposomes prepared from bovine myelin lipids. Using meticulous and elaborate experiments, they demonstrated that these liposomes have low proton permeability, although the authors did not indicate which lipid or lipids are responsible for this unusual permeability. Besides the unknown chemical composition and dependence on the extraction process, these liposomes exhibited limitations with regard to immunogenicity since they are a biological material, containing antigens, which are not removed at all.

Sterically stabilized liposomes with known chemical composition and low proton permeability are found in the literature, encapsulating some antineoplasic Camptothecin derivatives [1,3]. This pentacyclic alkaloid contains a lactone ring, which is necessary for its antitumor activity. This ring hydrolyzes under physiological conditions (pH 7.4) to yield a therapeutically inactive carboxylate form [1,10]. These reports have indicated that Camptothecin derivatives, such as CPT-11, encapsulated in sterically stabilized liposomes containing low internal pH can avoid this lactone hydrolysis [1,3]. Although these results are very important to the Camptothecins study in liposomes, the procedures used for quantification of the lactone form are questionable since they involve the suspension of the liposomes in acidic medium, shifting the equilibrium toward the lactone form. Since low proton permeability in these liposomes was not demonstrated, a rigorous physicochemical characterization is necessary for a better understanding of the system and an interpretation of in vivo results.

In this context, the aim of this work is the preparation of liposomes having a defined chemical composition of the

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lipidic membrane and low proton permeability, rigorously confirmed by physicochemical characterization of the maintenance of a transmembranic pH gradient.

The sterically stabilized liposomes were prepared as follows. DSPC[1], DSPE-PEG[2], DSPG[3] and cholesterol (Genzyme Pharmaceuticals), at 7 mM of total lipids, were dissolved in a mixture of chloroform and methanol (2:1, v/v), which is evaporated off at reduced pressure. The lipid film was hydrated with 10.0 ml of 10 mM lactate buffer (pH 4.0) with 10% sucrose or 30 mM trehalose at 65 °C. Large unilamellar vesicles (LUV) were prepared by extruding 10 times through two stacked polycarbonate membranes with 100-nm pores.

The maintenance of a transmembranic proton gradient in sterically stabilized liposomes was evaluated, according to proton migration through the lipid bilayer, by recording the fluorescence spectra of 9-aminoacridine (9AA), in buffers with different pH values.

The 9AA probe is a fluorescent pH indicator whose fluorescence is quenched in an acidic medium due to the protonation of the amino group ($pK_a = 4.53$). To check for the low proton permeability of these liposomes, the fluorescence quenching of 9AA was used to monitor the acidification of the intravesicular space. An aliquot of LUV liposomes, prepared in 10 mM lactate buffer with 10% sucrose or 30 mM trehalose, was added to a quartz cuvette with 2 ml of 10 mM citrate or HEPES buffer, with the external pH ranging from 4 to 7.8, and 6 μM 9AA, as described in Ref. [9]. The fluorescence spectra were obtained on an SLM-AMINCO Spectrofluorimeter (SPF-500C), with a xenon lamp (250 W) as excitation source, for 550 s, at 37 °C, with excitation and emission wavelengths of 400 and 455 nm, respectively.

Two liposome formulations were prepared: the first composition was DSPC/DSPE-PEG/DSPG/cholesterol at 36:5:23:36 molar percentages, respectively, in 10 mM lactate buffer (pH 4.12) containing 10% sucrose (Fig. 1a); the second one was DSPC/DSPE-PEG/DSPG/cholesterol at 36:5:21:38 molar percentages, respectively, in 10 mM lactate buffer (pH 4.16) containing 30 mM trehalose (Fig. 1b). The buffers used in these experiments were prepared in the respective sugar solutions.

As shown in Fig. 1, there is a rapid quenching of the initial fluorescence whose intensity was dependent on the imposed pH gradient (ΔpH); that is, as this intensity increases, the 9AA migration into the vesicle is faster (according to the slope of the quenching curve). After the initial quenching, a stable baseline was obtained, even at the

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[1] DSPC = 1,2-distearoyl-*sn*-glycerol-3-phosphocholine (from Genzyme Pharmaceuticals).
[2] DSPE-PEG = *N*-(carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, sodium salt (from Genzyme Pharmaceuticals).
[3] DSPG = 1,2-distearoyl-*sn*-glycerol-3-phosphoglycerol, sodium salt (from Genzyme Pharmaceuticals).
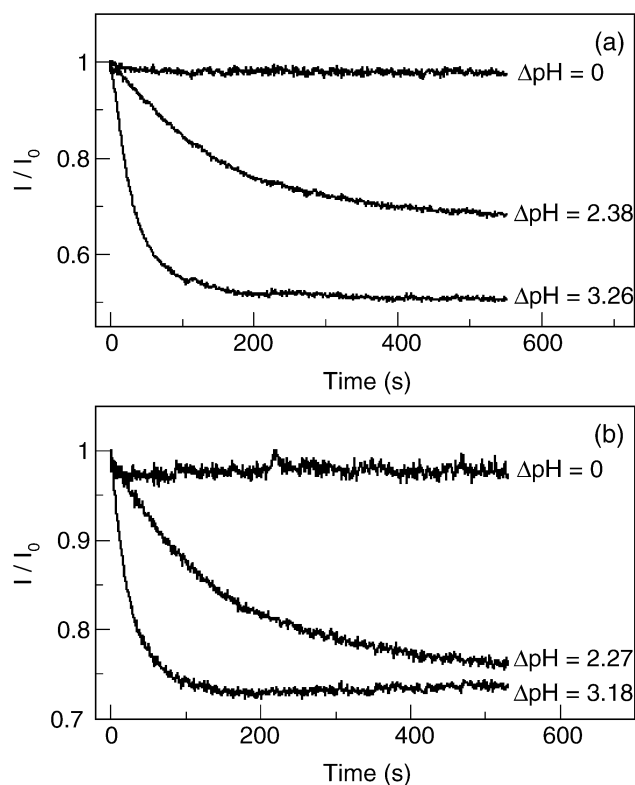

Fig. 1. pH gradient-dependent proton permeability of LUV composed of DSPC, DSPE-PEG, DSPG and cholesterol in lactate buffer containing (a) 10% sucrose and (b) 30 mM trehalose, monitored by the fluorescence quenching of 9AA, at 37 °C, for 550 s, with the external pH ranging from 4 to 7.8. The excitation and emission wavelengths were 400 and 455 nm, respectively. The buffers used in these experiments were prepared in the respective sugar solutions.

highest superimposed pH gradients. These results indicate that the established ΔpHs remain constant and therefore there is not any substantial efflux of protons from the intravesicular (acidic) space to the extravesicular one.

To certify that these liposomes are able to maintain a transmembranic proton gradient for a long period of time, the antineoplasic compound CPT-11 was incorporated into these liposomes. The liposomes were prepared according to the procedure described previously, in which the drug (10 μmol) was added to the chloroform/methanol mixture during the thin-film formation step. Nonincorporated CPT-11 was removed by using the Sephadex minicolumn centrifugation method [11]. Fluorescence assays were performed using an aliquot of LUV liposomes, prepared in 10 mM lactate buffer (pH 4) containing 10% sucrose, placed into a quartz cuvette with 2 ml of 10 mM HEPES buffer (pH 7.4) containing 10% sucrose. The fluorescence spectra were recorded at 37 °C, with an excitation wavelength of 370 nm.

Initially, we studied the hydrolysis kinetics behavior of free CPT-11. The fluorescence spectra of free CPT-11 (prepared in lactate buffer) in HEPES buffer (Fig. 2a) showed a red shift and an isostilbic point, indicating the presence of two fluorescent species in equilibrium, the lactone and carboxylate forms. The overlapped spectra

and the kinetic behavior of the lactone and carboxylate forms (according to the mean intensity of fluorescence profiles) were investigated using a chemometric method developed by Lawton and Sylvestre and applied to each experiment [12,13]. The method uses Principal Components Analysis, where the kernel is a Singular Value Decomposition [12].

Fig. 2b shows resolved fluorescence spectra and Fig. 2c mean intensity of fluorescence, as a function of time, of lactone (—) and carboxylate (- -) forms of free CPT-11, in 10 mM HEPES buffer (pH 7.4) containing 10% sucrose.
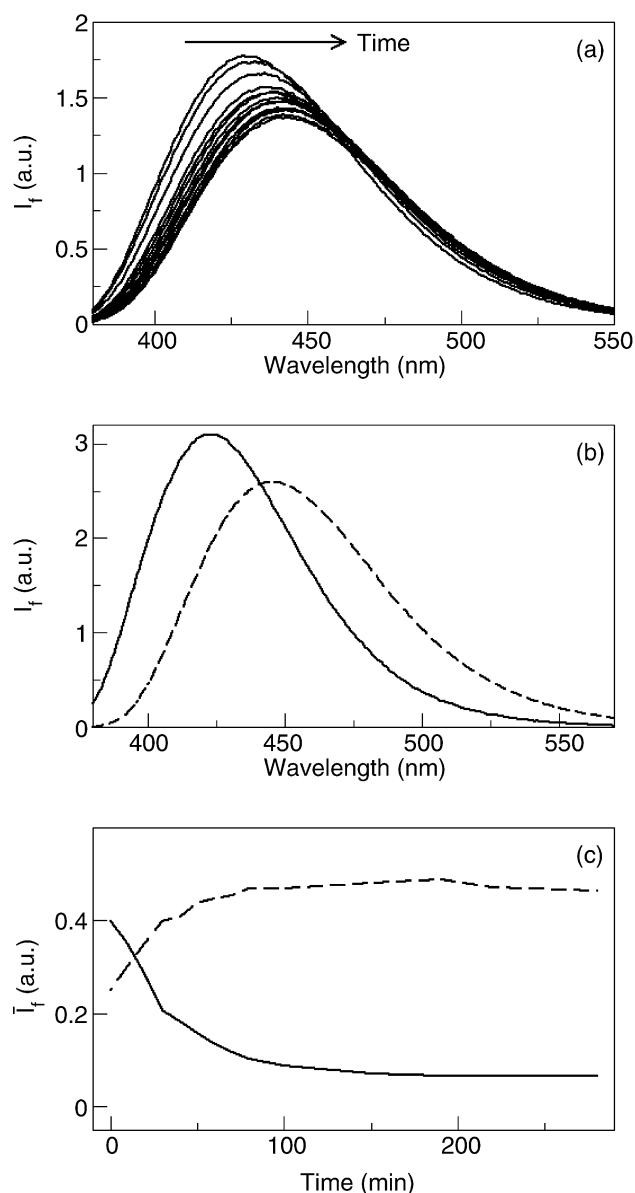

Fig. 2. (a) Fluorescence spectra of free CPT-11, as a function of time (280 min), in 10 mM HEPES buffer (pH 7.4) containing 10% sucrose, at 37 °C, with an excitation wavelength of 370 nm. (b) Resolved fluorescence spectra and (c) the mean intensity of fluorescence profiles, as a function of time, of lactone (—) and carboxylate (- -) forms of free CPT-11.
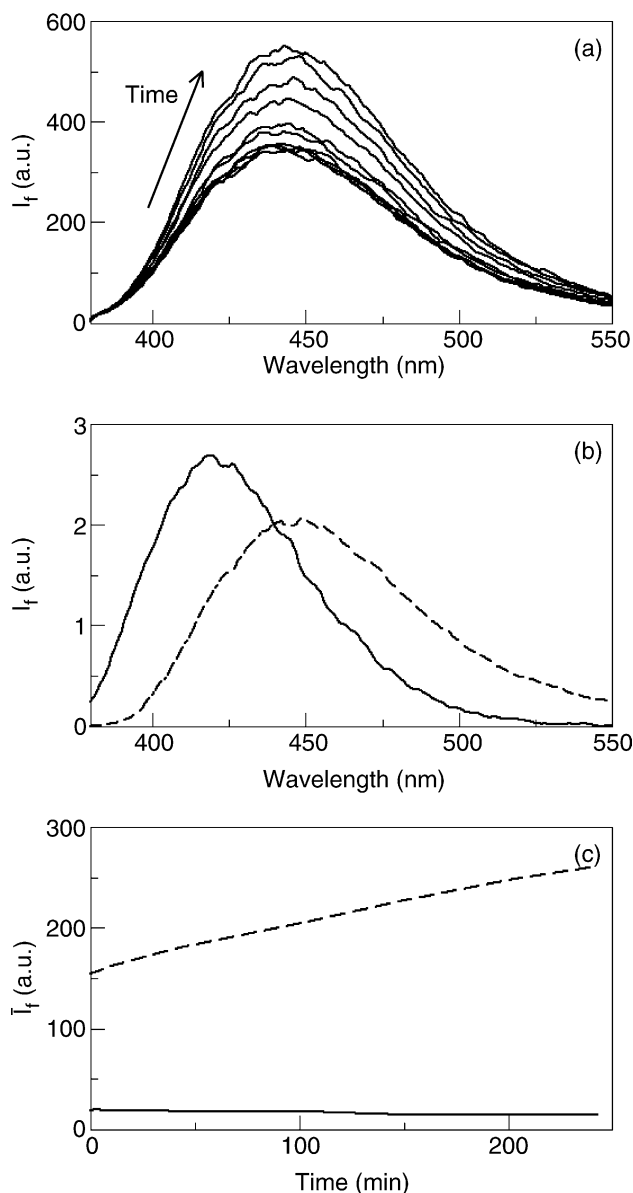

Fig. 3. (a) Fluorescence spectra of CPT-11:LUV, as a function of time (243 min), in 10 mM HEPES buffer (pH 7.4) containing 10% sucrose, at 37 °C, with an excitation wavelength of 370 nm. (b) Resolved fluorescence spectra and (c) the mean intensity of fluorescence profiles, as a function of time, of lactone (—) and carboxylate (- -) forms of CPT-11:LUV.

The free CPT-11 spectral results (Fig. 2b) show that there are two different species, lactone (—) and carboxylate (- -) forms. Evaluating the mean intensity of fluorescence profiles of both species (Fig. 2c), we observe that the lactone form hydrolyzes quickly in aqueous solution.

A similar kinetic study was performed in sterically stabilized liposomes containing CPT-11 (CPT-11:LUV), which consisted of DSPC/DSPE-PEG/DSPG/cholesterol at 34:5:22:39 molar percentages, respectively, in 10 mM lactate buffer (pH 4) containing 10% sucrose. CPT-11:LUV fluorescence spectra in HEPES buffer (Fig. 3a) show a slight red shift, an isostilbic point and an enhancement of fluorescence intensity.

The chemometric treatment indicated an unexpected result, compared to free CPT-11, as shown in Fig. 3b,c. The mean intensity of fluorescence profiles indicate that the lactone form remained constant during 243 min, although the fluorescence of carboxylate form was enhanced. If hydrolysis occurs, the fluorescence of lactone form should decrease and, on the other hand, the fluorescence of carboxylate should increase concomitantly. Therefore, our results suggest the stabilization of lactone form, confirmed by the low and constant fluorescence of lactone form related to its presence in lipidic bilayers at high concentrations, and, consequently, fluorescence quenching due to high concentration has occurred. In addition, the enhanced fluorescence of the carboxylate form is related to its entrance from the interface to deeper into the bilayer, raising its fluorescence due to lower quenching. This assumption was confirmed by using conductivity measurements (data not shown). In this experiment, the charge density on the liposome surface decreased as a function of time, indicating the entrance of carboxylate species (negatively charged) into the bilayer.

According to these experiments, we conclude that these liposomes can maintain a stable lactone form, avoiding its hydrolysis in physiological pH, i.e., this liposomal formulation is able to maintain a transmembranic proton gradient for a long period of time. This liposomal formulation is important for the encapsulation of pH sensitive bioactives for applications in the pharmaceutical field, bringing benefits to human health.

Acknowledgements

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