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Development of Doxorubicin hydrochloride loaded pH-sensitive liposomes: investigation on the impact of chemical nature of lipids and liposome composition on pH-sensitivity

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Abstract

This study investigates the impact of the chemical nature of lipids and additive on the formulation and properties of pH sensitive liposomes. The objective is to understand the respective role of the formulation parameters on the liposome properties in order to optimize the conditions for efficient encapsulation of doxorubicin (DOX). These liposomes should be stable at physiological pH, and disrupt in slightly acidic media such as the tumor microenvironment to release their DOX load. The major challenge for encapsulating DOX in pH sensitive liposomes lies in the fact that this drug is soluble at low pH (when the pH-sensitive liposomes are not stable), but the DOX aqueous solubility decreases in the pH conditions corresponding to the stability of the pH-sensitive liposomes. The study of pH-sensitivity of liposomes was conducted using carboxyfluorescein (CF) encapsulated in high concentration, *i.e.* quenched, and following the dye dequenching as sensor of the liposome integrity. We studied the impact of (i) the chemical nature of lipids (dioleoyl phosphatidyl ethanolamine (DOPE), palmitoyl-oleoyl phosphatidyl ethanolamine (POPE) and dimyristoyl phosphatidyl ethanolamine (DMPE)) and (ii) the lipid / stabilizing agent ratio (alpha-tocopheryl succinate), on the pH sensitivity of the liposomes. Optimized liposome formulations were then selected for the encapsulation of DOX by an active loading procedure, *i.e.* driven by a difference in pH inside and outside the liposomes. Numerous experimental conditions were explored, in function of the pH gradient and liposome composition, which allowed identifying critical parameters for the efficient DOX encapsulation in pH-sensitive liposomes.

Keywords

Liposomes; pH-sensitivity; phosphatidyl ethanolamine lipids; alpha-tocopheryl succinate; carboxyfluorescein; doxorubicin.

1. Introduction

Cancer is second only to the cardiovascular diseases as a cause of mortality. The clinical use of chemotherapeutic agents to treat cancer is successful in many cases. However, the lack of selectivity of chemotherapeutic agents which cause severe side effects and the emergence of multidrug resistance (MDR) are two major drawbacks for the effective use of these agents in clinic [1]. MDR is a complex phenomenon resulting from synergism of many factors. One of the most important factors is the change of pH gradient across the cell membrane, i.e. acidification of the tumoral extracellular (pHe) fluid and alkalization of the cytosol (pHi), which results from the Warburg effect [2–4].

Liposomes are considered as the most advanced type of particulate drug carriers and have gained importance as the mainstream drug delivery system. The importance of liposomes lies in the fact that hydrophilic, lipophilic as well as amphiphilic drugs can be entrapped in the liposomes [5–7]. Of particular interest are fusogenic liposomes that show triggered phase transitions and release properties promoted by various chemical and physical stimuli, *e.g.*, temperature, pH, light etc. [8–11]. pH-sensitive liposomes are of prime importance because they undergo phase transition and acquire fusogenic properties in acidic environment, leading to the release of their aqueous contents [12]. This property is of particular interest for delivery of anticancer drugs since the extracellular pH of cancer tissues is slightly acidic due to the high metabolic activity of cancer cells [13–15].

Different classes of pH-sensitive liposomes have been proposed according to their triggering mechanism [16–21]. The most advanced liposomes use lipids with phosphatidyl-ethanolamine (PE) as polar head in their composition. However, pure PE lipids do not form stable liposomes and are thus associated with an additional amphiphilic molecule that stabilizes the liposomes bilayers, such as alpha-tocopheryl succinate (α -TOS), oleic acid, palmitoylhomocysteine, or cholesteryl hemisuccinate (CHEMS). These stabilizers are in ionized form (negatively charged) at physiological pH and thus intercalate in between the phosphatidylethanolamine (PE) molecules and favor the lamellar organization, resulting in the formation of liposomes. As these liposomes are exposed to acidic environment, the carboxyl group of the stabilizer is protonated resulting in the reversion of the PE molecules into inverted hexagonal phase, destabilization of the liposomes and thus the release of the contents of the liposomes [12,22–24].

The objective of the present study was to develop and design a novel efficient pH-sensitive formulation to form doxorubicin (DOX) loaded liposomes that should be stable at physiological pH and collapse in slightly acidic media such as the cancerous microenvironment to selectively release their doxorubicin hydrochloride (DOX) within tumor tissues [25].

To this end, in the first part of our study we optimized the formulation of pH-sensitive liposomes, by investigating the impact of formulation parameters, such as the chemical nature and composition of the lipids, on the properties of liposome (size, encapsulation efficiency and pH-sensitivity). A variety of liposomes was prepared using (i) –PE containing lipids (dioleoylphosphatidyl ethanolamine, DOPE, palmitoyloleoylphosphatidyl ethanolamine, POPE, dimyristoylphosphatidyl ethanolamine, DMPE), and (ii) the stabilizing agent, α -TOS at different ratios. The pH-sensitivity was assessed by measuring the release of carboxyfluorescein, CF from liposomes at different pH in the range 5.5-7.4 and for different incubation times, according to a fluorescence methodology based on the self-quenching of CF [26]. CF was encapsulated at millimolar concentration in the internal aqueous phase during liposome preparation, leading to a strong fluorescence quenching and thus a low emission of CF molecules in the liposomes. Once the liposome membrane is disrupted, CF fluorophores are released into the buffer. This leads to a decrease in the concentration and fluorescence quenching of CF molecules in the liposomes, and thus, an increase in their emission.

The second part of the work involves the encapsulation of DOX in pH-sensitive liposomes and study of the effect of nature and concentration of lipid and stabilizer on doxorubicin encapsulation. DOX is a widely used efficient anti-cancer drug [27], but its clinical use is limited by its cardiotoxicity and myelosuppression [28]. Doxorubicin has high anti-tumor activity but specificity is very low, which results in the serious side effects. Interestingly, DOX entrapped in liposomal formulation has shown reduced cardiotoxicity and improved specificity for the tumor area [24,29–33]. However, being a weak base, its solubility in aqueous buffers changes with pH, which makes its encapsulation, in pH-sensitive liposomes, difficult. The neutral form of DOX is membrane permeable at alkaline pH and becomes membrane impermeable when charged at acidic pH. Therefore, the encapsulation of DOX in the liposomes is based on a pH gradient between inner and outer water phase. For example, DOX has been encapsulated in the liposomes using transmembrane sulfate-or phosphate-or citrate-gradient [34–42] with acidic pH inside and physiological pH outside. DOX in its neutral form diffuses into the liposomes and gets

protonated, which prevents the leakage of the positively charged DOX once encapsulated [34–40,43]. Moreover, the solubility of DOX increases at low pH [35], while pH-sensitive liposomes become unstable and may disrupt at acidic pH [44]. In this context, encapsulating DOX in pH-sensitive liposomes is a complex problem, addressed by some reports in the literature [44] with formulations composed of DOPE / HSPC / CHEMS / CHOL (respectively dioleoylphosphatidyl ethanolamine / hydrogenated soy phosphatidylcholine / cholesteryl hemisuccinate / cholesterol). The purpose of the present study is more general, exploring the impact of the formulation parameters to understand and optimize the pH sensitivity of liposomes as well as the conditions compatible with the best encapsulation of DOX. The original system chosen here focuses on; i) – PE lipids (DOPE, POPE and DMPE) in association with α -TOS and CHOL, and ii) the active loading of DOX in function of different pH gradients through the liposome bilayer.

2. Materials and Methods

2.1. Materials

Dioleoyl phosphatidyl ethanolamine (DOPE), palmitoyl-oleoyl phosphatidyl ethanolamine (POPE) and dimyristoyl phosphatidyl ethanolamine (DMPE) were purchased from Avanti-Polar Lipids, Inc. Alpha-tocopheryl succinate (α -TOS), carboxyfluorescein (CF), triton X-100, phosphate buffered saline (PBS) and chloroform were purchased from Sigma-Aldrich. Doxorubicin hydrochloride was purchased from Alfa Aesar, and Sephadex™ G-25M PD10 column from GE Healthcare. All other chemicals were of analytical grade.

2.2. Preparation of CF-loaded Liposomes

The liposomes were prepared by the polycarbonate membrane extrusion method. Liposomes containing CF were obtained using three different ratios between lipids and α -TOS (90:10, 80:20 and 70:30 respectively). Briefly, specified amounts of lipid and α -TOS were dissolved in 1 mL of chloroform – with the exception for DMPE which was dissolved in chloroform / methanol (2:1) for solubility reasons – in a small round bottom flask to make final lipid concentration of 10 mM. Thereafter, the solvent was evaporated using the rotary evaporator and a dried thin film was formed at the bottom of the flask. The film was further dried under vacuum for 1 hour to ensure complete removal of solvent. The lipid film was then rehydrated with 1 mL

carboxyfluorescein solution (50 mM) in phosphate buffered saline (pH 7.4), followed by sonication for 5-10 seconds and then was let 2 hours for proper hydration of the film and formation of the suspension of multilamellar vesicles (MLVs). The suspension was then vortexed for 5 minutes and finally passed through a 100 nm polycarbonate membrane (17 times), using a Liposofast[®] extruder, to form large unilamellar vesicles (LUVs). The non-encapsulated CF was separated from the liposomes by size exclusion chromatography (PD10 Sephadex[®] G-25M column), pre-equilibrated in phosphate buffered saline (pH 7.4).

2.3. Preparation of DOX-loaded Liposomes

2.3.1. Passive Loading

The liposomes were prepared according to the same protocol as described above, except that the thin film was rehydrated with 1 mL DOX solution in PBS (pH 7.4). Then, non-encapsulated DOX was separated from the liposomes suspension by size exclusion chromatography as described above, with a column equilibrated with PBS (pH 7.4).

2.3.2. Active loading by Sodium Phosphate and pH gradient

In this method, liposomes were prepared by using sodium phosphate buffer (0.2M NaH₂PO₄·2H₂O + 0.2M Na₂HPO₄·12 H₂O) at different pH values (7.0, 7.2, 7.4 and 7.8). After formation of liposomes, the external pH was increased, to promote DOX diffusion inside the liposomes, by addition of specified amounts of NaOH 1 M, up to reach pH values of 7.4, 7.8, 8.5 or 9.0. Each pH value corresponds to a single experiment that allowed investigating the impact of pH gradient on the active loading (see below). Then, the DOX-saline solution was added to the liposomes, with DOX concentration at 2 mM, and lipid concentration at 10 mM. The liposomes / DOX were then incubated overnight at room temperature (20-25°C), and then free DOX was separated by size exclusion chromatography using PBS (pH 7.4) as eluent, as described above.

2.4. Characterization

2.4.1. Size measurements

Size distribution and polydispersity indices were measured by dynamic light scattering (DLS) with a Malvern apparatus (NanoZS[®], Malvern, Orsay, France). Mean particle size was

assimilated to z-average hydrodynamic diameter and the width of size distribution to polydispersity index (PDI). DLS measurements were performed using a helium/neon laser, 4 mW, operated at 633 nm, with the scatter angle fixed at 173° and temperature maintained at 25°C on diluted sample. DLS data were analyzed using a cumulants-based method assuming spherical shape.

2.4.2. Determination of encapsulation efficiency (EE)

The absorbance values of liposomes encapsulating CF or DOX were measured by using Cary 400 and Cary 4000 Scan UV-Visible Spectrophotometers. The liposomes encapsulating the molecules of interest (CF or DOX) were diluted 100x and their absorbance was measured in the range between 300 and 600 nm. The encapsulation efficiency (EE) was obtained from the absorbance values at 492 nm and 500 nm for CF and DOX respectively, according to Eq. (1),

$$EE = \frac{m_x}{m_x^0} \times 100 \quad (1)$$

where, x refers to DOX or CF, m_x is the mass encapsulated in the liposomes (measured by visible spectrometry), and m_x^0 is the total mass used.

2.4.3. Fluorescence assay

The pH sensitivity of liposomes was evaluated by using the CF quenching assay. All fluorescence measurements were done by using a Fluorolog[®] spectrofluorimeter (Horiba, France). Excitation and emission wavelengths were set at 480 nm and 517 nm, respectively. CF-loaded liposomes (10 µL) were added to cuvettes containing each 1 mL of PBS at different pH (5.5, 6.0, 6.4 and 7.4), and kept for incubation. Fluorescence intensity was measured after various incubation times (5, 10, 15 and 30 min). Since the quantum yield of CF is pH dependent, the pH was readjusted to 7.4 by addition of specified amount of NaOH 1 M, in order to compare the changes in quenching efficiency, and fluorescence was measured again. Finally, 10 µL of Triton X-100 was added in each cuvette to disrupt all liposomes and fully release CF molecules, and the fluorescence was measured again. The percent of CF release was calculated using:

$$\%CF \text{ release} = \frac{F_{pH} - F_0}{F_{TX} - F_0} \times 100 \quad (2)$$

where, F_{pH} is the fluorescence intensity following the incubation at a given acidic pH , F_0 is the fluorescence intensity after incubation at $pH = 7.4$, and F_{TX} is the fluorescence intensity after addition of Triton X-100.

3. Results and Discussion

3.1. CF-loaded pH sensitive liposomes

3.1.1. Effect of lipid composition on the size of the liposomes

The size of the liposome suspensions encapsulating the CF, made with DOPE, POPE and DMPE, and at different ratio lipid / α -TOS is reported in Fig. 1 (a). Liposomes made with DMPE (having the smallest chain length) are the smallest, while those made with DOPE (longest chain) are the largest. Furthermore, these results show that the proportion of α -TOS in the formulation also impacts the liposome size, as increasing the α -TOS content induces a global decrease of the size (except for DMPE: α -TOS 80:20). The size range of the suspensions depends on the nature of the lipid, i.e., varies from 190 nm to 160 nm for DOPE, from 170 nm to 140 nm for POPE, and from 130 nm to 108 nm for DMPE. All samples show a good monodispersity, with PDI values < 0.15. For all compositions, the size range remains compatible with the parenteral administration route.

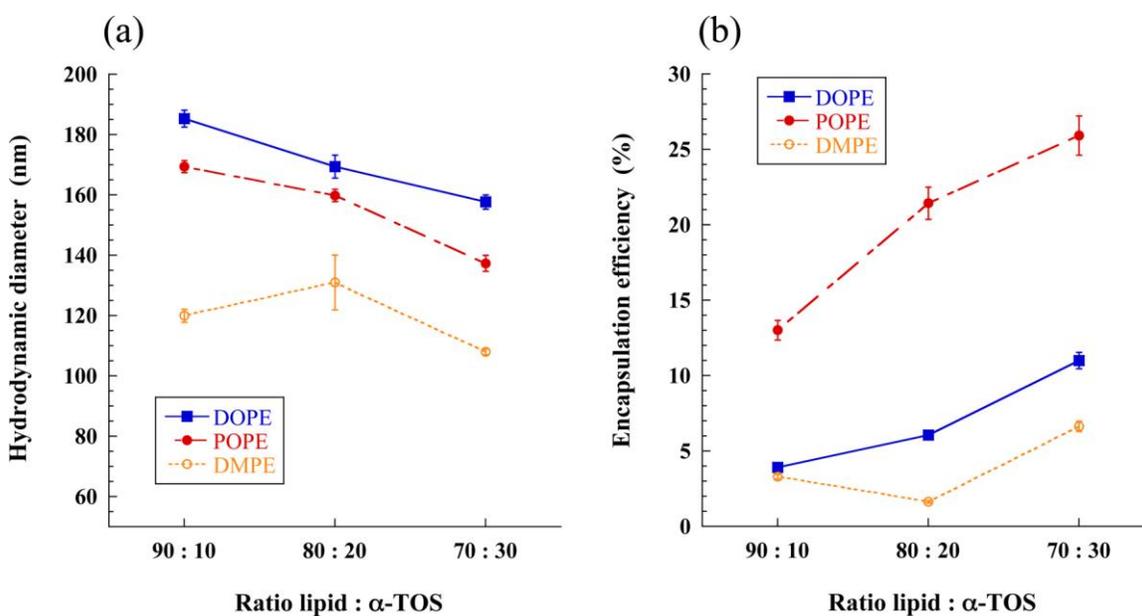


Figure 1: Effect of the nature of the lipid and the lipid: α -TOS ratio on (a) the average size of liposomes, and (b) the efficiency of CF encapsulation.

3.1.2. CF encapsulation efficiency

The absorbance of the CF-loaded liposomes was measured after separation by size exclusion chromatography (PD10 columns). As the concentration of CF loaded in the liposomes was constant (50 mM solution) in all the formulations, the measurements of absorbance before and after the separation of the free dye reflect globally the % of CF inside the liposomes. The results reported in Fig. 1 (b) show that the best encapsulation properties were obtained by using POPE, followed by DOPE. Additionally, an increasing α -TOS content increases the encapsulation efficiency (except for DMPE: α -TOS 80:20). This can be explained by the α -TOS impact on the liposome size (Fig. 1 (a)), and the increase in the total number of liposomes produced (since lipid concentration is constant and size decreases, thus number of liposomes increases). To prove this point, the number of vesicles of each formulation was calculated based on the liposome size, values of encapsulation efficiency and concentration of the CF solution inside the vesicles. Results are summarized in Table S1 in supplementary information section. These new results show that, assuming that the dye concentration is similar and constant in all formulations, the encapsulation efficiency is only related to the size and number of vesicles. It is noteworthy that the encapsulation efficiency of these formulations with CF does not exceed 25%, possibly due to the very high CF concentration (50 mM) required for the quenching-based method. It follows therefrom that the percentage of CF encapsulated in liposomes is 10%, 25% and 5% for liposomes made with DOPE, POPE and DMPE, respectively. These differences in values are likely related to variations in the number and size of liposomes for the different formulations as well as to the ability of the lipids to form stable liposomes with α -TOS.

3.1.3. Effect of lipid composition on pH-sensitivity/Release of CF:

As described above, the integrity of the lipid bilayer was monitored by a fluorescent assay based on the self-quenching of concentrated CF in the liposome core. Once the bilayer is permeabilized, CF is released into the buffer, which reduces the quenching of the fluorophore and results in a fluorescence intensity increase of the liposome core. It should be noted that the CF fluorescence is pH dependent and decreases in acidic pH. Therefore, all comparison of

fluorescence intensities was performed after readjusting pH at 7.4. Since the liposomes are stable at this pH, this readjustment should not influence the liposome permeation measurement. Finally, Triton X-100 was used to completely destroy the remaining intact liposomes, giving rise to the reference signal that corresponds to the complete CF release.

Figure 2 reports the CF release as a function of the incubation pH and lipid composition for (a) DOPE, (b) POPE, (c) DMPE, containing different fractions of α -TOS. These data highlight the clear sensitivity of these liposomes to acidic pH that results in their gradual destruction as pH is lowered. The nature of the lipid does not strongly impact on this behavior, as all of them were very sensitive to change in pH. But POPE appears less sensitive to slightly acidic pH and more sensitive to $\text{pH} \leq 6$. Finally, the stabilizer α -TOS also modifies the pH sensitivity of the liposomes, as the pH-sensitivity decreases when α -TOS concentration increases. This appears fully logical since α -TOS stabilizes the liposomes and thus, decreases the membrane disruption. For all three lipids, among the three different lipid/ α -TOS ratios, the 90:10 ratio showed the highest pH-sensitivity. At this ratio, the CF was released very quickly (almost 40-60%) from the liposomes at pH 6.4, that corresponds to the pH of tumor microenvironment [14,15], and was fully released at pH 5.5.

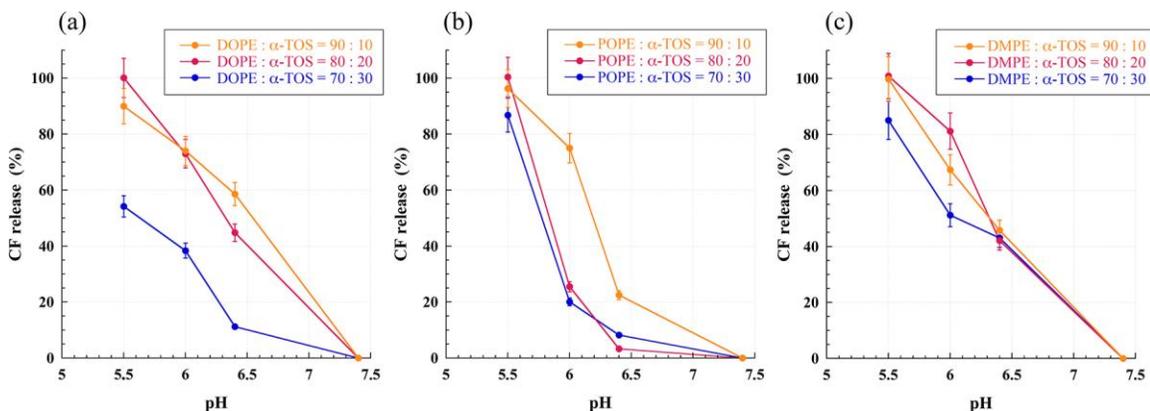


Figure 2: Evaluation of the pH sensitivity of CF-loaded liposomes, after 30 min incubation in PBS at different pH, as a function of the nature of lipid (a) DOPE, (b) POPE, (c) DMPE, and as a function of the lipid: α -TOS ratio.

3.1.4. Effect of incubation time on the release of CF from the liposomes

Figure 3 reports the impact of incubation time on the CF release for DOPE, POPE and DMPE liposomes containing 10% or 30% of α -TOS. The time of incubation shows almost no influence

on liposomes containing 10% of α -TOS, where the pH-induced membrane disruption and the release of the liposomes content are almost immediate.. In contrast, the incubation time plays an important role in the case of less sensitive DOPE and POPE liposomes (i.e., with 30 % α -TOS). In these cases, a prolonged incubation of pH sensitive liposomes in acidic conditions promotes the bilayer permeation and / or liposome destruction, and thus increases the amount of CF release. When incubated at pH = 5.5, the % of released CF increases from 30% (after 5 min) to 55% (after 30 min) for DOPE: α -TOS, from 50% (5 min) to 85% (30 min) for POPE: α -TOS liposomes and finally from 60 % (5 min) to 85 % (30 min) for DMPE: α -TOS. .

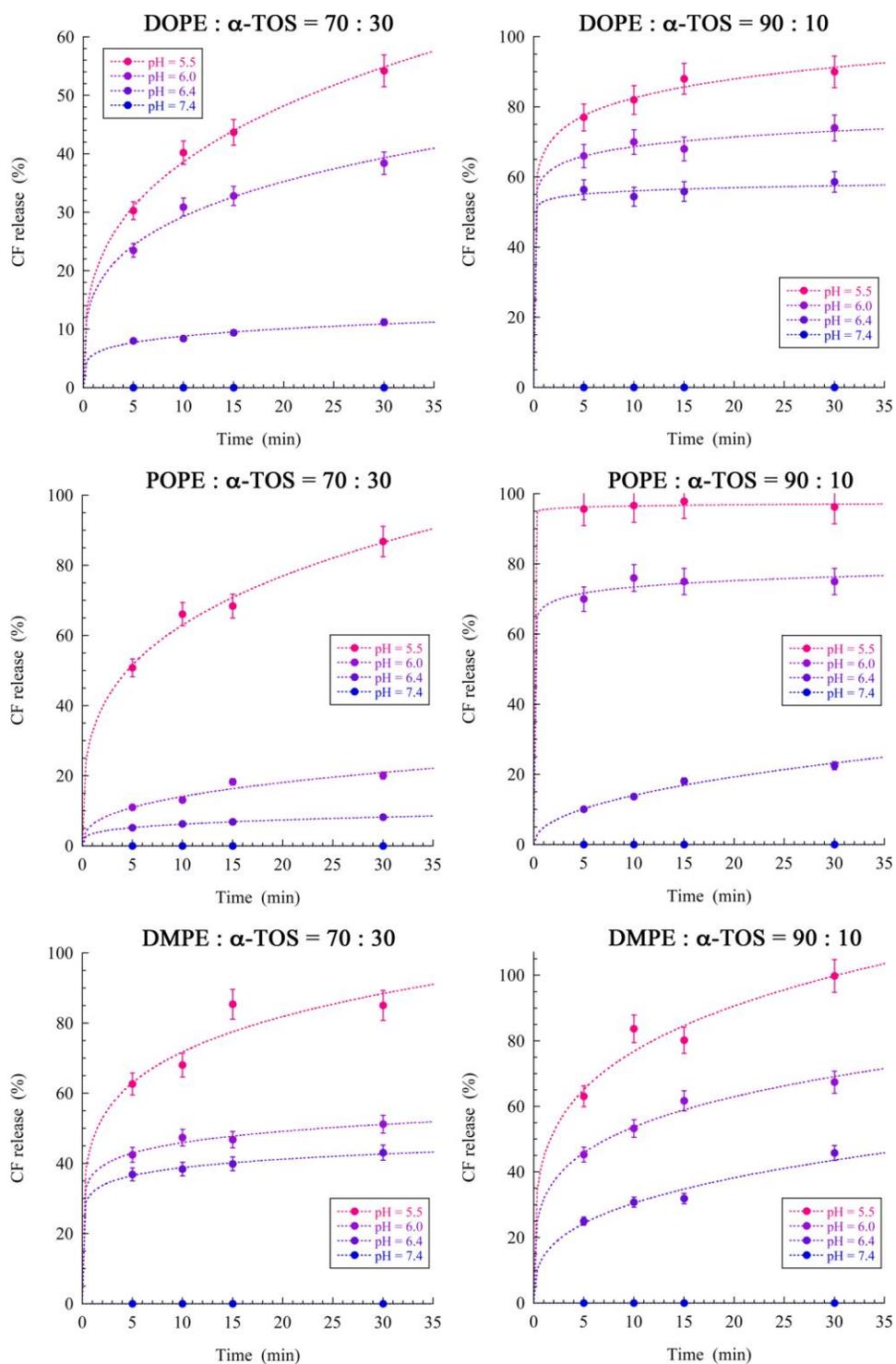


Figure 3: Effect of incubation time on the release of CF from liposomes at different pH, for different lipids (DOPE, POPE and DMPE) and two different lipid:α-TOS ratios, 70:30 and 90:10.

3.2. DOX-loaded pH-sensitive liposomes

Based on the results obtained above, we adapted the formulation processes to the encapsulation of DOX, which has a pH-dependent solubility. All the assays described with CF were performed with liposomes prepared by a passive loading method, where the lipid multilayers were rehydrated with an aqueous buffer already containing CF. Then after extrusion, the non-encapsulated CF molecules were removed by size exclusion chromatography.

In order to formulate DOX-loaded liposomes, we first tried to use the same passive loading method, using liposomes containing different stabilizers (α -TOS, CHEMS, CHOL and their combination). However, when rehydrating the lipid film with a DOX solution at neutral or alkaline pH, the liposomes could not be formed, likely due to electrostatic repulsive interactions between the liposome components and the DOX. We then used an active loading method to encapsulate DOX. This technique is based on the pH gradient between the external and internal aqueous phase. The most commonly reported buffer solutions for hydrating the lipid film are citrate buffer at pH 4.0 (300 mM), ammonium sulfate buffer at pH 5.5 (120 mM) and magnesium sulfate buffer at pH 3.5 (300 mM) [41]. Herein, we hydrated the lipid film at neutral to alkaline pH using phosphate buffer.

Different pH gradients of phosphate buffer were tested to optimize the encapsulation of DOX (Table 1). Part I of Table 1 shows the assays performed with passive loading, which were not working as liposomes did not form. Part II of Table 1 describes the assays with the active loading technique using POPE and DOPE / α -TOS system at different lipid: α -TOS ratios, and different values of internal / external pH. The formulations associated with “+” result represent the best formulations with efficient formation of liposomes and significant encapsulation of DOX (20 % to 100 %). The formation of liposomes was verified by DLS measurements and the DOX encapsulation was checked by the absorbance measurements of all the formulations. The highest load of DOX was obtained with “POPE: α -TOS 80:20” and “POPE/DOPE: α -TOS 70:30” using pH 7.4 / 9.0 gradient. Finally, the part III of Table 1 shows additional assays performed using different stabilizing agents and also by using a combination of stabilizing agent and CHOL. Our data show successful formation of liposome and encapsulation of DOX using α -TOS in association with POPE and CHOL, as well as for the combination of POPE/DOPE with CHEMS.

Table 1: Optimization of conditions for getting efficient loading of DOX in different formulations of liposomes. Hydrating and external buffers were PBS in both cases.

	Composition of liposomes	Loading technique	pH of hydrating buffer	pH of external buffer	Result
I	POPE/DOPE: α TOS (90:10)	Passive	7.4	---	(-)
	POPE/DOPE: α TOS (70:30)	Passive	7.4	---	(-)
	POPE/DOPE: α TOS:CHOL (65:20:15)	Passive	7.4	---	(-)
II	POPE/DOPE: α TOS (80:20)	Active	7.0	7.4	(-)
	POPE/DOPE: α TOS (80:20)	Active	7.4	8.0	(-)
	POPE/DOPE: α TOS (80:20)	Active	7.4	8.5	(-)
	POPE/DOPE: α TOS (80:20)	Active	7.8	8.5	(-)
	POPE/DOPE: α TOS (80:20)	Active	7.0	9.0	(-)
	DOPE: α TOS (80:20)	Active	7.4	9.0	(+/-)
	POPE: α TOS (80:20)	Active	7.4	9.0	(+)
	POPE/DOPE: α TOS (90:10)	Active	7.4	9.0	(-)
	POPE/DOPE: α TOS (70:30)	Active	7.4	9.0	(+)
III	POPE/DOPE:CHEMS (70:30)	Active	7.4	9.0	(+)
	DOPE: α TOS:CHOL (65:20:15)	Active	7.0	9.0	(-)
	DOPE: α TOS:CHOL (65:20:15)	Active	7.4	9.0	(+/-)
	POPE: α TOS:CHOL (65:20:15)	Active	7.0	9.0	(+)
	POPE: α TOS:CHOL (65:20:15)	Active	7.4	9.0	(+)
	POPE/DOPE:CHEMS:CHOL (65:20:15)	Active	7.4	9.0	(+/-)

The notation POPE/DOPE: α -TOS means that two experiments were performed, one being with POPE: α -TOS and the other one with DOPE: α -TOS, but results were identical.

(-) = Not working: either DOX precipitated or no liposome was formed

(+) = Liposome formed and DOX was encapsulated

(+/-) = Liposomes were formed and DOX was encapsulated but quickly released within 1-2 minutes

The formulations in Table 1 associated with “+/-” results provide efficient liposome formation and DOX encapsulation, but lead to DOX leaking after size exclusion chromatography using PBS 7.4 as an eluent. This might be related to the inadequate strength of the membrane to retain the DOX in the liposomes. This phenomenon was observed by the precipitation of the DOX within few minutes after the separation of the liposomes encapsulating DOX from the free DOX by size exclusion chromatography.

The general overview emphasized in Table 1 was further investigated through the measurement of encapsulation efficiencies for the best formulations, reported in Fig. 4a. The encapsulation efficiency of DOX in pH sensitive liposomes depends on the nature of the -PE lipid (higher with POPE). And among different liposomal formulations of POPE, higher encapsulation efficiencies are resulted with α -TOS and α -TOS/CHOL combination as compared to CHEMS and CHEMS/CHOL combination. The graphs represent the results for two different pH gradients inside and outside the liposomes. In case of pH gradient with pH 7 inside and 9 outside the liposomes, the DOPE did not form liposomes at pH 7. But there was liposome formation in case

of POPE at this pH. That's why the results have been shown only for POPE in case of the pH gradient with pH 7 inside and pH 9 outside the liposomes. In the other half of the graph the results have been shown for the experiments conducted with pH 7.4 inside and pH 9 outside the liposomes. Importantly, the two most interesting formulations are "POPE: α -TOS 80:20" and "POPE: α -TOS:CHOL 65:20:15", which provide encapsulation efficiencies around 100%. This result is likely due to the presence of lipid POPE, as we have got higher encapsulation efficiencies (25%) using POPE in case of CF as well (Fig. 1b). The reason could be the better affinity of the POPE to form liposomes and encapsulate CF/DOX under these conditions as compared to other lipids used. Interestingly, compared to the encapsulation efficiencies obtained with CF (Fig. 1 (b)), the values with DOX are much higher, due to the active loading methodology. The presence of cholesterol is also very important because cholesterol considerably decreases the leakage of the encapsulated drugs in the extracellular environment or throughout the circulation [22] and helps to achieve immediate release of the encapsulated drugs when used for triggered release applications due to its non-bilayer structure forming properties [45].

DOX being neutral at alkaline pH migrates into the liposomes through the bilayer. Two phenomena are likely involved in an efficient encapsulation and trapping of DOX in the liposomes (Fig. 4a). The first one is the protonation of the drug inside the liposomes. The resulting charged DOX is unable to cross the bilayer and stays thus inside the liposomes [43,46,47]. The second phenomenon involved in the active loading of DOX is its precipitation due to an increase of its concentration in the liposome above the saturation threshold. Li *et al.* [47] showed that DOX starts aggregating at 0.5-1.5 mM concentrations in citrate and sulfate buffers even if its concentration is about 100 times lower than its normal aqueous solubility threshold and the DOX release from the liposomes containing DOX fibers was relatively slow. Cullis and coworkers [46,48] proposed that DOX is predominantly bound to the inner monolayer, leading to invaginations of the membrane. Both these phenomena contribute towards retention of DOX inside the liposomes. Literature also reported that, due to its amphiphilic nature, part of the DOX can be entrapped in the lipid bilayer of the liposome, thus, further increasing the DOX loading [49].

The size of the liposomes was measured after extrusion both before and after active DOX loading. The data in Fig. 4 (b) shows that "empty" liposomes present a relatively constant size

whatever their composition. After drug loading, the majority of samples showed only a slight increase in their size. A significant increase in size was only observed in CHEMS-containing liposomes (DOPE:CHEMS 70:30 and POPE:CHEMS 70:30 liposomes), likely as a result of the sensitivity of CHEMS to the prolonged exposure to alkaline pH, resulting in the aggregation and increase in the average size of the liposomes.

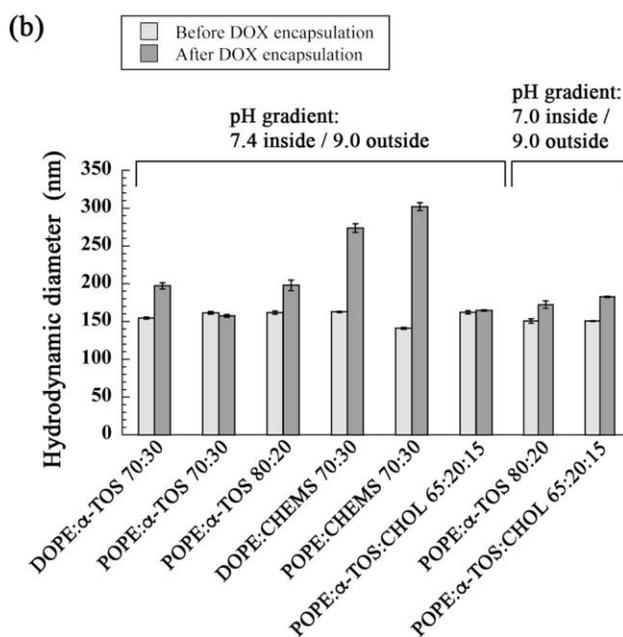
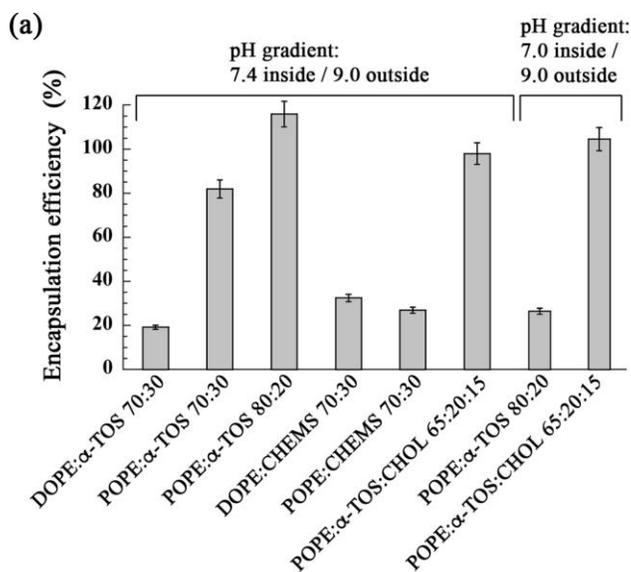


Figure 4: Characterization of the formulations of pH sensitive liposomes encapsulating DOX. (a) Encapsulation efficiency and (b) hydrodynamic diameter (before and after DOX encapsulation) as a function of the nature of the lipid and stabilizing agent, and their respective proportions.

The presence of CHOL seems to be important for stability of the liposomes and for the retention of the DOX inside liposomes at physiological pH. In order to confirm the pH sensitivity for this new formulation containing CHOL (*i.e.* the system “POPE: α -TOS:CHOL 65:20:15”), we performed the experiments to form liposomes with the same composition but using passive loading technique. We used the CF solution in PBS (pH 7.4) to load CF into the liposomes and then measured the release of CF by a fluorescent assay explained above in section 2.4.3. The results are reported in Fig. 6, after 5 min, 10 min, 15 min and 30 min of incubation at different pH. It clearly appears that, in that case (*i.e.* POPE: α -TOS:CHOL 65:20:15), liposome formulation containing CHOL remain significantly sensitive to pH like the formulations without CHOL described above.

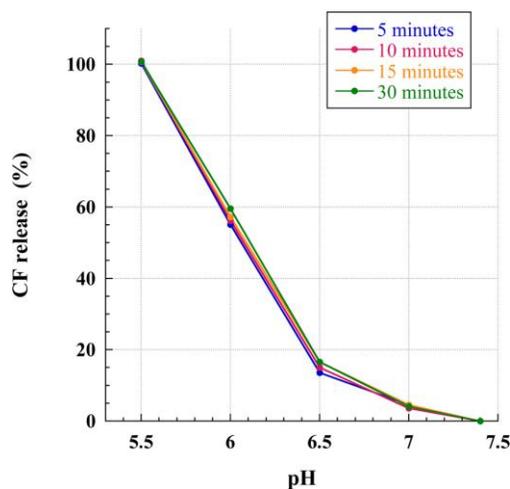


Figure 5: pH sensitivity of CF-loaded liposomes, after different incubation times (5 min, 10 min, 15min and 30 min) in PBS at different pH, for the system “POPE: α -TOS:CHOL 65:20:15”.

Finally, in contrast to the liposomes passively loaded with CF in the absence of CHOL (Figure 3), the incubation time has no impact on the disruption of the CHOL containing liposomes. For a given pH, the dye is almost completely released after 5 minutes, thus ensuring immediate release of dye/drug at the desired pH. Therefore, this last, cholesterol-containing formulation suits perfectly for the aimed applications of selective pH-sensitive nanocarrier.

4. Conclusion

This study investigated the formulation and optimization of pH-sensitive liposomes as a function of the chemical nature of –PE lipids and stabilizing agents and the lipid: stabilizer ratio. The ultimate objective was to find the most efficient system to encapsulate doxorubicin in pH-sensitive liposomes. In the first part of the study, we have evaluated the pH-sensitivity of the nanocarriers with a fluorescent method based on the encapsulation of CF as a model dye. Different lipids (DOPE, POPE and DMPE) were used, associated with α -TOS as stabilizing agent at different lipid: α -TOS ratios. These combinations were found to form stable liposomes at pH 7.4, with the higher encapsulation efficiency obtained with POPE. The integrity of liposomes was significantly pH-dependent with a possible modulation as a function of the concentration of α -TOS. As the concentration of α -TOS increases, the breakdown of liposomes and release of CF are slowed down. Incubation time also has an impact on the release of the dye from the liposomes because incubation for longer periods increases the extent of the liposome disruption in acidic pH. The second part of the study focused on the encapsulation of DOX in pH sensitive liposomes. We showed that DOX was not compatible with the passive loading approach, and showed good encapsulation tendencies by active loading technique (but only in specific pH ranges). Different pH gradients were studied, along with the variation of the nature and amounts of lipids and stabilizing agents. Values of the pH gradient was shown to be a crucial parameter in the DOX loading. The encapsulation efficiency of DOX in pH sensitive liposomes depends on the nature of the –PE lipid (higher with POPE) (Fig. 4a). And among different liposomal formulations of POPE, higher encapsulation efficiencies are resulted with α -TOS and α -TOS/CHOL combination as compared to CHEMS and CHEMS/CHOL combination. In function of the composition, the presence of cholesterol also increases the encapsulation of DOX and also helps in the retention of the DOX inside the liposomes.

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