Colloids and Surfaces B: Biointerfaces 164 (2018) 50–57

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Long term stability and interaction with epithelial cells of freeze-dried pH-responsive liposomes functionalized with cholesterol-poly(acrylic acid)

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ARTICLE INFO

Article history:
Received 7 November 2017
Received in revised form 3 January 2018
Accepted 15 January 2018
Available online 3 February 2018

Keywords:
pH-sensitive polymer-liposome complexes
Poly(acrylic acid)
Caged-liposomes
Storage stability
Cellular interaction

ABSTRACT

Liposomes are exceptional carriers for therapeutic drug delivery. However, they generally suffer from poor cell penetration, low half-life in bloodstream and loss of functionality during storage. To overcome these problems some strategies can be applied, such as functionalization with polymers and the use of protective molecules during dehydration processes.

This work reports a complete study about the stability, including freeze-drying in the presence of trehalose, storage and internalization into HEp-2 cells, of stable formulations of pH sensitive polymer-liposome complexes (PLC) composed of soybean lecithin and crosslinked/non-crosslinked poly(acrylic acid) with a cholesterol end-group (CHO-PAA).

The results showed that the average hydrodynamic particle size of the complexes persisted unaffected for approximately 75 days after freeze-drying in the presence of 10% w/v trehalose. The efficiency of calcein encapsulation and release profiles in physiologic conditions exhibited no significant alterations when stored for 0 and 1 month, and for 2 and 3 months of storage the calcein release increased with time. The stored complexes were efficiently uptaken into HEp-2-cells, as determined by confocal microscopy. In all cases, the percentage of viable cells was above 90%, as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, indicating no potential toxicity. Finally, transepithelial transport assays demonstrated that both fresh and 2 months-stored complexes could transport their calcein content through HEp-2 monolayers over time.

1. Introduction

Drug-delivery is essential in pharmaceutical sciences, and therefore, drug delivery systems are continuously developed. In the formulation of such systems, high performance, stimuli-response, cell penetration and storage stability are crucial aspects [1]. Among the numerous types of drug carriers, liposomes have been widely recognized and extensively used for the treatment of numerous diseases and for the development of intelligent drug delivery systems [2]. Due to their biocompatibility, biodegradability, non-immunogenic character, amphiphilicity, drug stabilization effect, potential reduction of drug toxicity and cost effective production in large scale [3–6], liposome formulations have been applied in several areas [7–9]. For this reason, the stability of liposomes is crucial, and all attempts to prevent fusion, aggregation and leakage, the most challenging problems to be faced to, are welcome. However, conventional liposome formulations have low stability in bloodstream, since they are attacked by the phagocytic cells and the reticuloendothelial system, and are also generally unstable during storage due to their physical and chemical instability in aqueous dispersions [10–14]. Furthermore, unsaturated lipids are susceptible to oxidative degradation, and both unsaturated and saturated lipids are susceptible to hydrolysis to form lysolipids and free fatty acids [15]. Consequently, countless approaches for liposome preservation have been investigated, such as freeze-drying, freezing, spray-drying and supercritical fluid technology. Among them, freeze-drying is the most widely used [11].

The incorporation of some molecules into the lipid bilayers has also been reported as helpful to enhance their stability. Cholesterol (CHO) is a paradigmatic case because it weakens the interactions between the acyl chains of phospholipids, and therefore stabilizes liposomes during long-term storage [16,17]. Besides that, the incor-

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https://doi.org/10.1016/j.colsurfb.2018.01.018
0927-7765/

poration of sugars into lipid bilayers is an adequate strategy to protect liposomes during freezing and freeze-drying. Small sugars are able to interact with the polar heads of lipid bilayers by replacing water molecules, thus dehydrated lipids behave as if they were hydrated, and leakage, fusion or aggregation are precluded [18]. Among these sugars, trehalose is one of the most efficient ones [19]. Taking all this into account, to ensure the physical stability of liposomes as drug delivery systems, some factors should be considered, such as the lipid bilayer integrity, size distribution, and drug retention/release [12].

To enhance the *in vivo* performance, surface modification of liposomes with hydrophilic biocompatible polymers is widely reported as an effective solution because this strategy prolongs their integrity in bloodstream and improves cell penetration [4,9,13]. These types of modified liposomes are known as polymer-liposome complexes (PLC). The addition of stimuli-sensitive polymers can also provide special features to the PCL, namely their capacity to release their contents in response to environmental changes as pH or temperature [20,21]. Stimuli-responsive liposomes can sense environmental conditions, thus releasing their content in ischemic tissues, in endocytic compartments where the medium has lower or higher pH than the physiological, or in inflammatory sites where temperatures are above the physiological [22,23].

We recently reported the design, formulation and characterization of a stable pH-sensitive PLC [24]. The polymer-liposome conjugate contained a lipid core of soybean lecithin (LC) and stearylamine, functionalized with poly(acrylic acid) (PAA), a pH-sensitive biocompatible polymer with mucoadhesive properties [25]. To improve the anchoring to lipid bilayers, a cholesterol (CHO) endgroup was incorporated (CHO-PAA). Synthesis was performed by control/living radical polymerization (LRP), specifically by atom transfer radical polymerization (ATRP), which allows a tight control of the final properties of the obtained polymers, viz. molecular weight and dispersity, and does not require extreme reaction conditions [26,27]. The polymeric chains of the complexes were also covalently crosslinked through the carboxylic groups of PAA to form caged-liposomes. This crosslinked PLC (C-PLC), proved to be more stable and release their content rapidly in an acidic medium [24].

In line with the previous work and aiming at filling a crucial gap from the application point of view, the stability of PLC and C-PLC during storage has been addressed and is here reported. The complexes were freeze-dried in the presence or absence of trehalose, and stored at 4 °C for 3 months. The average size and the capacity of the complexes to retain and release drugs was evaluated during storage. Internalization and transepithelial transport by human epithelial cells was evaluated with both fresh and freeze-dried PLC and C-PLC formulations.

2. Materials and methods

2.1. Materials

The following materials were used as received: soybean lecithin (granular, Acros Organics, Geel, Belgium), stearylamine (90%, Acros Organics, Geel, Belgium), chloroform (99.4%, VWR Chemicals, PA, USA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Amresco®, OH, USA), N-cyclohexyl-N′-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (97%, Fluka, WI, USA), ethylenediamine (99%, Merck, Darmstadt, Germany), calcein (Sigma-Aldrich Co. MO, USA), Triton X-100 (Sigma-Aldrich, MO, USA), trehalose (99%, Acros Organics, Geel, Belgium), Dulbecco's Modified Eagle Medium (DMEM, Microvet SRL, Buenos Aires, Argentina), To-Pro®-3 iodide (Thermofisher Scientific, NJ, USA), dimethylsulfoxide (99.7%, VWR Chemicals, PA, USA), fetal bovine serum (Natocor, Córdoba, Argentina), non-essential amino acids (GIBCO BRL Life Technologies, Rockville, MD, USA), penicillin–streptomycin solution (GIBCO BRL Life Technologies, Rockville, MD, USA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, MO, USA).

2.2. Methods

2.2.1. Polymer synthesis

Cholesterol-poly(acrylic acid) (CHO-PAA) was synthesized by ATRP. This polymerization procedure allows a stringent control of the molecular weight and dispersity (Đ). The synthesis and characterization of CHO-PAA were performed according to [24].

2.2.2. PLC and C-PLC formulation

Liposomes were prepared using the hydration film method [14]. For all experiments, calcein was encapsulated into the vesicles and used as fluorescent dye. For bare liposomes, soybean lecithin and stearylamine in a 5% stearylamine/soybean lecithin molar ratio were dissolved in chloroform (2.9 mM). Chloroform was then removed using a nitrogen stream. The lipid films were rehydrated in a calcein solution (60 mM, pH 7.0) prepared in HEPES (50 mM, pH 7.0), vigorously stirred and incubated for 24 h above the melting transition temperature (*ca.* 37 °C).

A 0.3 mM CHO-PAA solution prepared in HEPES was added to the bare liposomes in a CHO-PAA/soybean lecithin molar ratio of 10%. The formulation was vigorously stirred, and incubated for 24 h at 37 °C to allow the incorporation of CHO-PAA into the lipid bilayer.

For the C-PLC, the encapsulation must be performed only after the formation of those complexes because the crosslinking step does not occur in the presence of calcein. Thus, the lipid films were rehydrated in HEPES (50 mM, pH 7.0), vigorously stirred and incubated for 24 h at 37 °C. A CHO-PAA solution (0.3 mM), in a CHO-PAA/soybean lecithin molar ratio of 10%, was added to the bare liposomes. The formulation was vigorously stirred, and incubated for 24 h at 37 °C. The obtained empty liposomes were used to prepare C-PLC by adding *N*-cyclohexyl-*N*′-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate and ethylenediamine in HEPES (both at 100 mM) according to the molar ratio of carboxylic groups present in each formulation. The formulations were incubated at 37 °C for 24 h. A 60 mM calcein solution was added to the empty C-PLC and incubated for 48 h at 37 °C. The encapsulation of calcein into C-PLC was achieved by diffusion. Non-encapsulated calcein was removed by washing the obtained PLC and C-PLC with HEPES (50 mM, pH 7.0) three times, according to [25,28].

2.2.3. Preservation

Both the calcein-loaded PLC and C-PLC were suspended in HEPES (50 mM, pH 7.0) or in 10% (w/v) trehalose prepared in HEPES. The fresh suspensions were refrigerated at 4 °C, frozen at −20 °C, freeze-dried and stored for 3 months at 4 °C. The freeze-drying process was carried out at −50 °C and 0.04 mbar in an Alpha 1–2 LD Plus (CHRIST, Osterode am Harz, Germany) on samples previously frozen at −20 °C for 48 h.

2.2.4. Particle size measurements

Particle size measurements were performed in a Malvern Instrument Zetasizer Nano-Z (Malvern Instruments, Malvern, UK) at 37 °C. The average hydrodynamic particle size (Z-size) was determined by dynamic light scattering at backward scattering (173°) with the Zetasizer 6.20 software.

2.2.5. Retention capacity and leakage experiments

Release profiles and capacity of calcein retention through the storage period for all formulations were determined fluorometrically in a Synergy HT fluorescence microplate reader (Bio-Tek Instruments, Winoski, Vermont, USA), with an excitation and emission wavelengths of 485/20 nm and 528/20 nm, respectively. The release of the fluorescent probe was monitored at pH 7 for 30 h at 37 °C. After adding Triton X-100 (20 % v/v solution) to promote total lysis, the fluorescence intensity resulting from the calcein release was recorded along time. The extent of content release was expressed as a percentage, according to Eq. (1):

$$\%Release = \frac{(F - F_i)}{(F_t - F_i)} \times 100 \quad (1)$$

where F is the fluorescence intensity of the sample after each incubation time, F_i is the initial fluorescence intensity of the sample, and F_t is the total fluorescence intensity of the sample recorded after leakage with Triton X-100 [25].

The capacity of drug retention upon storage was expressed as the encapsulation efficiency (EE) of PLC and C-PLC over the storage time. For each formulation, EE (%) was expressed in molar concentration of calcein per molar concentration of lipid (Eq. (2)).

$$EE\,(\%) = \frac{[Calcein]}{[Lipid]} \times 100 \quad (2)$$

To determine the molar concentration of lipids in the complexes, a commercial kit (CHO-POD enzymatic colorimetric from Spinreact, Portugal) was used according to the instructions of the manufacturer [29,30].

2.2.6. Culture of HEp-2 cells

Human epithelial HEp-2 cells were cultured in DMEM supplemented with 10% (v/v) heat-inactivated (30 min/60 °C) fetal bovine serum, and 1% (w/v) non-essential amino acids and 1% (v/v) penicillin–streptomycin solution (100 U/mL penicillin G, 100 μg/mL streptomycin). Cultured cells were incubated at 37 °C in a 5% CO_2, 95% air atmosphere.

2.2.7. Uptake of PLC and C-PLC by HEp-2 cells

HEp-2 cells were seeded in 24-well culture plates (density *ca.* 2×10^5 cells/well) and incubated to early post confluence in the conditions indicated above. Monolayers were washed with phosphate saline buffer [PBS (K_2HPO_4 0.144 g/L; NaCl 9.00 g/L; Na_2HPO_4 0.795 g/L), pH 7], and fresh medium containing calcein-loaded complexes were added to each well. After being incubated for 30 min, cells were washed twice with PBS to remove free non-internalized complexes. Cells were removed by trypsination and transferred to FACS tubes containing PBS. The acquisition was performed in a FACSCalibur flow cytometer by using Cell Quest software (Becton Dickinson, Mountain View, CA, USA). Green (FL1) and red (FL2) fluorescence of 5000 events were analysed for each sample.

2.2.8. Confocal laser scanning microscopy analysis

Cell internalization of the fresh and stored calcein-loaded PLC and C-PLC was determined using a confocal laser-scanning microscope (Leica TCS SP5 Leica Microsystems, Wetzlar, Germany). Cells cultured on glass coverslips (Assistent, Glaswarenfabrik KG, Sondheim, Germany) were incubated for 30 min with DMEM containing PLC and C-PLC. After incubation, coverslips were washed twice with PBS to remove free non-internalized liposomes. To assess the localization of acidic intracellular compartments (endosomes/lysosomes) cells were incubated for 30 min with Lysotracker DND-99 (10 μM) (Molecular Probes, Oregon, USA). Afterwards, cells were fixed with 3% (w/v) paraformaldehyde and mounted with anti-fading mounting media (Dako, Agilent Technologies, Carpinteria, C.A. USA) with the addition of To-Pro-3® Iodine (2 μM) as nuclear staining (Termofisher Scientific, NJ. USA). Image analysis was done using the Leica Application Suite X (Leica Microsystems) and Fiji – ImageJ (NIH, USA) software.

2.2.9. Cytotoxicity assays

Cell viability was determined by assessing mitochondrial dehydrogenase activity, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. HEp-2 cells were seeded in 48-well plates at 1×10^5 cells per well and incubated to early post confluence. Fresh and freeze-dried PLC and C-PLC (concentrations 75 and 750 μM, respectively) were added to the cells using three wells per group, and incubated for 24 h. Afterwards, cells were washed twice with PBS and the medium was replaced with DMEM (without phenol red dye) containing 0.5 mg/mL MTT. After 2 h incubation, 0.2 mL dimethylsulfoxide were added to each well and agitated for 20 min at 25 °C on a plate shaker to solubilize the cells and the formed formazan crystals. The optical density (OD) values were registered in a Synergy HT fluorescence microplate reader at 490 nm (Bio-Tek Instruments, Winoski, Vermont, USA). Cell viability was determined according to Eq. (3),

$$Cell\ Viability\,(\%) = \frac{\mathrm{ODt}}{\mathrm{ODc}} \times 100\% \quad (3)$$

where OD_t is the optical density of the cells treated with liposomes and OD_c is the optical density of the non-treated control cells.

2.2.10. Trans epithelial transport of PLC and C-PLC in HEp-2 cells

HEp-2 cell monolayers were obtained by seeding 0.2 mL of HEp-2 cells onto polycarbonate membrane filters (8.0 μm pore size, 1.12 cm^2 growth area) inside transwell cell culture chambers (Corning Costar Cambridge, MA) at a density of 4×10^5 cells/cm^2, and were cultured according to the procedure described above. After 7 days, HEp-2 cell monolayers were confirmed by determining transepithelial electrical resistance using a Millicell-ERS system (MilliCell Corporation, Billerica, MA, USA). Cells whose transepithelial electrical resistance values were around 150–300 (Ω cm^2) were used for transepithelial transport studies. Before the experiment, monolayers were washed twice with PBS and then, the apical and basolateral compartments were filled with 0.2 and 0.8 mL of fresh DMEM without fetal bovine serum. PLC and C-PLC were added to the apical compartment, the transwell plate was incubated at 37 °C and 100 μL sample solution in the basolateral compartment was taken at 2, 4 and 6 h with equal volume of supplemented DMEM. At each time point, the total fluorescence in the basolateral compartment was determined by adding Triton X-100 (20% v/v solution). The percentage of transepithelial transport was expressed as a percentage according to Eq. (4):

$$\%Transepithelial\ transport = \frac{(F_{\mathrm{BA}})}{(F_{\mathrm{BAt}})} \times 100 \quad (4)$$

where F_{BA} is the total fluorescence intensity in the basolateral compartment after each incubation time, and F_{BAt} is the total fluorescence intensity in the basolateral compartment after Triton treatment. To assess monolayer integrity, transepithelial electrical resistance of HEp-2 monolayers was determined after each time point.

3. Results

The effect of trehalose as protectant of PLC and C-PLC during freeze-drying was investigated (Fig. 1). Liposomes freeze-dried in the presence of trehalose showed values of Z-sizes significantly lower than those of the unprotected samples (see Fig. S1 for further details). In comparative terms, the Z-sizes of liposomes freeze-dried with trehalose did not change significantly (values within the range

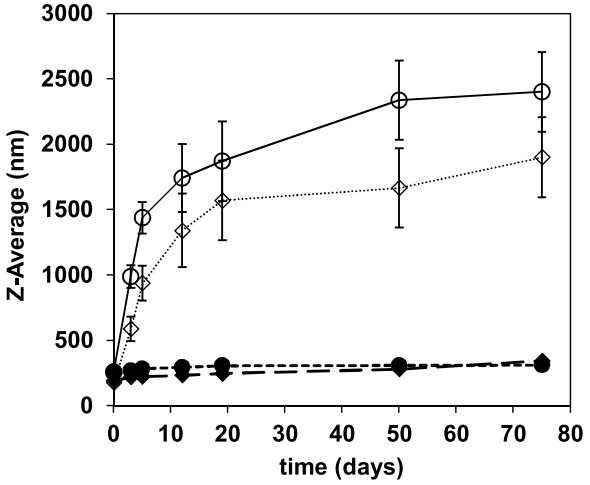

Fig. 1. Z-size of fresh and freeze-dried complexes in the presence and absence of trehalose along 75 days of storage at 4 °C. Circles: PLC; Diamonds: C-PLC. Full symbols, liposomes freeze-dried with trehalose; empty symbols: liposomes freeze-dried without trehalose.

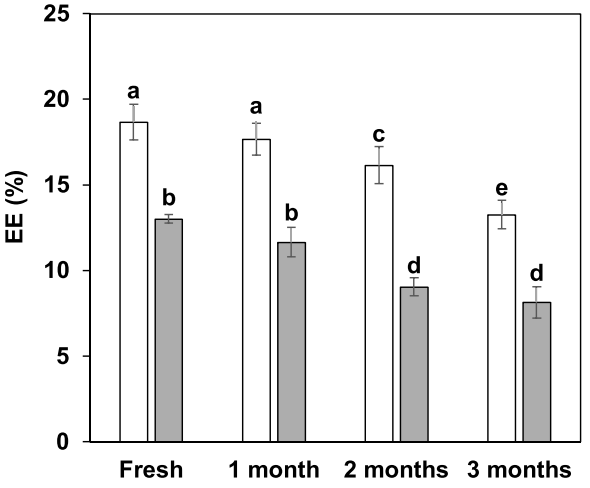

Fig. 2. Efficiency of encapsulation (EE%) of the fresh and freeze-dried polymer lipid complexes protected with trehalose after 0, 1, 2 and 3 months of storage at 4 °C. White bars: PLC; gray bars: C-PLC. Different letters indicate significant differences ($P < 0.05$).

200–350 nm) over the storage period of (75 days at 4 °C). No significant differences between PLC and C-PLC were observed. On the contrary, the Z-size of the liposomes freeze-dried without lyoprotectant increased by 3–4 times after 60 days of storage (Fig. 1). Moreover, PLC were significantly larger than C-PLC. Taking these results into account, only liposomes freeze-dried with trehalose were used in further studies.

Fig. 2 shows the efficiency of calcein encapsulation of PLC and C-PLC freeze-dried in the presence of trehalose, after 1, 2 or 3 months of storage. The efficiency of encapsulation of PLC was significantly higher than that of C-PLC for both fresh (0 months) and stored samples (Fig. 2). In addition, no calcein release was observed after one month of storage, but larger storage times led to an increase of leakage (Fig. 2). Noteworthy, C-PLC was more efficient in retaining calcein at all storage times.

The calcein release from fresh and freeze-dried PLC and C-PLC stored for 1, 2 and 3 months is shown in Fig. 3. For fresh samples, both PLC and C-PLC exhibited low calcein release, which increased with the time of storage. It is important to notice a different behaviour for PLC and C-PLC. Although both types of complexes stored up to 1-month showed a similar time-dependent calcein

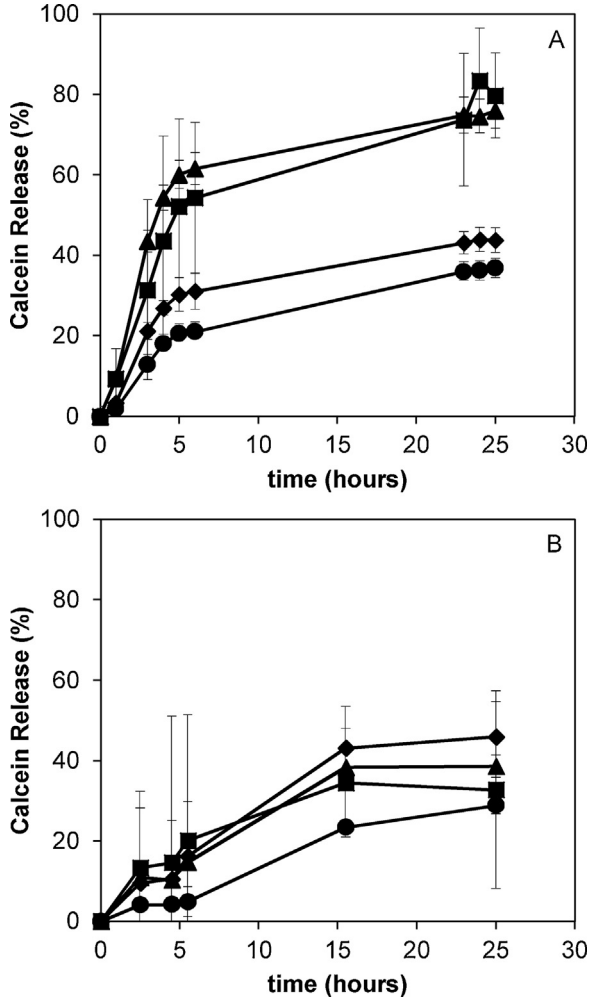

Fig. 3. Calcein release from fresh and freeze-dried polymer lipid complexes protected with trehalose after 0, 1, 2 and 3 months of storage. Circles: fresh; Diamonds: 1 month; Triangles: 2 months; Squares: 3 months. A: PLC; B: C-PLC;.

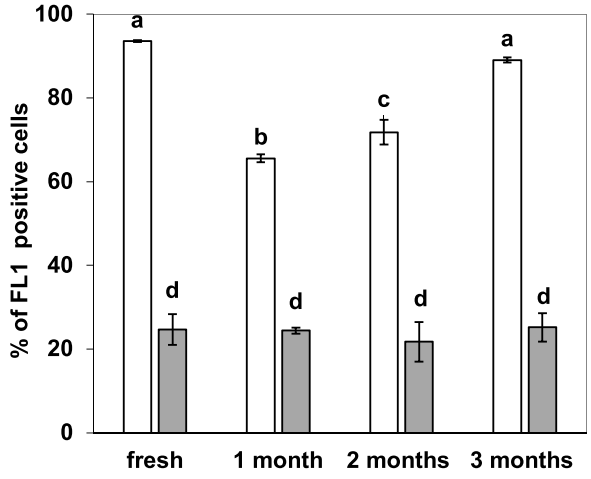

Fig. 4. Percentage of calcein positive HEp-2 cells after incubation with fresh or stored PLC or C-PLC. Values were obtained by flow cytometry. White bars: PLC; gray bars: C-PLC. Different letters indicate significant differences ($P < 0.05$).

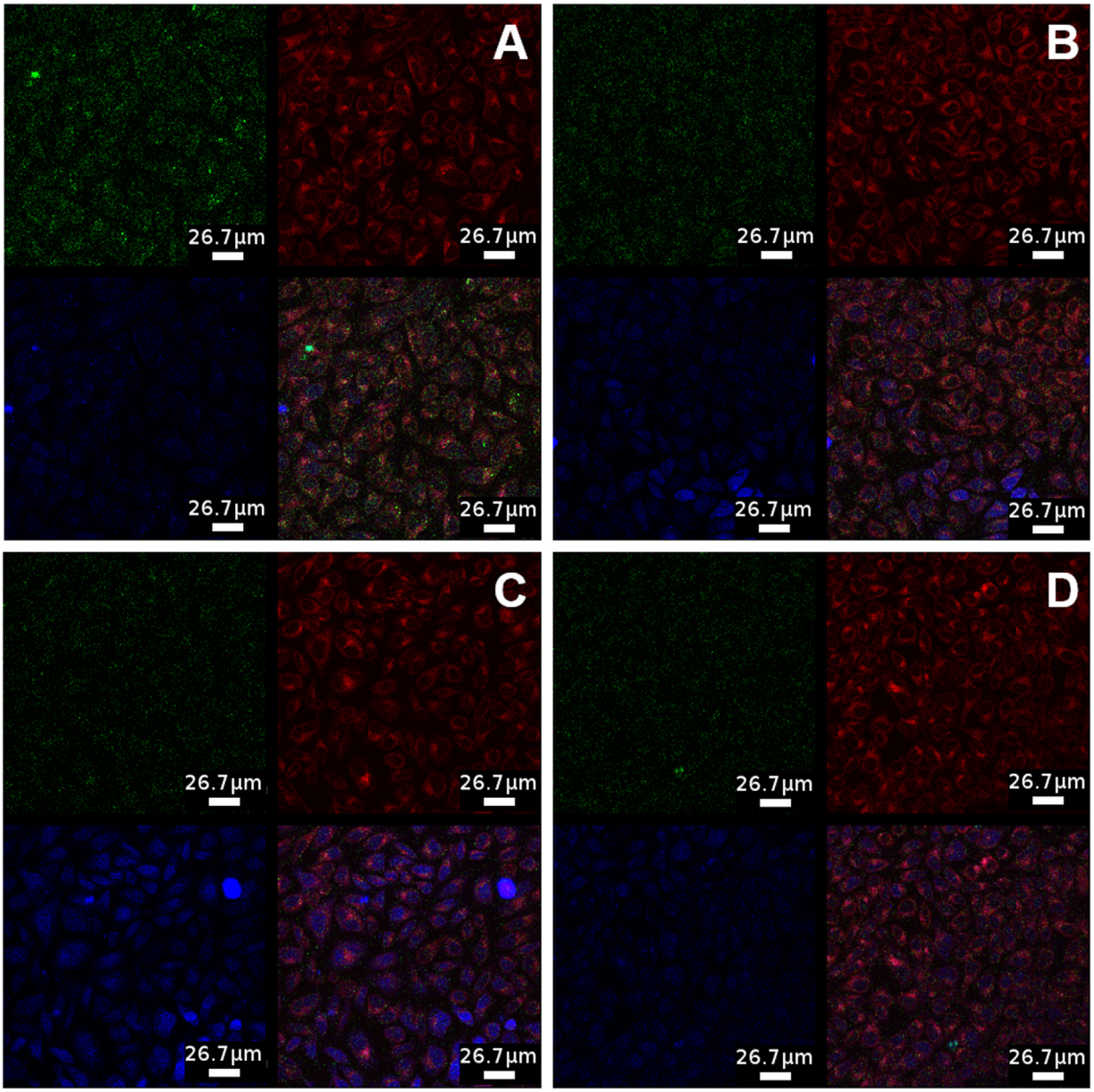

Fig. 5. Confocal images of HEp-2 cells incubated with calcein loaded PLC and C-PLC. Cells were incubated for 30 min with different complexes samples. A: fresh PLC; B: PLC stored for 2 months; C: fresh C-PLC; D: C-PLC stored for 2 months. Green fluorescence corresponds to calcein loaded liposomes. Red fluorescence corresponds to cellular acid compartments stained with Lysotracker. Blue flurorescence corresponds to nucleus stained with To-Pro® -3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

release (calcein release ~30% after 25 h in both cases) (Fig. 3A, B), there was a significantly faster kinetics of calcein release in PLC stored for 2 and 3 months (around 70% after 25 h) as compared with C-PLC (Fig. 3A and B).

Flow cytometry assays were performed to evaluate the capacity of the interaction of the HEp-2 cells with PLC and C-PLC liposomes. Irrespectively of the time of storage, the interaction of liposomes with epithelial cells was significantly higher for PLC than for C-PLC (Fig. 4). The percentage of FL1 (+) cells ranged from 65.50 to 93.50% for PLC and was around 24.01 for C-PLC. This indicates that the percentage of cells interacting with liposomes [FL-1 (+) cells] was 2.7–3.8 fold higher for PLC than for C-PLC (Fig. 4).

Confocal images of HEp-2 cells incubated with PLC and C-PLC showed that liposomes were internalized by eukaryotic cells although C-PLC showed a lower intensity of the green fluorescence, thus indicating less internalization (Fig. 5). These results are in good correlation with the flow cytometry measures, which suggests a better incorporation of PLC into the eukaryotic cells (Fig. 5). There were no differences between the internalization of fresh or freeze-dried PLC and C-PLC formulations. Both types of complexes were mainly located in the cell cytoplasm and there was no apparent colocalization with acidic compartments (Fig. 5A and B).

The potential toxicity of the complexes was assessed through MTT assays. Two different concentrations of PLC and C- PLC (*ca.* 75 µM and 750 µM) were incubated with HEp-2 cells cultures for 24 h (Fig. 6). In all cases, the percentage of viable cells was above 90%.

The transepithelial transport of fresh and 2 months-stored PLC and C-PLC was also investigated (Fig. 7). The results indicate the percentage of complexes transported across the HEp-2 cell monolayers over time (Fig. 7). There was a time-dependent increase of fluorescence in the basolateral compartment, thus indicating that

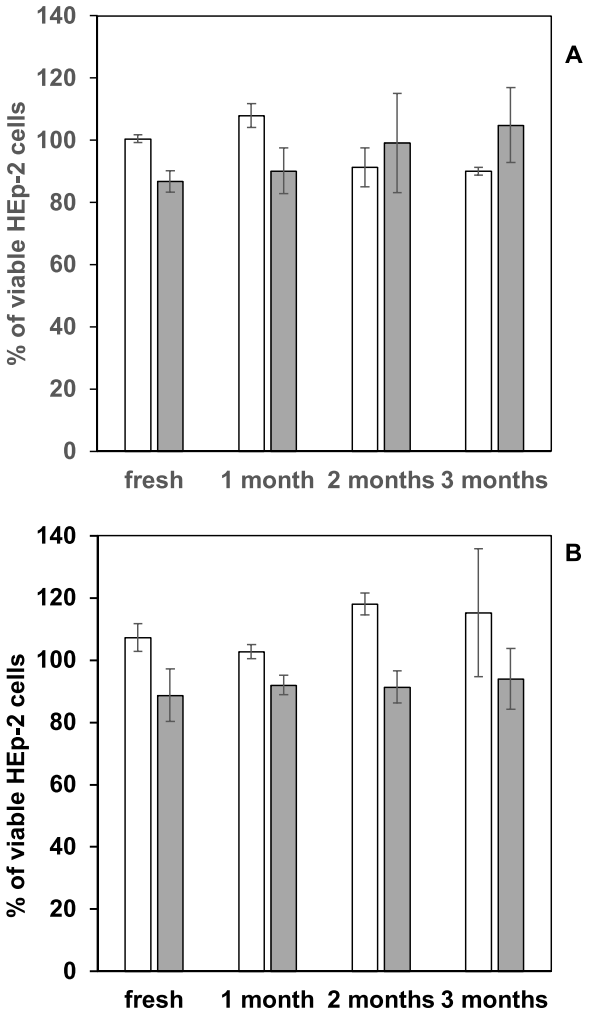

Fig. 6. Viability of HEp-2 cells after the incubation with fresh or freeze-dried PLC/C-PLC stored for 1, 2 or 3 months. White bars: 75 μM; Gray bars: 750 μM. Different letters indicate significant differences ($P < 0.05$). A: PLC; B: C-PLC.

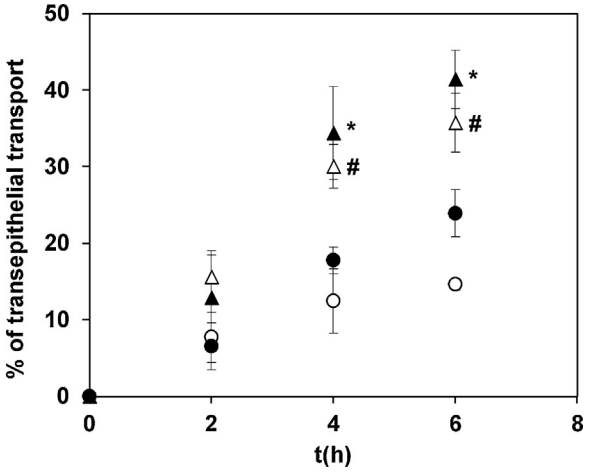

Fig. 7. Transepithelial transport across HEp-2 cells for fresh and 2 month stored PLC and C-PLC. # indicates significant differences ($P \leq 0.05$) between fresh PLC and fresh C-PLC; * indicate significant differences ($P \leq 0.05$) between PLC and C-PLC stored for 2 months. Full symbols: stored liposomes, empty symbols fresh liposomes, circles: PLC, triangles: C-PLC.

liposomes were transported across HEp-2 monolayers. Although after 2 h of incubation, no significant differences were observed between both formulations, after 4 and 6 h, C-PLC were more efficiently translocated than PLC liposomes ($P \leq 0.05$). Storage of complexes for up to 2 months did not modify the ability to be translocated to the basolateral compartment. It is worth to note that no differences in transepithelial resistance were observed at all the time-points studied (data not shown).

4. Discussion

Drug delivery systems enable an adequate transport, retention and release of drugs by the target cells. Therefore, their adequate design is of great importance. A stable pH-sensitive PLC functionalized with PAA bound to cholesterol was previously used with success [24]. In this work a complete study on the stability of such complexes, including freeze-drying, storage and interaction with eukaryotic cells, was carried out.

The Z-size of the complexes over the storage time showed extremely high values for liposomes freeze-dried in the absence of trehalose (Fig. 1). On the contrary, the Z-size of the protected liposomes was significantly lower (<400 nm), thus indicating that there was no burst, aggregation or coalescence during storage [14]. The use of sugars as lyoprotectants is a well-known strategy to prevent liposomes from a large variety of adverse conditions [31]. In fact, the protective effect of sugars has been ascribed to their capacity to preclude leakage and fusion of liposomes by replacing water molecules from the lipid membranes [32]. Furthermore, as trehalose has a high vitreous transition temperature (T_g: 107 °C), it also contributes to prevent aggregation during storage at 4 °C (Fig. 1) [11].

When analysing the efficiency of encapsulation, both freeze dried PLC and C-PLC showed a significant release of calcein after 2 months of storage, but that release was lower than 15% (Fig. 2). This observation has already been reported for other drug delivery systems and can be ascribed to leakage [33,34]. The release profiles of calcein in physiologic conditions (*ca.* 37 °C pH 7.0) (Fig. 3) were consistent with the results obtained for efficiency of encapsulation (Fig. 2). The calcein release increased as a function of time, and PLC and C-PLC displayed no significant differences when stored for 0 and 1 month. In addition, it must be considered that the maximal calcein release observed for C-PLC was ~45% after 25 h (Fig. 3B). This value is comparable to those reported for slow and controlled delivery systems [35,36].

It is known that PAA polymer has mucoadhesive properties [37], and therefore PLC and C-PLC formulations can be used to deliver drugs to mucosal surfaces, such as the intestinal tract, the respiratory tract or the sensory cavities. Despite that, considering that mild acidic conditions promote the content release of PLC and C-PLC, the intestinal tract would not be an appropriate target. The passage through the low pH of the stomach would lead to the release of the entire content of the vesicles before arriving to the intestine. For this reason, a non-intestinal cell line (HEp-2 cells) was selected to study the interaction of PLC and C-PLC with eukaryotic cells. HEp-2 is an immortalized epithelial cell line often used as model of respiratory epithelium because it was traditionally thought to arise from a laryngeal tumour. However, nowadays it is well-known that HEp-2 cells derive from Hela cells, and thus, can be considered as a general model of an epithelium. The study of the interaction of HEp-2 cells with calcein loaded liposomes by flow cytometry showed higher percentages of HEp-2-calcein (+) cells for PLC than those for C-PLC in all the conditions assayed (Fig. 4). However, these values could underestimate the actual situation. In fact, C-PLC had an efficiency of calcein encapsulation 1.66-1.85 times lower than that of PLC. Therefore, the incorporation of a same amount of both types of complexes led to different fluorescence

values. As the flow cytometry analysis defines a threshold to consider a fluorescent event as positive, it is possible that cells which had previously incorporated a smaller quantity of C-PLC were no fluorescent enough to pass the threshold. Considering that the Z-sizes (Fig. 1) and ζ – potential (−34.98 for C-PLC and −31.58 for PLC [24]) were similar for both formulations, it could be hypothesized that the interaction of epithelial cells with PLC and C-PLC would not be so different. The lower fluorescence values for C-PLC could be ascribed to its lower efficiency for encapsulating calcein. An accurate determination of the liposome internalization could be achieved by loading them with a traceable compound like a drug. It is important to point out that the internalization of freeze-dried and fresh C-PLC was very similar whereas that of PLC showed a more variable behaviour (Fig. 4). As C-PLC were more stable in both storage conditions and rehydration (Fig. 2), no significant differences in the uptake were observed for fresh and stored liposomes.

Above considerations prompted us to conduct studies by confocal microscopy. This experimental approach demonstrated the high incorporation of both calcein loaded PLC and C-PLC (Fig. 5). Confocal images showed a uniform distribution of the green fluorescence throughout the cytoplasm for both types of complexes. Moreover, there were no differences between fresh and stored complexes for both formulations. However, it is important to point out that the green fluorescence was less evident in cells incubated with C-PLC, probably due to their lower efficiency of encapsulation. It is known that the internalization of liposomes and nanoparticles in most cells occurs primarily through active endocytic or phagocytic mechanisms [38]. Therefore, PLC and C-PLC can probably employ an endocytic mechanism to be internalized by HEp-2 cells. As liposomes did not co-localized with intracellular acidic compartments (Lysotracker staining) they would probably be endocyted through a non-degradative pathway.

The potential adverse effects elicited by the liposomal formulations on HEp-2 cell monolayers were evaluated by the MTT assay. To be consistent with the conditions used in the internalization and transepithelial assays, two different concentrations of the stored and fresh formulations were employed (75 and 750 μM). After 24 h of incubation, no cytotoxic effect on HEp-2 cells was observed for none of the concentrations and formulations assayed (Fig. 6). It is known that liposomes composed of phosphatidylcholine and cholesterol do not have adverse effects on epithelial monolayers, even at high concentrations [39,40]. PAA is also considered as a safe polymer, as no evidence of *in vitro* cytotoxicity was reported in terms of cell morphology, cell proliferation and cell viability [24]. In contrast, several studies revealed that cationic liposomes containing stearylamine could have cytotoxic effects inducing apoptosis in lymphocytes and macrophage cell lines [41]. However, the formulations investigated in this work exhibited no side effects on HEp-2 cells. PLC and C-PLC had 5% molar ratio of stearylamine, which improves the incorporation of PAA into the lipid bilayers without changing the negative charges of liposomes [24]. Other studies employing stearylamine at 5% molar ratio in cationic liposomes demonstrated an increase of transepithelial permeation due to the action of stearylamine without affecting Caco-2 cell viability [42]. In conclusion, the use of 5% molar ratio stearylamine in PLC and C-PLC proved to be safe for HEp-2 epithelial cell line.

PLC and C-PLC could transport their calcein content across de HEp- 2 monolayers (Fig. 7). There were no differences between the transport capacity of fresh and stored liposomes for both formulations. Interestingly, C-PLC showed a greater capacity to release their content into the basolateral compartment. C-PLC were smaller and slightly less negative than PLC (146.83 *vs* 214.73 nm and −31.58 *vs* −34.98 respectively) [43]. These characteristics could favour their interaction with HEp-2 cells, decreasing the repulsion with the negative charged surface of eukaryotic cells and promoting the passage through the monolayer. HEp-2 cells have the capacity to form monolayers and the epithelial cells are linked by tight junction unions [14,44]. Under physiological conditions, the tight junction gap among epithelial cells is less than 10 nm, and can only be opened up to 20–30 nm under abnormal physiological conditions [45]. The particle size of PLC and C-PLC was greater than 100 nm, indicating that intercellular transport through the tight junction gap is not possible. Considering that liposomes are internalized in HEp-2 cells (Fig. 5), their transport through transcellular passage is a possible mechanism. However, a paracellular route cannot be rouled out sinceHep-2 cells are not a 100% confluent cell line.

5. Conclusion

Non-functionalized liposomes are often not effective in releasing their content at the target site, and, if not protected, also suffer from several instabilities on storage.

Stable PLC were successfully established through the incorporation of a pH-sensitive polymer. To understand the real potential of these complexes, a complete study on their stability and the interaction with epithelial cells of fresh and stored complexes was accomplished, shedding light on the mechanisms behind these processes.

Considering the size, capacity of encapsulation, stability during storage, non-cytotoxicity, capacity of internalization and transport of the stored liposomes, these formulations appear as interesting pH-sensitive liposomes, with great potential as delivery systems in biomedical applications.

Acknowledgments

This work was supported by the European Commission [H2020-MSCA-RISE Action. Code: 777657 (PREMIUM)], the Argentinean Agency for the Scientific and Technological Promotion (ANPCyT) [Projects PICT (2014)/0912; PICT start-up (2016)/4808; PICT Raíces 2013-1205; the National University of La Plata (UNLP)]. Fabricio Lareu is acknowledged for technical assistance. A.H., P.F.P. and A.G.-Z are members of the research career CONICET. P. F. P. is Professor of Microbiology at the Department of Biological Sciences, Faculty of Exact Sciences (UNLP). P.A. is a postdoctoral fellow in CIEPQPF and would like to thank FCT for her grant (REF: SFRH/BPD/69410/2010).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.colsurfb.2018.01.018.

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