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Stabilization of polymer lipid complexes prepared with lipids of lactic acid bacteria upon preservation and internalization into eukaryotic cells

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ABSTRACT

The physicochemical characterization of polymer liposome complexes (PLCs) prepared with lipids of lactic acid bacteria and poly(N,N-dimethylaminoethyl methacrylate) covalently bound to cholesterol (CHO-PDMAEMA) was carried out in an integrated approach, including their stability upon preservation and incorporation into eukaryotic cells.

PLCs were prepared with different polymer:lipid molar ratios (0, 0.05 and 0.10). Zeta potential, particle size distribution and polydispersity index were determined. The optimal polymer:lipid ratio and the stability of both bare liposomes and PLCs were evaluated at 37 °C and at different pHs, as well as after storage at 4 °C, −80 °C and freeze-drying in the presence or absence of trehalose 250 mM. Internalization of PLCs by eukaryotic cells was assessed to give a complete picture of the system.

Incorporation of CHO-PDMAEMA onto bacterial lipids (ratio 0.05 and 0.10) led to stabilization at 37 °C and pH 7. A slight decrease of pH led to their strong destabilization. Bacteria PLCs showed to be more stable than lecithin (LEC) PLCs (used for comparison) upon preservation at 4 and −80 °C. The harmful nature of the preservation processes led to a strong decrease in the stability of PLCs, bacterial formulations being more stable than LEC PLCs. The addition of trehalose to the suspension of liposomes stabilized LEC PLC and did not have effect on bacterial PLCs.

In vitro studies on Raw 264.7 and Caco-2/TC7 cells demonstrated an efficient incorporation of PLCs into the cells. Preparations with higher stability were the ones that showed a better cell-uptake.

The nature of the lipid composition is determinant for the stability of PLCs. Lipids from lactic acid bacteria are composed of glycolipids and phospholipids like cardiolipin and phosphatidylglycerol. The presence of negatively charged lipids strongly improves the interaction with the positively charged CHO-PDMAEMA, thus stabilizing liposomes. In addition, glycolipids and phosphatidylglycerol act as intrinsic protectants of PLCs upon preservation.

This particular lipid composition of lactic acid bacteria makes them natural formulations potentially useful as drug delivery systems.

Abbreviations: ANOVA, analysis of variance; ATRP, atom transfer radical polymerization; CHO-PDMAEMA, cholesterol covalently bound to poly(N,N-dimethylaminoethyl methacrylate); CIDCA133PD5, PLCs prepared with bacterial lipids whose polymer:lipid ratio is equal to 0.055; CIDCA133PD10, PLCs prepared with bacterial lipids whose polymer:lipid ratio is equal to 0.1; CL, cardiolipin; *Đ*, dispersity; DMEM, Dulbecco's Modified Eagle Medium; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; FACS, flow cytometry; FEBS, fetal bovine serum; GLY, glycolipid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LEC, lecithin; LECPD5, polymer lipid complexes prepared with lecithin whose polymer:lipid ratio is equal to 0.05; LECPD10, polymer lipid complexes prepared with lecithin whose polymer:lipid ratio is equal to 0.10; PBS, phosphate buffered saline; PdI, polydispersity index; PG, phosphatidylglycerol; PL, phospholipid; PLCs, polymer lipid complexes; POPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; RDRP, reversible deactivation radical polymerization.

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1. Introduction

The lipid composition of liposomes intended for drug delivery systems depends on different factors and is far from being established. Before arriving to the target cells liposomes must overcome different barriers. For this reason, defining appropriate liposomal formulations suitable for drug delivery represents a challenge. Different formulations were investigated in terms of lipid composition, lipid:drug ratio, drug concentration, drug entrapment, filterability and short and long-term physical and chemical stabilities [1,2]. It has been reported that lipids containing unsaturated fatty acids [*e.g.*: 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)] stabilize liposomes because of their low gel to lipid-crystalline phase transition temperatures, forming flexible liposomes, which efficiently interact with hydrophobic molecules. Liposomes containing cardiolipin (CL) and/or other negatively charged lipids can better interact with hydrophilic drugs through electrostatic interactions and may also form complexes with lipophilic drugs through hydrophobic interactions [1,2].

Sugars like sucrose or trehalose have a stabilizing effect on membrane integrity because of their capacity to prevent fusion and leakage of liposomes [3–6]. This ability of sugars aids to retain encapsulated drugs when liposomes are intended for drug delivery. Indeed, the sugar:lipid molar ratio is critical in preserving the integrity of the liposomes subjected to processes that may alter their stability. It has been reported that the optimum sugar:lipid molar ratio to prevent aggregation and fusion must be higher than 6:1–8:1 [1].

Bacterial lipids are mainly found in cell membranes and have a crucial role in the response of bacteria exposed to dehydration [7,8]. Indeed, the capacity of a given strain to overcome dehydration associated with preservation processes (*i.e.*: freezing, freeze-drying) is directly related with the composition of the lipid membrane [8,9]. In particular, lipids of lactic acid bacteria are composed of CL and phosphatidylglycerol (PG) as main phospholipids (PL), and three different glycolipids (GLY) [9]. The unsaturated/saturated fatty acids ratio is also related with the stability of bacteria exposed to different kinds of stress [8,9]. In spite of this peculiar lipid composition including both negatively charged phospholipids (CL and PG) and sugars (as GLY) together with a high sugar:lipid molar ratios [8,9], bacterial lipids have never been considered as natural formulations potentially useful for drug delivery.

When liposomes are to be used for drug delivery, internalization is an important issue in the process of intracellular release. After cell internalization, liposomes are exposed to the acidic environment of the lysosomes and therefore, their stability upon pH changes must be carefully considered. The design of pH-sensitive liposomes represents an appropriate strategy to overcome this problem. These liposomes are tailored to release their content only under mild acidic conditions, such as those found in lysosomes or in tumor cells which have a low intracellular pH [10–13]. Different amphiphiles have been used in the design of pH-sensitive liposomes. The addition of stimuli-responsive polymers to liposomes constitutes one of the most promising strategies developed because of the possibility of render almost any liposomal composition sensitive to pH [14]. In this sense, alkylated N-isopropylacrylamide based copolymers and poly(glycidol) derivatives have been designed to confer pH-sensitivity to liposomes [15,16]. In our previous work, it was demonstrated that cholesterol anchored to the stimuli-responsive polymer poly(N,N-dimethylaminoethyl methacrylate) (CHO-PDMAEMA), a weak polyelectrolyte (pK_a *ca.* 8.0), synthesized by atom transfer radical polymerization (ATRP) stabilizes lecithin liposomes (LEC) at pH 7 and facilitates their incorporation by eukaryotic cells [17]. ATRP is a kind a reversible deactivation radical polymerization (RDRP) technique that allowed us to obtain polymers for potential biomedical applications [18–24]. This approach demonstrated adequate perspectives to be used for drug delivery and encouraged us to go ahead on the development of even better systems.

As the development of such systems represents an active field in which much space for innovation still exists, the aim of this work was to develop an integrated framework in the formulation of stimuli-responsive liposomes. This approach includes the incorporation of CHO-PDMAEMA to lipids extracted from lactic acid bacteria, never used as drug delivery systems up to now. The optimal polymer:lipid ratio and the stability of both bare liposomes and PLCs was evaluated at 37 °C and at different pHs, as well as after storage at 4 °C, −80 °C and freeze-drying in the presence or absence of trehalose. Finally, the incorporation of PLCs by eukaryotic cells was assessed to gain further insights on the possibility of using these PLCs as drug delivery systems.

2. Materials and methods

2.1. Lipid extraction

Lactobacillus delbrueckii subsp. *lactis* CIDCA 133 belongs to the CIDCA culture collection [8]. Stock cultures were stored at −80 °C, and microorganisms were cultured twice in MRS broth [25] (Biokar Diagnostics, Beauvais, France) at 37 °C for 16 h. Bacteria were harvested by centrifugation at 10,000 × *g* for 10 min and washed twice with phosphate buffered saline (PBS) [K_2HPO_4 0.144 g/L; NaCl 9.00 g/L; Na_2HPO_4 0.795 g/L]. Pellets were used for lipid extraction, according to the modified Bligh and Dyer method [26]. Briefly, cell pellets were suspended in chloroform–methanol–water (1:2:0.8 ratio) (4.75 mL/g of cell culture) for 12 h at 4 °C and then centrifugated at 8000 × *g*, 10 min at 10 °C. The supernatant was collected and a second extraction was performed on the pellet. Both supernatants were mixed and chloroform-water (1:1) was added (12.5 mL/g of cell culture). The final mixture was centrifuged at 8000 × *g* for 20 min. The chloroform phase was collected and dried under vacuum in a rotavapor (Rotavapor® RE 120, Büchi, Flawil, Switzerland). Lipids were resuspended in chloroform (concentration 4 mg/mL) and stored at −20 °C for up to 2 weeks.

For comparison, all the assays described in this section were also carried out with lecithin (LEC) (Calbiochem, Merck KGaA, Darmstadt, Germany), which was stored as chloroformic solution (6.25 mM).

2.2. Polymers synthesis

Cholesterol-poly(2-(dimethylamino)ethyl methacrylate) (CHO-PDMAEMA) was synthesized by atom transfer radical polymerization (ATRP). This polymerization procedure allows a stringent control of the molecular weight and dispersity (*Đ*). The synthesis and characterization of CHO-PDMAEMA were performed according to our recent work [17].

2.3. Bare liposomes and PLC preparation

Chloroformic solutions of CHO-PDMAEMA and bacterial lipids were used to prepare bare liposomes and PLCs. Three different CHO-PDMAEMA:lipid molar ratios were assayed: 0, 0.05 and 0.10. The solvent was then evaporated under nitrogen flow. The dried lipid:polymer films were then rehydrated with a buffered solution of 10 mM HEPES (Sigma–Aldrich, Sintra, Portugal), pH 7.0 and vigorously stirred at 37 °C. For the experiments carried out in the presence of trehalose, trehalose (Merck, Darmstadt, Germany) prepared in 10 mM HEPES (pH 7.0) was added to the suspensions of liposomes (final concentration of trehalose: 250 mM).

2.4. Characterization techniques

2.4.1. Zeta (ζ) potential measurement

All measurements were recorded at 37 °C using a Zetasizer Nano Z (Malvern Instruments, Malvern, UK). The average hydrodynamic particle size (*Z*-average) and polydispersity index (PdI) were determined by using dynamic light scattering at backward scattering (173°) with the Zetasizer 6.20 software. ζ-Potential was determined using a combination of measurement techniques: electrophoresis and laser Doppler velocimetry (laser Doppler electrophoresis). The ζ-potential values were provided directly by the instrument. An average value of ζ-potential was obtained from at least 20 determinations for each sample, at pH 7.0 and 37 °C.

2.4.2. Leakage experiments

Calcein-loaded bare liposomes and PLCs were prepared by hydration of the polymer/lipid films in 60 mM of calcein (Sigma–Aldrich, Sintra, Portugal) prepared in 10 mM HEPES (pH 7.0). Non-entrapped calcein was removed by washing and resuspending the liposome pellets in 10 mM HEPES (pH 7.0), or in 10 mM HEPES (pH 7.0) containing 250 mM trehalose, for experiments carried out in the presence of this sugar (see below).

The release of calcein from liposomes was determined fluorometrically on a Synergy HT fluorescence microplate reader (Bio-Tek Instruments, Winoski, Vermont, USA) with an excitation and emission wavelengths at 485/20 nm and 528/20 nm, respectively. The release of the fluorescent probe was monitored for 24 h at 37 °C at pH 7.0. Calcein release was also evaluated at 37 °C and at a pH ranging from 2 to 12.

After adding Triton X-100 (1 μL, 10% w/v solution) to provoke 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 the equation:

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

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 the rupture of vesicles with Triton-X.

2.4.3. Preservation

PLCs washed with and without trehalose were refrigerated (4 °C), frozen (−80 °C) and freeze-dried, and stored for 28 days. The freeze-drying process was carried out at −50 °C and 0.04 mbar in a Heto FD4 (Heto Lab Equipment, Denmark) on liposomes and PLCs previously frozen at −80 °C for 48 h.

2.4.4. Cell culture

The monocyte/macrophage murine cell line Raw 264.7 and the human enterocyte-like cells Caco-2/TC7 were cultured in Dulbecco's Modified Eagle Medium (DMEM, GIBCO BRL Life Technologies, Rockville, MD, USA) supplemented with: 10% (v/v), heat-inactivated (30 min/60 °C) fetal bovine serum (FBS, PAA Laboratories, GmbH, Pasching, Austria), 1% (v/v) non-essential amino acids (GIBCO BRL Life Technologies, Rockville, MD, USA), and 1% (v/v) penicillin–streptomycin solution (100 U/mL penicillin G, 100 μg/mL streptomycin, GIBCO BRL Life Technologies, Rockville, MD, USA). Cultured cells were incubated at 37 °C in a 5% CO_2 95% air atmosphere.

2.4.5. PLCs uptake by eukaryotic cells

Flow cytometry (FACS) were used to assess uptake of bacteria PLCs by Raw 264.7 or Caco-2/TC7 cells. Cells were seeded in 24-well culture plates at a density of 2×10^5 cells/mL and incubated 48 h (Raw 264.7) or 5 days (Caco-2/TC7) at 37 °C as indicated. Monolayers were washed with PBS and fresh medium containing calcein loaded PLCs was added to each well. After incubating for 1 h, cells were washed twice with PBS to remove free non-internalized vesicles. Cells were removed by trypsination (Caco-2/TC7) or scraping (Raw 264.7) afterward they were transferred to FACS tubes containing PBS. FACS was performed in a FACScalibur flow cytometer by using Cell Quest software (Becton Dickinson, Mountain View, CA, USA). For each analysis the green fluorescence (FL1) of 10,000 events was acquired and plotted against forward light scatter (FSC-H).

2.4.6. Statistics

All assays were performed in triplicate and in three independent assays. Analysis of variance (ANOVA) was carried out for all the assays, using the statistical program InfoStat 2008 (Infostat Group/FCA. National University of Cordoba. Ed. Brujas, Cordoba, Argentina). Means were compared by the Tukey's test ($P < 0.05$).

3. Results

Table 1 shows the physicochemical properties of both bare liposomes and PLCs prepared with lipids from strain CIDCA 133. The corresponding values of LEC vesicles are shown for comparison [17]. The PdI obtained for all the studied vesicles was lower than 0.5. The incorporation of CHO-PDMAEMA onto the liposomes led to a decrease of about 50–60% in the liposome size and positively charged the lipid vesicles. The incorporation of trehalose increased the particle size when compared to the corresponding cases not treated with trehalose.

Fig. 1A shows calcein release from CIDCA 133 bare vesicles as well as from PLCs at 37 °C and pH 7. The incorporation of polymer strongly stabilized liposomes at the studied conditions, being CIDCA133PD10 slightly more stable after prolonged incubation (240 min). Stability of liposomes was strongly dependent on pH. The presence of CHO-PDMAEMA in the PLCs stabilized them at pH 7. Upon a slight decrease of pH, the PLCs destabilized. No differences were observed between CIDCA133PD5 and CIDCA133PD10 (Fig. 1B).

The stability of PLCs upon preservation at 4 °C, −80 °C and freeze-drying was also evaluated (Fig. 2). To assess the influence of the lipid composition on the stability of PLCs upon preservation, the assays were also carried out on LEC PLCs for comparison (Fig. 2B). For both CIDCA133 and LEC PLCs, preservation at 4 °C and −80 °C were found to be the most suitable conditions as they showed the lowest values of calcein release (Fig. 2). In these conditions, calcein release was less than 20% for CIDCA133PD5, CIDCA133PD10, and for LECPD5. LECPD10 was less stable as indicated by the 40% of calcein release at 4 °C, and 60% at −80 °C (Fig. 2B). The presence

Table 1
Physical characterization of bare liposomes and PLCs at 37 °C.

	Particle size (nm)	PdI	ζ-Potential (mV)
CIDCA133	110.6 ± 1.4	0.348 ± 0.009	−13.6 ± 2.9
CIDCA133PD5	57.3 ± 0.8	0.467 ± 0.016	12.8 ± 0.8
CIDCA133PD10	51.1 ± 0.6	0.430 ± 0.011	11.8 ± 0.8
LEC[a]	198.7 ± 1.8	0.485 ± 0.013	−7.7 ± 0.2
LECPD5[a]	77.3 ± 1.9	0.487 ± 0.014	12.5 ± 2.3
LECPD10[a]	80.1 ± 1.0	0.464 ± 0.015	13.3 ± 0.4
250 mM Trehalose			
CIDCA133	269.0 ± 2.1	0.466 ± 0.187	−28.6 ± 0.3
CIDCA133PD5	131.4 ± 2.3	0.374 ± 0.057	18.6 ± 0.1
CIDCA133PD10	126.1 ± 1.5	0.495 ± 0.009	15.8 ± 0.3
LEC	184.5 ± 3.9	0.314 ± 0.066	−48.4 ± 1.5
LECPD5	161.9 ± 0.5	0.339 ± 0.074	34.2 ± 1.2
LECPD10	207.3 ± 4.0	0.439 ± 0.201	35.9 ± 0.8

[a] Information taken from our previous work Alves et al. [17].

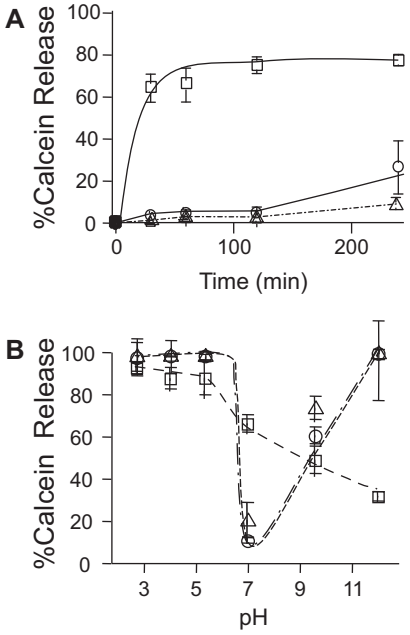

Fig. 1. Calcein release profiles from bacterial PLCs prepared with lipids extracted from *Lactobacillus delbrueckii* subsp. *lactis* CIDCA 133 and different molar concentrations of CHO-PDMAEMA: 0% (squares), 5% (circles) and 10% (triangles). (A) Calcein release along time at 37 °C and pH 7.0 and (B) Calcein release at different pHs after 2 h at 37 °C. Each result is the mean ± standard error of the mean of three independent experiments.

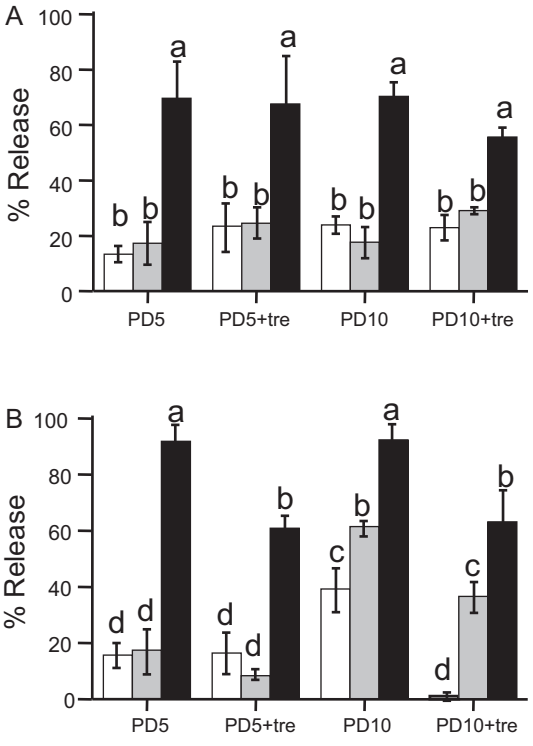

Fig. 2. Calcein release from (A) CIDCA 133 and (B) LEC PLCs after 7 days of preservation at 4 °C, −80 °C and freeze-drying. White bars represent preservation at 4 °C, gray bars, preservation at −80 °C and black bars, preservation after freeze drying process. Two independent ANOVA were performed, one for CIDCA 133 PLCs and the other one, for LEC PLCs. Each result is the mean ± standard error of the mean of three independent experiments. Different letters (a, b, c and d) denote statistically significant differences ($P < 0.05$).

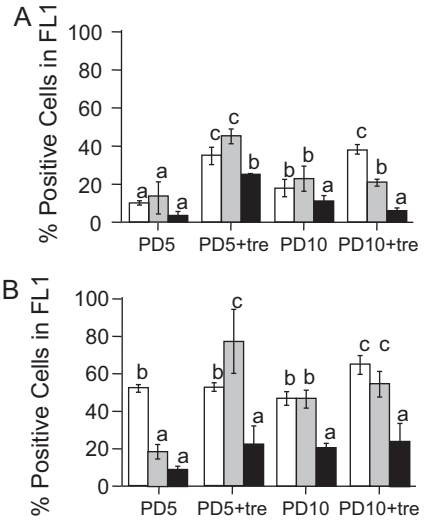

Fig. 3. Calcein uptake results obtained by FACS for CIDCA 133PD5 and CIDCA 133PD10 with: (A) Caco2/TC7 cells; (B) Raw 264.7 cells. White bars represent preservation at 4 °C, gray bars, preservation at −80 °C and black bars, preservation after freeze drying process. Two independent ANOVA were performed for Caco2/TC7 and Raw 264.7 cells. Two ANOVA were performed independently, one for the Caco2/TC7 calcein uptake (script A) and the other one, for the Raw 264.7 calcein uptake (script B). Each result is the mean ± standard error of the mean of three independent experiments. Different letters denote statistically significant differences ($P < 0.05$).

of trehalose in the liposome formulations significantly stabilized LECPD10 PLCs when stored at 4 °C and −80 °C ($P < 0.05$).

Freeze-drying had an adverse effect on both types of PLCs. A calcein release of 70% was obtained for bacterial PLCs with no significant differences between CIDCA133PD5 and CIDCA133PD10 ($P < 0.05$). Destabilization of LEC PLCs was even higher since calcein release was more than 90% for both LECPD5 and LECPD10 ($P < 0.05$). The presence of trehalose in the formulation significantly improved the entrapment of calcein in LEC PLCs ($P < 0.05$) to attain values of ~60% (similar to those of freeze-dried CIDCA133 PLCs) (Fig. 2A and B). No further stabilization was observed for CIDCA133 PLCs freeze-dried with trehalose ($P < 0.05$) (Fig. 2A).

Raw 264.7 and Caco-2/TC7 cells were able to incorporate CIDCA133 calcein loaded PLCs (Fig. 3A and B). The incorporation was higher when PLCs were stored at 4 °C and −80 °C. In contrast, for freeze-dried PLCs, the percentage of calcein positive cells was in average 2.4 times lower for Caco-2/TC7 cells (Fig. 3A) and 3.1 times lower for Raw 264.7 cells (Fig. 3B). In general, PLCs stored at 4 °C, −80 °C and freeze-dried in the presence of trehalose were more easily incorporated into eukaryotic cells than the corresponding PLCs prepared without trehalose.

4. Discussion

The use of liposomes as drug delivery systems requires the assessment of their physical and chemical stability, the most appropriate preservation conditions, and also their incorporation into eukaryotic cells.

The low PdI of both bare liposomes and PLCs indicated uniformity in particle size (Table 1). The positive charge of PLCs (around 12 mV) might be explained considering the cationic character of CHO-PDMAEMA, which confers a positively charged surface when incorporated in the liposome formulation. It is interesting to note that bare liposomes prepared with bacterial lipids were more negative than bare LEC liposomes (−13.6 ± 2.9 *vs* −7.7 ± 0.2 mV) (Table 1 and Ref. [17]). The composition of bacterial lipids, mainly including negatively charged phospholipids (CL and PG) and GLY, explains their higher negative charge [8]. This also contributes for a better

interaction with the positively charged CHO-PDMAEMA. Lambruschini et al. [27] observed that the surface potential-area isotherms obtained in DPPC monolayers increases upon injection of trehalose in the lipid subphase. In the presence of trehalose, the rise of surface potential occurs at significantly larger area per molecule than in ultrapure water. This increase of the lipid area modifies the superficial potential and thus, supports the changes in zeta potential observed in Table 1 in this work.

The incorporation of CHO-PDMAEMA strongly stabilized bacteria-derived liposomes at pH 7 at both polymer:lipid ratios assayed (Fig. 1A). In our previous work, it was shown that in the case of LEC liposomes, LECPD5 are more stable than the corresponding bare liposomes; and LECPD10 and bare liposomes have similar stabilities [17]. On the other hand, the weak polyelectrolyte character of PDMAEMA (pK_a *ca.* 8.0) can explain its destabilization at pHs lower than 7.0, which supports their potential use as stimuli-responsive systems, in mildly acidic environments [17]. CIDCA133PD also destabilized upon slight changes of pH, supporting their use as stimuli-responsive systems as well (Fig. 1B).

Preservation is an important issue for systems to be used as pharmaceutical formulations. In fact, unstable liposomes during storage represent a serious limiting factor for their applicability as drug delivery systems. It has been reported that the main causes leading to liposomes instability include hydrolysis or oxidation of phospholipids and variations in the vesicle size (originated in fusion and/or aggregation), which lead to leakage [28]. Freezing or freeze-drying procedures are generally used to maintain the long-term stability of liposomes [29]. Hence, the behavior of bacteria PLCs after freezing and freeze-drying process was investigated. Storage at 4 and −80 °C showed to be considerably less drastic processes than freeze-drying. This was reflected in the lower calcein release of PLCs stored under these conditions (Fig. 2A and B). The most stable as prepared formulations (CIDCA133PD5, CIDCA133PD10 and LECPD5 according to Fig. 1) were also more stable upon preservation at 4 and −80 °C (Fig. 2).

The presence of trehalose had a protective effect on LECPD10 (Fig. 2B). The stabilizing effect of sugars is based on their capacity to preserve membrane integrity during dehydration/rehydration processes like freeze-drying [3,9]. One of the mechanisms proposed for sugar protection is the replacement of water molecules through the formation of hydrogen bonds sugar-phospholipids [3,9]. This avoids dehydration and maintains membranes as if they were hydrated. Hence, stable liposomes are able to retain the encapsulated drug (or compound). In the absence of protectants, leakage of internal aqueous content from liposomes usually occurs [3–5]. This explains the protective effect of trehalose on LECPD10 upon preservation at 4 and −80 °C (Fig. 2B).

The sugar:lipid molar ratio is very important when a sugar is used as protectant, and it has been suggested that the optimal relation is 6:1–8:1 [1]. In addition, the sugars bound to phospholipids head groups (that is, sugars of GLY), have demonstrated a protective role on freeze-dried liposomes [30]. GLY are one of the main components of bacterial lipids [8,9] and the sugar:lipid ratio for lipids of strain CIDCA 133 is 11:1 [8]. Moreover, the presence of phosphatidylglycerol (PG) also contributes for the stabilization of PLCs considering the protective effect of glycerol [31]. This explains their higher stability upon preservation in the absence of trehalose (Fig. 2A). The particular composition of bacterial lipids was previously referred as responsible for leakage prevention after interacting with disrupting peptides like defensins [32].

After freeze-drying, both CIDCA133 and LEC PLCs became less efficient in retaining calcein (Fig. 2A and B). Even though, CIDCA133 PLCs without trehalose remained more stable than LEC PLCs. The presence of trehalose during freeze-drying stabilized LEC PLCs and showed significant effect ($P < 0.05$) on CIDCA PLCs. The presence of GLY and PG explains, also in this case, the greater stability of bacterial formulations. It is well known that culture conditions (*i.e.*: culture medium, temperature, osmolarity, *etc.*) strongly affect the lipid composition of lactic acid bacteria affect [9]. For example, growing microorganisms in suboptimal conditions leads to an increase of the sugar:lipid molar ratio [9]. Therefore, the alteration of the growth conditions can be used as a strategy to modulate the composition of bacterial lipids and improve even more, the stability of bacterial lipids.

As expected, monocytic cells (Raw 264.7) incorporated higher quantity of calcein than enterocyte-like cells (Caco-2/TC7) because of their phagocytic capacity (Fig. 3A and B). It is known that clathrin-mediated endocytosis plays a main role in the internalization of liposomes by professional phagocytic cells [33,34], which are generally more efficient than epithelial cells in liposomal uptaking [35].

FACS analysis demonstrated that the calcein uptake by eukaryotic cells correlated well with the stability of liposomes (Fig. 2). In fact, liposomes preserved at 4 and −80 °C (the most stable ones) were highly incorporated whereas freeze-dried liposomes were not (Fig. 3A and B). Interestingly, the calcein uptake of bacterial liposomes previously exposed to ±80 °C for 5 min (lysed liposomes) dramatically dropped (4.7 ± 0.3 for Caco-2/TC-7 and 0.8 ± 0.1 for Raw 264.7) (data not shown). These results indicate that the integrity of the liposomes is essential for an efficient incorporation of the fluorescent probe into the cells. Membrane disruption of freeze-dried liposomes explains the poor calcein transfer observed in freeze-dried liposomes (Fig. 3A and B).

The electrostatic interactions between positively charged vesicles and the negatively charged eukaryotic cell membranes and cell surface proteoglycans facilitates cell uptake [36]. It has been reported that cationic liposomes are associated with efficient cellular delivery of drug cargoes [37,38] and routinely applied for *in vitro* gene delivery [39]. The good interaction between the positively charged PLCs and eukaryotic cells can be explained on this basis.

5. Conclusion

The composition of lactic acid bacteria lipids, naturally including both negatively charged lipids and sugars plays an important role in stabilizing bacteria PLCs upon preservation. From one side, the negative charge of bacterial lipids favors the interaction with CHO-PDMAEMA, leading to stable stimuli-response systems. From the other side, GLY act as intrinsic protectants upon preservation processes, thus maintaining the membrane integrity. This membrane integrity demonstrated to be essential for an efficient incorporation of calcein into both Raw 264.7 and Caco-2/TC-7 cells.

Considering that physical–chemical properties of liposomes often limit their use in medical applications, the results obtained support the use of natural formulations potentially useful as stimuli-responsive drug delivery systems.

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