Effect of cholesterol-poly(N,N-dimethylaminoethyl methacrylate) on the properties of stimuli-responsive polymer liposome complexes

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ABSTRACT

The development of new polymer–liposome complexes (PLCs) as delivery systems is the key issue of this work. Three main areas are dealt with: polymer synthesis/characterization, liposome formulation/characterization and evaluation of the PLCs uptake by eukaryotic cells.

Poly(N,N-dimethylaminoethyl methacrylate) (PDMAEMA) with low molecular weight and narrow polydispersity was synthesized by Atom Transfer Radical Polymerization (ATRP). The polymers were synthesized using two different bromide initiators (cholesteryl-2-bromoisobutyrate and ethyl 2-bromoisobutyrate) as a route to afford PDMAEMA and CHO-PDMAEMA.

Both synthesized polymers (PDMAEMA and CHO-PDMAEMA) were incorporated in the preparation of lecithin liposomes (LEC) to obtain PLCs. Three polymer/lipid ratios were investigated: 5, 10 and 20%. Physicochemical characterization of PLCs was carried out by determining the zeta potential, particle size distribution, and the release of fluorescent dyes (carboxyfluorescein —CF— and calcein) at different temperatures and pHs.

The leakage experiments showed that CHO covalently bound to PDMAEMA strongly stabilizes PLCs. The incorporation of 5% CHO-PDMAEMA to LEC (LEC_CHO-PD5) appeared to be the stablest preparation at pH 7.0 and at 37 °C. LEC_CHO-PD5 destabilized upon slight changes in pH and temperature, supporting the potential use of CHO-PDMAEMA incorporated to lecithin liposomes (LEC_CHO-PDs) as stimuli-responsive systems.

In vitro studies on Raw 264.7 and Caco-2/TC7 cells demonstrated an efficient incorporation of PLCs into the cells. No toxicity of the prepared PLCs was observed according to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. These results substantiate the efficiency of CHO-PDMAEMA incorporated onto LEC to assist for the release of the liposome content in mildly acidic environments, like those found in early endosomes where pH is slightly lower than the physiologic.

In summary, the main achievements of this work are: (a) novel synthesis of CHO-PDMAEMA by ATRP, (b) stabilization of LEC by incorporation of CHO-PDMAEMA at neutral pH and destabilization upon slight changes of pH, (c) efficient uptake of LEC_CHO-PDs by phagocytic and non-phagocytic eukaryotic cells.

1. Introduction

Liposomes are attractive materials for drug delivery because of their biocompatibility, non-immunogenicity, non-toxicity, and ability to entrap both hydrophilic and hydrophobic compounds [1]. However, for the delivery process to be efficient, they should be able to entrap drugs and release them at the target cell, which in several cases is still an unsolved problem. The factors involved in liposome leakage are not always clear, although an acidic environment might be the main responsible. To improve the leakage process, different approaches have been employed. Among them, the design of stimuli-sensitive liposomes capable of releasing their contents in response to environmental changes (*e.g.*: temperature, pH) has been described as a promising approach [2].

Thermosensitive liposomes can be prepared by using lipids whose membranes undergo a gel-to-liquid crystalline phase transition some degrees above physiological temperature [3]. In general, a thermosensitive liposome formulation shows maximal drug release when exposed to temperatures of 40–45 °C within 30–60 min. Therefore, an external thermal stimulus is required to

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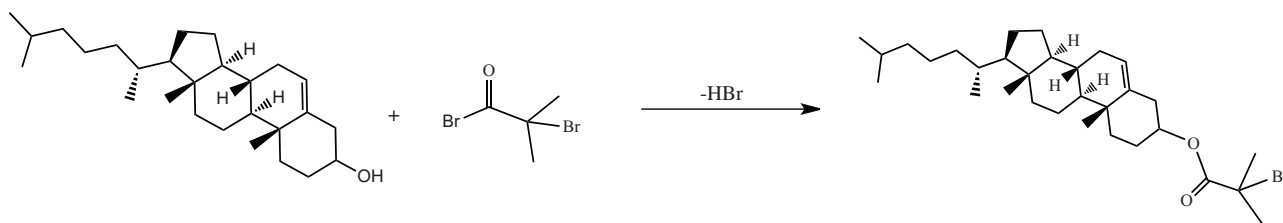*E-mail address:* angoza@qui.uc.pt (A. Gómez-Zavaglia).

Fig. 1. Schematic representation of the synthesis of cholesteryl-2-bromoisobutyrate (CHO-Br).

trigger the thermosensitive liposomes in the target region (*i.e.*: tumor and adjacent regions, local infections [2,4]).

pH-sensitive liposomes, on the other hand, release their contents in ischemic tissues or into the endosomal compartments, where the medium has pHs lower than physiologic. To prepare pH-sensitive vesicles, different methods can be employed: (a) combinations of polymorphic lipids with mildly acidic amphiphiles that act as stabilizers at neutral pH; (b) synthesis of caged lipid derivatives, in which an acid-induced hydrolysis of specifically engineered chemical bonds results in an increased presence of membrane destabilizing lipid components [5]; (c) use of pH-titratable polymers to destabilize membranes due to a change of the polymer conformation at low pH [2,3].

The attachment of functional polymers to liposomes by means of hydrophobic anchors has emerged as a strategy to prepare these stimuli-responsive systems. Several amphiphiles have been used in the design of stimuli-sensitive liposomes [6–9]. Copolymers of alkylated N-isopropylacrylamide with titratable monomers (*e.g.*: acrylic or methacrylic acid), can be used to obtain temperature-pH-sensitive polymer-incorporated liposomes [10]. In addition, poly(methacrylic acid-co-stearyl methacrylate) coatings have also been used to obtain pH-sensitive liposomes [11]. The attachment of poly(2-ethylacrylic acid) to the liposome surface also leads to destabilization at low pH [2].

The optimization of the efficiency of stimuli-responsive drug delivery systems by incorporating a polymeric material into the liposomal membrane demands a rigorous control of the polymeric synthesis. In this regard, the tailored synthesis of macromolecules based on control/*living* radical polymerization (LRP) method is a suitable approach [12]. In particular, ATRP is very well established for different families of monomers [13], and has several advantages over other methods, such as, mild work conditions, fast kinetics, and very high tolerance to different functionalities [13]. In addition, the polymers can have different architectures, topologies and compositions, providing a high degree of control over polymer microstructure [14].

Poly(N,N-dimethylaminoethyl methacrylate) (PDMAEMA) is an amphiphilic molecule composed of methacrylate, a substituent containing poly(2-ethylacrylic acid), that may lead to the destabilization of liposomes in response to environment temperature or pH changes. In spite of its capacity to respond to environmental stimuli, PDMAEMA may easily dissociate from the liposome surface, returning to the less stable state. This is in part attributed to the properties of PDMAEMA hydrophobic anchor (chain length and/or saturation degree) [5]. This problem could be overcome by covalently linking cholesterol (CHO) to PDMAEMA to obtain CHO-PDMAEMA. The role of CHO in enhance the anchoring of a polymer/molecule into the liposomal membrane is already known [15,5]. Therefore, CHO would facilitate the anchoring of the thermo and pH-sensitive N,N-dimethylaminoethyl methacrylate (DMAEMA) polymer into the liposomal membrane. In a recent study [16,17], CHO-PDMAEMA was obtained by oxyanion-initiated polymerization, a complex and time consuming method. However, the ATRP method of synthesis is much simpler and uses mild conditions of polymerization along with a rigorous control of the polymer

properties. To the best of our knowledge, the synthesis of CHO-PDMAEMA by ATRP was never reported in the literature.

In this work, the stimuli-responsive polymer PDMAEMA was synthesized by ATRP. In order to evaluate the CHO effect on the polymer, and consequently on its incorporation in the liposome, two different structures were synthesized: PDMAEMA and CHO-PDMAEMA. Lecithin was selected to be used for liposome formulation because of its naturally presence in cell membranes allowing a good bioacceptability [18]. In order to define the most appropriate formulation for drug delivery, the stability at different pHs and temperatures allowed the selection of the most suitable polymer/lipid ratio. The endocytosis and cytotoxicity of PLCs have also been evaluated to give a complete picture of this potentially useful drug delivery system.

2. Materials and methods

2.1. Polymers synthesis

2.1.1. Cholesteryl-2-bromoisobutyrate synthesis

The initiator was obtained by esterification of CHO with 2-bromoisobutyryl bromide (Sigma–Aldrich, Sintra, Portugal) [19] as presented in Fig. 1. Briefly, 1 g sample of 4-(dimethylamino)pyridine (DMAP, Sigma–Aldrich, Sintra, Portugal) (previously recrystallized from toluene) in 10 mL of dry methylene chloride (CH$_2$Cl$_2$, ACROS, JMGS, Portugal) was mixed with 0.7 mL of triethylamine (TEA, Sigma–Aldrich, Sintra, Portugal) (dried over CaH$_2$ and vacuum distilled). The solution was transferred into a 250 mL three-neck round-bottom flask equipped with condenser, dropping funnel, gas inlet/outlet, and a magnetic stirrer. After cooling to 0 °C, 3 mL of 2-bromoisobutyryl bromide in 10 mL of dry CH$_2$Cl$_2$ was added. 1 g of CHO (Sigma–Aldrich, Sintra, Portugal) in 50 mL of dry CH$_2$Cl$_2$ was added dropwise to the formed yellow dispersion, during 1 h under dry nitrogen; subsequently the temperature was allowed to rise to room temperature. The reaction was kept under stirring for 20 h. Afterwards, the mixture was washed with a saturated aqueous sodium chloride solution, dried over magnesium sulphate, followed by evaporation of half of the solvent. The cholesteryl-2-bromoisobutyrate (CHO-Br) initiator was precipitated in ethanol (ACROS, JMGS, Portugal) and finally filtered and dried in vacuum. The final product was obtained in the form of a white powder and characterized using ^{1}H nuclear magnetic resonance (^{1}H NMR) (400 MHz, CDCl$_3$): $\delta = 0.73$ (s, 3 H, cholesteryl CH$_3$), $\delta = 0.89$ (d, 6 H, cholesteryl CH$_3$), $\delta = 0.95$ (d, 3 H, cholesteryl CH$_3$), $\delta = 1.07$ (s, 3 H, cholesteryl CH$_3$), $\delta = 0.95$–2.40 (m, 28 H, cholesteryl CH and CH$_2$), $\delta = 1.97$ (s, 6H, CH$_3$CBr), $\delta = 4.60$ (m, 1H, CHO), $\delta = 5.40$ (s, 1H, CH=C).

2.1.2. Polymers synthesis

PDMAEMA (Sigma–Aldrich, Sintra, Portugal) was synthesized by ATRP. Two polymers were synthesized: a CHO terminated PDMAEMA (CHO-PDMAEMA) and PDMAEMA. These polymers were tailored to have the same molecular weight. The procedure was the same for both polymers, the only difference being the initiator used: for CHO-PDMAEMA the previously
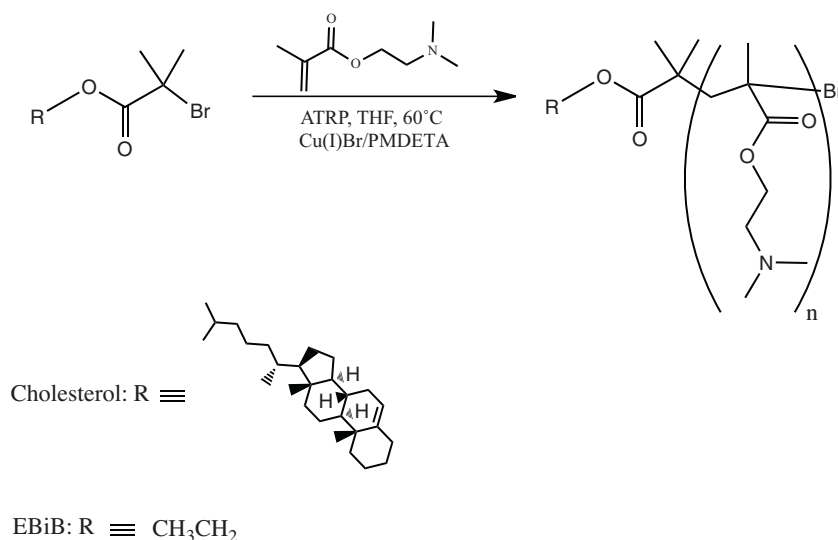
Cholesterol: R ≡

EBiB: R ≡ CH₃CH₂

Fig. 2. Schematic illustration of the ATRP of DMAEMA.

obtained cholesteryl-2-bromoisobutyrate (CHO-Br) was used, while for PDMAEMA was employed a standard LRP initiator, ethyl 2-bromoisobutyrate (EBiB, Sigma–Aldrich, Sintra, Portugal). Therefore, the proper initiator was used in the ATRP of DMAEMA using copper (I) bromide as catalyst (Cu(I)Br, Sigma–Aldrich, Sintra, Portugal) and n,n,n′,n′,n-pentamethyldiethylenetriamine as ligand (PMDETA, Sigma–Aldrich, Sintra, Portugal) in tetrahydrofuran (THF, Sigma–Aldrich, Sintra, Portugal) at 60 °C. Succinctly, the monomer (DMAEMA, 12.7 mmol, freshly passed through a Al_2O_3 column), and the initiator (CHO-Br, 0.41 mmol) were added to a 25 mL Schlenk flask equipped with a magnetic stirrer and was frozen and bubbled with N_2 to eliminate oxygen. Then, catalyst (Cu(I)Br, 0.41 mmol), the ligand (PMDETA, 0.82 mmol) and the solvent (THF, 2.1 mL, previously bubbled with N_2) were added under N_2 atmosphere to the Schlenk flask, which was then sealed and deoxygenated under reduced pressure and then filled with N_2. The reaction proceeded at 60 °C for 24 h. The schematic reaction of this procedure is presented in Fig. 2.

After 24 h, the polymerization was stopped by dissolving the reaction mixture in THF. Then, the product was purified by passing the solution though a basic Al_2O_3 column to remove the catalysts. Finally, the solution was precipitated in n-hexane (Sigma–Aldrich, Sintra, Portugal) and the obtained powder was filtered and dried under vacuum.

2.2. Characterization

2.2.1. NMR
^{1}H NMR spectra were obtained using a Bruker Avance III 400 MHz spectrometer. CDCl₃ sample solutions were analyzed in tubes of 5 mm of diameter, using tetramethylsilane (TMS) as internal standard.

2.2.2. Gel permeation chromatography (GPC)
The number-average molecular weight (M_n) and dispersity ($Đ = M_w/M_n$) of CHO-PDMAEMA and PDMAEMA were determined by GPC analysis using a Viscotek (Dual detector 270, Viscotek, Houston, USA), with THF as eluent at 30 °C (1.0 mL/min), previously calibrated with narrow polystyrene standards. Number-average molecular weight ($M_{n,GPC}$) and Đ of synthesized polymers were determined by TriSEC calibration by using OmniSEC software.

2.3. Stabilization assays

2.3.1. Liposomes and polymer–lipid complexes (PLCs) preparations
Liposomes and PLCs were prepared by the reverse-phase evaporation procedure [20]. Briefly, lecithin (Calbiochem, Merck KGaA, Darmstadt, Germany) was dissolved in chloroform (6.25 mM); PDMAEMA and CHO-PDMAEMA were also dissolved in chloroform (0.4 mM). Then, different PDMAEMA and CHO-PDMAEMA/lecithin molar ratios (0, 5, 10 or 20%) were prepared from their chloroformic solutions, and further dried under a nitrogen stream. The dried lipid/polymer film was then rehydrated with a buffered solution of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma–Aldrich Co., St. Louis, MO) or phosphate saline buffer [PBS; (K_2HPO_4 0.144 g/L; NaCl 9.00 g/L; Na_2HPO_4 g/L)], pH 7.0 and vigorously stirred above the transition temperature (37 °C).

2.3.2. Particle size and zeta (ζ) potential measurement
All measurements were recorded at 37 °C using a Malvern Instrument Zetasizer Nano-Z (Malvern Instruments, Malvern, UK). The average hydrodynamic particle size (Z-average) and polydispersive 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. For both measurements, liposomes and PLCs were dispersed in PBS (pH 7; 37 °C). Different pHs evaluations were adjusted with NaOH 0.1 M and HCl 0.1 M. An average value of ζ-potential was obtained from at least 20 determinations for each sample.

2.3.3. Leakage experiments
The previously obtained polymer/lipid films were hydrated in 29 mM 5(6)-carboxyfluorescein (CF, Sigma–Aldrich Co., St. Louis, MO) CF, prepared in PBS (pH 7.0). At these concentrations, CF in liposomes is self-quenched. Non-entrapped CF was removed from the external medium by washing and resuspending the liposome pellets in 10 mM PBS (pH 7.0), according to Hollmann et al. [21]. Calcein-loaded liposomes were used for experiments carried out at pHs different from 7.0. They were prepared in the same way as

the CF-loaded ones, but using 60 mM calcein (Sigma–Aldrich Co., St. Louis, MO) and 10 mM HEPES (pH 7.0) for liposome resuspension.

Release of CF and calcein from liposomes was determined fluorometrically on a Synergy HT fluorescence microplate reader (Bio-Tek Instruments, Winoski, Vermont, USA) with excitation and emission wavelengths at 485/20 nm and 528/20 nm, respectively. The release of fluorescent probes was monitored for 37 °C and 45 °C. CF-loaded liposomes and PLCs were used to evaluate the stability of the liposomes along time at physiological pH (pH 7.0). Calcein-loaded vesicles where exposed to different pH environments to evaluate the stability at different pHs at 37 °C.

The fluorescence intensity resulting from the release of the encapsulated CF or calcein from the vesicles was recorded along time. After the last record, 2 μL Triton X-100 (10%, w/v) (Sigma–Aldrich Co., St. Louis, MO) were added to each sample to induce its total lysis (100% release of CF or calcein). The extent of probe release was expressed as a percentage calculated from Eq. (1)

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

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 after the addition of Triton-X100.

2.3.4. Cell cultures

The monocyte/macrophage murine cell line Raw 264.7 and the human enterocyte Caco-2/TC7 cell line 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.

2.3.5. Cytotoxicity assays

Cell viability was determined by assessing mitochondrial dehydrogenase activity by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma–Aldrich Co., St. Louis, MO). Cells were seeded in duplicate in 24-well plates at 1×10^5 cells per well and incubated for 48 h or 5 d for Raw 264.7 and Caco-2/TC7, respectively. Afterwards, polymer and PLCs with different polymer/lipid ratios were added and cells were incubated for 24 or 48 h. Next, cells were washed twice with PBS and the medium was replaced by DMEM (without phenol red dye) containing 0.5 mg/mL MTT. After 3 h incubation, 0.2 mL DMSO were added per well to solubilize formazan crystals. The 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. (2).

$$\text{Cell viability (\%)} = \frac{(OD_t)}{(OD_c)} \times 100\% \tag{2}$$

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

2.3.6. Cellular uptake by eukariotic cells

Fluorescence microscopy and flow cytometry (FACS) were used to assess intracellular uptake of lisosomes 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. After incubation, monolayers were washed with PBS and fresh medium containing calcein loaded liposomes was added to each well. After incubating for 1 h (Raw 264.7) or 3 h (Caco-2/TC7) incubation, samples were discarded and the cells were washed twice with PBS to remove free non-endocytosed vesicles. For fluorescence analysis the cells were maintained in 0.1 mL PBS and examined in an inverted fluorescence microscope (Nikon, Eclipse Ti-U). Cells were observed by epifluorescence with a 30× objective and with excitation and emission wavelengths at 485/35 nm and 555/15 nm, respectively.

For FACS analysis after the incubation with liposomes, Caco-2/TC7 cells were washed and trypsinized for 5 min with trypsin-EDTA (0.25%). Trypsinization was stopped by diluting the liposomes with culture medium and cells were suspended and transferred to FACS tubes containing PBS. Raw 264.7 were detached by gently pipetting. FACS was performed in a FACS calibur 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).

2.3.7. 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). Comparison of means was tested using Tukey methods, and if $P < 0.05$, the difference was considered statistically significant.

3. Results

3.1. Characterization of PDMAEMA and CHO-PDMAEMA

PDMAEMA is a weak polyelectrolyte (pK_a ca. 8.0) that can be protonated in acid aqueous solutions. The ATRP synthesis of PDMAEMA allowed a high degree of control over the molecular weight and polymer microstructure, using relatively mild synthesis conditions. Fig. 2 depicts the schematic reaction corresponding to the ATRP of DMAEMA using CHO-Br and EBiB initiators. Fig. 3A and B shows the ^{1}H NMR spectra of PDMAEMA and CHO-PDMAEMA, respectively. The most intense bands were similar in both spectra and were ascribed to PDMAEMA [16,22]: (a) the methyl group at 0.9–1.1 ppm; (b) the peaks related with the $-CH_2-$ from PDMAEMA backbone at about 1.8–2.0 ppm; (c) the $-O-CH_2-$ peak at 4.1 ppm; (d) the $(CH_3)_2-N\langle$ peak at 2.6 ppm and (e) the $-CH_2-N\langle$ peak at 2.3 ppm. The presence of the cholesteryl-2-bromoisobutyrate initiator [16,19] from the cholesteryl CH_3 singlet at 0.7 ppm (g) and the double bound (CH=C from CHO) peak at 5.4 ppm (f), both of them labeled in the spectra, reveals the success of the polymerization reaction (Fig. 3B).

The good control over the polymer synthesis achieved by the ATRP method used is supported by the results shown in Table 1. Considering a theoretical molecular weight of about 5000 Da for
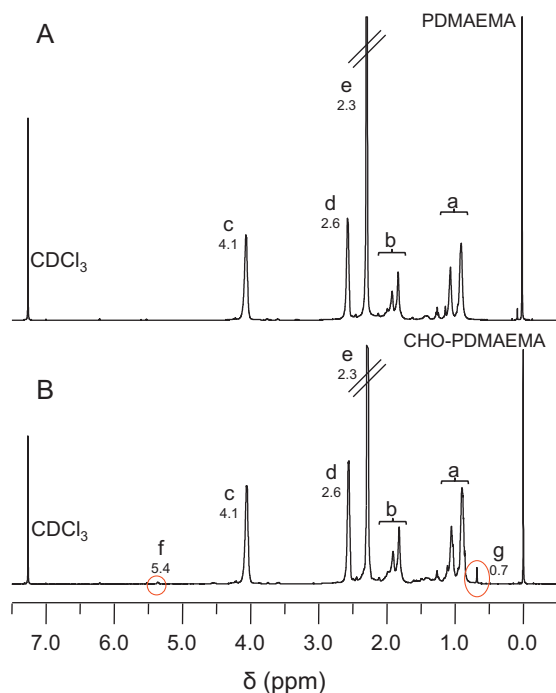
Table 1
Molecular weights (M_n), conversion and dispersity ($Đ$) of PDMAEMA and CHO-PDMAEMA synthesized by ATRP.

	PDMAEMA	CHO-PDMAEMA
$M_{n,th}$ (Da)[a]	5068	5409
Conversion (%)[b]	92.9	99.1
$M_{n,GPC}$ (Da)[c]	4067	5220
$Đ$ (M_w/M_n)[c]	1.19	1.17

[a] $M_{n,th} = M_{w,Initiator} + DP \times M_{wDMAEMA}$ (DP = 31).
[b] Conversion of DMAEMA measured by ^{1}H NMR.
[c] Measured by GPC in THF.

Fig. 3. (A) ^{1}H NMR spectrum of PDMAEMA in CDCl$_3$ at 400 MHz [(a) δ = 0.9–1.1 (−CH$_3$ from PDMAEMA), (b) δ = 1.8–2.0 (−CH$_2$− from PDMAEMA backbone), (f) δ = 2.3 [(CH$_3$)$_2$−N from PDMAEMA], (d) δ = 2.6 [−(CH$_3$)$_2$−N⟨ from PDMAEMA], (c) δ = 4.1 (−O−CH$_2$− from PDMAEMA)] (B): ^{1}H NMR spectrum of CHO−PDMAEMA in CDCl$_3$ at 400 MHz [(g) δ = 0.7 (cholesteryl CH$_3$ from CHO), (a) δ = 0.9–1.1 (−CH$_3$ from PDMAEMA), (b) δ = 1.8–2.0 (−CH$_2$− from PDMAEMA backbone), (f) δ = 2.3 [(CH$_3$)$_2$−N from PDMAEMA], (d) δ = 2.6 [−(CH$_3$)$_2$−N⟨ from PDMAEMA], (c) δ = 4.1 (−O−CH$_2$− from PDMAEMA), (f) δ = 5.4 (CH=C from CHO)].

PDMAEMA and CHO-PDMAEMA, a theoretical degree of polymerization (DP) was defined as 31. From the ^{1}H NMR spectra, the peak integration allowed determining an experimental DP of 35, which is close to the theoretical value and confirmed the target molecular weight. The reliability of the method became evident when analyzing the other different parameters presented in Table 1, including a Đ close to 1, the high percentage of conversion and the proximity between the theoretical molecular weight ($M_{n,th}$) and the experimental molecular weights obtained by GPC analysis ($M_{n,GPC}$) and ^{1}H NMR.

3.2. Physicochemical characterization of liposomes

The efficiency of PLCs prepared from lecithin and PDMAEMA polymer (LEC_PD) and with lecithin and CHO-PDMAEMA polymer (LEC_CHO-PD) to entrap CF at 37 °C was analyzed for three different polymer/lipid ratios: 5, 10 and 20%. CF release was evaluated after the maximum release was attained in all PLCs (Fig. 4). The release of CF for liposomes prepared from lecithin with 10 and 20% PDMAEMA polymer (LEC_PD10 and LEC_PD20, respectively) was above 90%. In the case of PLCs with polymer/lipid ratio equal to 5%, both polymers (PDMAEMA and CHO-PDMAEMA) exhibited an improved stability. However, the CF release in LEC_CHO-PD5 was ca. three times lower than LEC_PD5. These results show the higher stability of all LEC_CHO-PDs, denoting the stabilizing effect of CHO covalently bound to PDMAEMA. For this reason, further experiments were carried out only with LEC-CHO-PDs.

The CF release from LEC and LEC_CHO-PDs at 37 °C and pH 7 is shown in Fig. 5. These results allowed to evaluate the stability of the LEC and PLCs along time and the following conclusions could be drawn with respect to the formulations: (a) the incorporation of 5% CHO-PDMAEMA has a strong stabilizing effect; (b) the
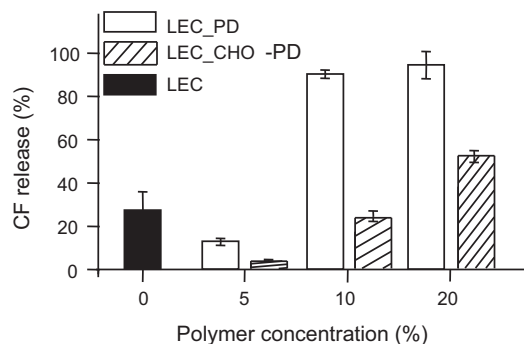
incorporation of 10% CHO-PDMAEMA has a neutral effect; (c) the incorporation of 20% of polymer has a strong destabilizing effect. According to these results, LEC_CHO-PD5 was selected to evaluate the effect of temperature and pH in the PLCs.

From Fig. 6A no significant differences were observed in the CF release from LEC at 37 and 45 °C. However, the CF release from LEC_CHO-PD5 at 45 °C increased from 4% to ca. 30% after ca. 120 min incubation, indicating the thermal destabilizing effect of CHO-PD5 on the PLCs.

The effect of pH as stimulus for destabilization of PLCs was evaluated for LEC_CHO-PD5 at 37 °C. Calcein release was determined at different pHs within the range 2.7–12.0. The highest stability of PLCs was observed at around pH 7.0 (Fig. 6B). In addition, a strong destabilization effect upon slight changes of pH was also evident. These results support the potential use of LEC_CHO-PDs as pH-sensitive systems.

The particle size and ζ-potential of LEC and LEC_CHO-PDs were evaluated at 37 °C and pH 7.0 (Table 2). For all the studied vesicles, the obtained PDI was lower than 0.5, indicating homogeneity in the population. In regard to their size, after the incorporation of CHO-PDMAEMA onto the liposomes, two noticeable changes were observed: PLC became about 50–60% smaller than the bare liposomes; and the negatively charged bare vesicles (−7.7 mV) became positive.

The analysis of the ζ-potential of these preparations along pH denoted an isoelectric point at pH ca. 7. Below this value, LEC_CHO-PD5 were positively charged and above, their charge became
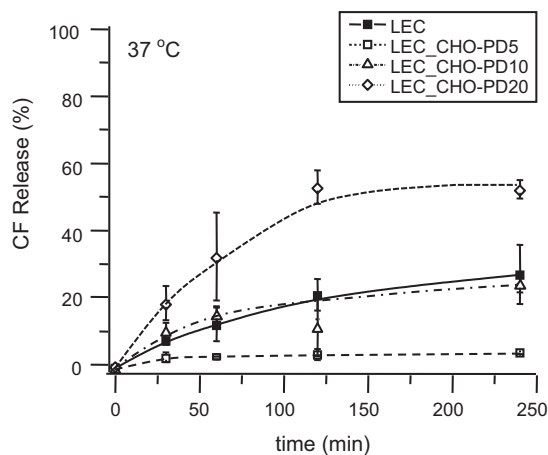

Fig. 4. CF release from PLCs prepared with different molar concentrations of PDMAEMA (white bars) and CHO-PDMAEMA (dashed bars) after 250 minutes incubation at 37 °C. LEC: black bars. Mean values ± SD of at least three independent experiments are shown.

Fig. 5. CF release from LEC (full squares), LEC_CHO-PD5 (opened squares), LEC_CHO-PD10 (opened triangles), LEC_CHO-PD20 (opened diamonds) PLCs prepared with different molar concentrations of CHO-PDMAEMA along time at 37 °C.
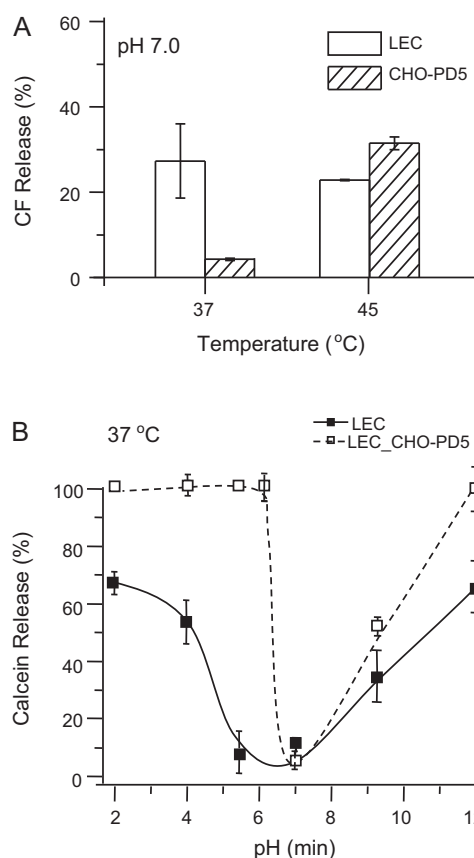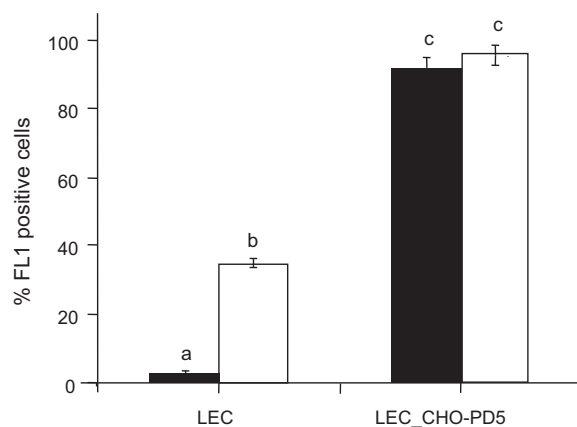
Fig. 7. Percentage of calcein positive cells after incubation of calcein loaded liposomes. Caco-2/TC7 cells (black bars), Raw 267.9 cells (white bars). Values were obtained by FACS. The lipid concentration in the incubation was of 87.5 M. Different letters indicate significant differences ($P < 0.05$).

The above results correlate with observations by fluorescence microscopy of cells incubated with calcein-loaded liposomes. Cells incubated with LEC exhibited weak fluorescence (Fig. 8A and C) whereas cells incubated with LEC_CHO-PD5 showed strong fluorescence (Fig. 8B and D)

MTT assays revealed that none of the LEC_CHO-PDs formulations was cytotoxic for the cell lines after 24 and 48 h, since all obtained results showed to have 100% viable cells.

4. Discussion

The development of systems for drug delivery requires a thorough investigation toward stability, toxicity, and internalization by eukaryotic cells to assure an adequate transport with the highest release of drugs in the target cells. In the last years, different strategies have been developed to the appropriate design of liposomes intended for drug delivery. Among them, stimuli-responsive systems prepared by the addition of some polymers in the liposome formulation appeared as a suitable strategy to guarantee stabilization during vesicles transport, and destabilization once arrived to the target cells.

For this purpose, the following goals were accomplished along this work: (a) synthesis of CHO-PDMAEMA by ATRP; (b) efficient incorporation of CHO-PDMAEMA on lecithin liposomes formulations with consequent stabilizing effect of the obtained PLCs at physiologic conditions and destabilization in response to environmental stimuli; (c) efficient uptake of the obtained PLCs by eukaryotic cells.

(a) To the best of our knowledge, the ATRP method was never used for the synthesis of CHO-PDMAEMA. The advantage over other methods, such as oxyanion-initiated polymerization [16,17] and reversible addition–fragmentation chain transfer (RAFT) polymerization [23], is mainly related with the mild conditions and simplicity of the polymerization. The stringent control of the polymer architecture, leading to Đ close to 1 and high percentage of conversion (Table 1), is another notable advantage of the LPR method. The resultant control of the homogeneity of the polymer in terms of both structure and length is of great importance because it permits a straightforward and more reliable interpretation of the biophysical studies carried out in this work.

The novel synthesized CHO-PDMAEMA shelled in the liposomal surface substantially reduced the potential dissociation of the polymer from the liposome, thus increasing its long-term
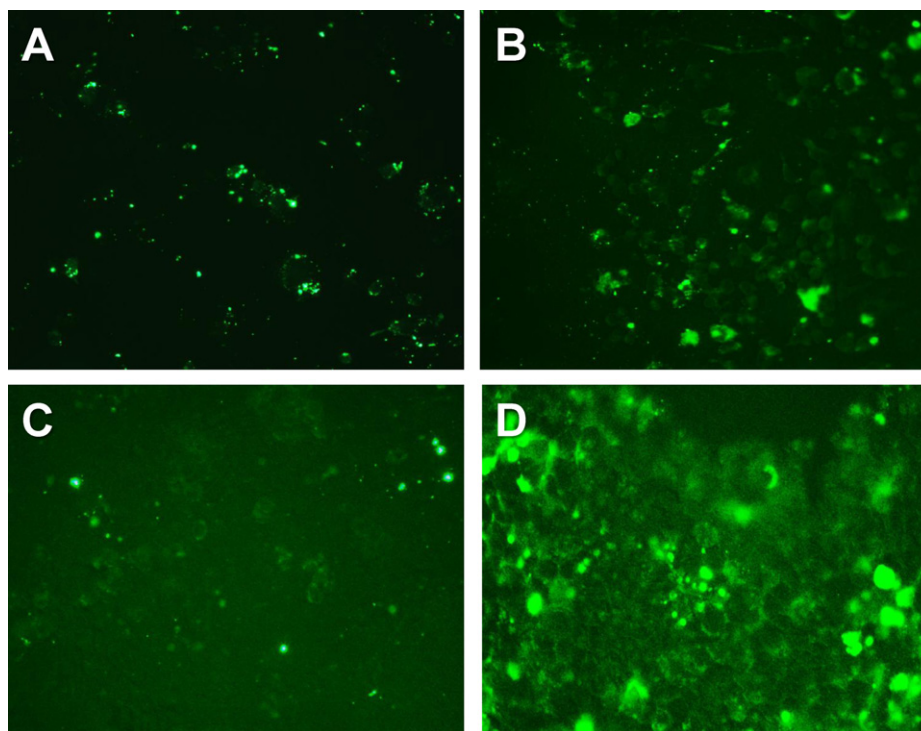
Fig. 6. (A) CF release from LEC (white bars) and LEC_CHO-PD5 (dashed bars) after 2 h at 37 and 45 °C. (B) Calcein release from LEC (full squares) and LEC_CHO-PD5 (opened squares) at different pHs after 2 h at 37 °C.

Table 2
Physical characterization of bare liposomes and PLCs at 37 °C and pH 7.0.

	Z-average[a] (nm)	Đ	ζ-potential (mV)
LEC	198.82 ± 1.75	0.485 ± 0.013	−7.74 ± 0.222
LEC_CHO-PD5	77.31 ± 1.29	0.487 ± 0.014	12.5 ± 2.310
LEC_CHO-PD10	80.17 ± 0.96	0.464 ± 0.015	13.3 ± 0.379
LEC_CHO-PD20	138.50 ± 0.97	0.447 ± 0.024	9.85 ± 0.528

[a] Z-average, also known as the cumulants mean. This mean parameter is defined as the "harmonic intensity averaged particle diameter".

negative. On the other hand, the isoelectric point of LEC was close to 6, since only LEC at pH 4 had positive charge (Table 3).

3.3. Liposome uptake by eukaryotic cells

The flow cytometry assays showed that the uptake of LEC_CHO-PD5 in both Raw 264.7 and Caco2/TC7 cells was significantly higher than that of LEC. Indeed, the percentage of FL1 (+) cells were 2.7 and 35.5 fold higher than controls for Raw 264.7 and Caco2/TC7 cells, respectively (Fig. 7).

Table 3
ζ-potential (mV) of LEC and LEC_CHO-PD5 at different pHs.

pH	LEC	LEC_CHO PD5
4	17.70 ± 0.231	7.23 ± 0.093
6	−2.94 ± 0.721	3.99 ± 0.966
7	−7.74 ± 0.222	12.5 ± 2.310
8	−3.18 ± 0.726	−5.25 ± 0.367
10	−23.7 ± 0.208	−8.26 ± 0.368

Fig. 8. Fluorescence micrographs of Raw 264.7 and Caco-2/TC7 cells incubated with liposomes and PLCs. Top panel represents Raw 264.7 cells, bottom panel represents Caco-2/TC7 cells. (A and C) Cells incubated with calcein LEC. (B and D) Cells incubated with calcein LEC_CHO-PD5.

stability (Fig. 4). As a whole, this kind of systems, also called polymer-incorporated liposomes, presents two advantages: (i) the presence of CHO stabilizes the lipid membrane; (ii) the CHO covalently linked to PDMAEMA precludes the excision of PDMAEMA from the formulations.

(b) Further analysis on LEC_CHO-PDs stability led us to conclude that, within the studied range of polymer/lipid ratios, the incorporation of 5% CHO-PDMAEMA in LEC led to the best PLC at 37 °C and pH 7.0 (Figs. 5 and 6B). At this pH, the obtained LEC_CHO-PD became positively charged (Tables 2 and 3). This can be explained considering that the cationic character of PDMAEMA induces a positively charged surface when incorporated in the liposome formulation. Moreover, it has been reported that a net positive charge of drug delivery systems may promote the association with negatively charged eukaryotic cell surfaces, as well as the destabilization of the plasma membrane or endosomal compartment promoted by the fusogenic properties of the lipids [24]. The exposure of positive charged liposomes to endosomal membranes is a prerequisite for electrostatic interaction between cationic nanoparticles and anionic lipids [25,26]. Therefore, the positive charged LEC_CHO-PDs appear as appropriate nanoparticles for drug release when exposed to negatively charged membranes, such as those from endosomes.

Fig. 5 depicts the stabilization effect of low concentrations (5%) of CHO-PDMAEMA on LEC formulation. Above this concentration, the excess of incorporated polymer might lead to a co-existence of saturated LEC_CHO-PDs with free CHO-PDMAEMA, which might result in a highly destabilizing factor (Fig. 7).

The complete destabilization observed at pH slightly below or above 7 (Fig. 6B) is derived from the change in PDMAEMA conformation under mild acidic or alkali conditions, strongly supporting the use of LEC_CHO-PDs as pH sensitive drug delivery systems [27]. From the pharmaceutical point of view, the significance of the PLCs' destabilization above pHs 7 is rather low, however it is also an interesting and expected result due to the weak polyelectrolyte character of the incorporated PDMAEMA. On the other hand, the increase of CF release at 45 °C indicates that the formulations investigated in this work also respond to temperature stimuli.

(c) The synthesis of CHO-PDMAEMA and physicochemical characterization of PLCs provided the basic requisites to support the biological assays performed in the last part of this work (Figs. 7 and 8), toward the evaluation of the toxicity of PLCs and their interaction with eukaryotic cells. The efficiency of LEC_CHO-PD5 to traffic into intracellular compartments is clearly noticed when compared with bare LEC liposomes (Figs. 7 and 8).

Destabilization of liposomes in mild acidic conditions (pH 5–6) such as those found in early endosomes represents an advantage for the delivery of labile macromolecules that may be degraded by the low pH and hydrolases or peptidases present in lysosomes [6]. Leakage experiments revealed an almost complete release of the fluorophore at pH 5.5 (Fig. 6B) suggesting a rapid delivery in eukaryotic cells after paghocytosis of the liposomes. It is known that PDMAEMA has cytotoxic effects on macrophages and epithelial cells [28] although LEC_CHO-PDs preparations showed no effects on the viability of Raw 264.7 and Caco-2/TC7 cells in the assayed conditions. The final concentration of CHO-PDMAEMA in liposomes was probably lower than 8 μg/mL, the minimal dose producing harmful effects.

The efficiency of CHO-PDMAEMA incorporated onto LEC demonstrated to be efficient in improving the stability at physiological conditions and at the same time in assisting the release of the liposome content in mildly acidic environments.

5. Conclusion

This work reports promising results regarding the development of stable liposomes with stimuli-responsive properties that can be loaded with therapeutic drugs. Three main characteristics were

afforded by the presence of CHO-PDs in liposomes: stabilization at neutral pH, increased uptake by phagocytic and non-phagocytic cells and release of content in mild acidic conditions. Our findings may lead to the possibility of targeting active compounds to specific intracellular compartments, thus improving biological effects.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2012.12.016.

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