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)

M.G. Simões [a,*], A. Hugo [b], P. Alves [a], P.F. Pérez [b,c], A. Gómez-Zavaglia [b], P.N. Simões [a]

[a] CIEPQPF, Department of Chemical Engineering, University of Coimbra, Polo II, Pinhal de Marrocos, P-3030-790 Coimbra, Portugal
[b] Center for Research and Development in Food Cryotechnology (CCT-Conicet La Plata, UNLP), RA-1900, Argentina
[c] Chair of Microbiology, Department of Biological Sciences, School of Exact Sciences, National University of La Plata, RA-1900, Argentina

ARTICLE INFO

Article history:
Received 7 November 2017
Received in revised form 3 January 2018
Accepted 15 January 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-

* Corresponding author.
E-mail address: monicasimoes@eq.uc.pt (M.G. Simões).

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.