Colloids and Surfaces B: Biointerfaces 157 (2017) 65–71

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Protocols

Influence of the environmental tonicity perturbations on the release of model compounds from large unilamellar vesicles (LUVs): A mechanistic investigation

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ARTICLE INFO

Article history:
Received 24 March 2017
Received in revised form 19 May 2017
Accepted 24 May 2017
Available online 26 May 2017

Keywords:
LUVs
Calcein
Rhodamine
Osmotic pressure
Tonicity
Release kinetics

ABSTRACT

In this work, the influence of environmental tonicity perturbations on the size and release kinetics of model markers from liposomes (calcein and rhodamine) was investigated. Large unilamellar vesicles (LUVs) were prepared from a mixture composed of organic solvents containing dissolved phosphatidylcholine and phosphate buffered saline (PBS, pH 7.4). Organic phase was removed by rotary evaporation and the obtained liposomal dispersions were extruded to reduce the liposomal sizes to approx. 400 nm. The LUVs were exposed to PBS of different tonicity to induce water migration, and consequently, generate an osmotic pressure on the vesicle membranes. The markers release kinetics were studied by the dialysis method employing Franz diffusion cells. LUVs appeared to be more susceptible to the osmotic swelling than the shrinking and the size changes were significantly more pronounced for calcein-loaded LUVs in comparison to rhodamine-loaded LUVs. The calcein release from LUVs was highly affected by the water influx/efflux, whereas rhodamine release was less affected by the tonicity perturbations. Mechanistically, it appeared that hydrophilic molecules (calcein) followed the water flux, whereas lipophilic molecules (rhodamine) seemed to be more affected by the changes in LUVs size and consequent alteration of the tightness of the phospholipid bilayer (where the lipophilic marker was imbedded in). These results demonstrate that the different tonicity (within the inner core and external environment of vesicles) can enhance/hamper the diffusion of a marker from LUVs and that osmotically active liposomes could be used as a novel controlled drug delivery system.

1. Introduction

Since their first description [1], several applications of liposomes have been proposed, among which their use as drug carrier remains the most studied [2,3]. Currently, liposomes account for a significant portion of the market share in nanomedicine [4].

Liposomes are spherical vesicles consisting of single or multiple phospholipid bilayers surrounding an aqueous core. Due to the amphiphilic nature of the phospholipids, liposomes have the ability to entrap both hydrophilic and lipophilic compounds [5]. The liposomal membranes are semipermeable, allowing neutral, poorly polarized and small molecules to diffuse through it, whereas ions, highly polarized small molecules and large molecules will not [6,7].

Liposomes are appealing for drug delivery because of their ability to solubilize poorly water-soluble compounds [8,9]. Chemical entities confined into liposomes are less subjected to early degradation and elimination, and an improved drug distribution compared to non-entrapped drugs can be achieved [10]. The shape and properties of liposomes are similar to human cells, which provide good compatibility and low toxicity [10].

As all systems composed by semipermeable membranes, liposomes shape and size can be subjected to osmosis. Osmotic pressure (π) is defined as the force that applies on the surface of a semipermeable membrane when water diffuses through it due to the different tonicity between the solutions on the two side of the membrane. In these conditions, water diffuses from the side with lowest solute concentration (higher chemical potential) to the regions of highest solute concentration (lower chemical potential) until the equilibrium is reached [11]. If liposomes are exposed to a hypertonic environment, water effluxes through the phospholipid bilayer to the external environment, generating a positive

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http://dx.doi.org/10.1016/j.colsurfb.2017.05.062
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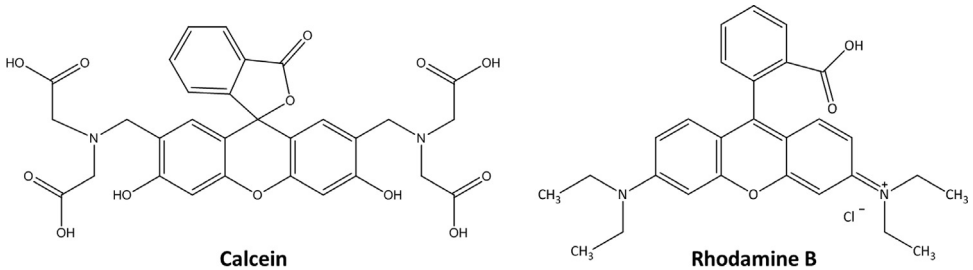

Fig. 1. Molecular structure, molecular weight (MW), partition coefficient (Log P) and dissociation constant (pKa) of calcein and rhodamine B.

osmotic pressure and induces size reduction [12]. On the contrary, when liposomes are exposed to a hypotonic environment, water influxes from the external environment, generating a negative osmotic pressure and size enlargement [12].

The influence of osmotic stress on lipid bilayers has been investigated on different vesicle models such as multilamellar vesicles [6,13] and large unilamellar vesicles (LUVs) [14–16]. Unilamellar vesicles are reported to be an appropriate model system to study the permeability properties associated with osmotic swelling or shrinking [6,13,15]. However, to the best of our knowledge, the influence that the environmental tonicity perturbations have on the release of chemical entities from liposomes has not been clarified yet.

The aim of this study was to verify if, and to which extent, the environmental tonicity perturbations affect the release kinetic of model substances from LUVs.

For this reason, two chemically different fluorescent markers (calcein and rhodamine) were used to study their release kinetics form LUVs. Fluorescent markers of different solubility were chosen to simplify the mechanistic study [17–20]. Calcein is a hydrophilic marker that can be entrapped in the inner aqueous core of LUVs [19], whereas rhodamine is a lipophilic marker (Log P of 2 [21]) that is expected to be incorporated in the phospholipid bilayer of LUVs.

2. Materials and methods

2.1. Materials

Calcein and rhodamine B (Fig. 1 reports the chemical structure of both markers together with meaningful physicochemical characteristics [19,21–23]), disodium hydrogen phosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$), sodium chloride (NaCl), sodium hydroxide (NaOH), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4{\cdot}H_2O$), chloroform, ethanol (96%, v/v), methanol and non-ionic surfactant Triton X-100 were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Soy phosphatidylcholine S100 (SPC) was a gift from Lipoid GmbH (Ludwigshafen, Germany).

2.2. Phosphate buffer saline (PBS) preparation

A 74 mM phosphate buffer saline (PBS) solution (buffer 300) was prepared dissolving 22.5 g $NaH_2PO_4{\cdot}H_2O$ and 36.8 g $Na_2HPO_4{\cdot}2H_2O$ in 5 L distilled water. The pH was adjusted to 7.4 with NaOH, whereas NaCl was employed to fix tonicity at 300 mOsm (Semi-Micro Osmometer K-7400, Knauer, Berlin, Germany). Buffer 300 was diluted with distilled water in order to obtain two other solutions (buffer 190 and 65 respectively, Table 1) with lower tonicity.

Table 1
Measured tonicity, pH (mean ± SD, n = 3) and calculated concentrations of the different types of PBS used.

PBS type	Tonicity (mOsm)	pH	Concentration (mM)
300	300 ± 4	7.40 ± 0.02	74
190	187 ± 3	7.46 ± 0.02	44
65	65 ± 1	7.56 ± 0.01	15

2.3. Liposomes preparation

LUV dispersions were prepared following a method previously described [24,25] with some modifications. In brief, 0.2 mL methanol was mixed with 1 mL SPC/chloroform solution (200 mg/mL) in a 50 mL round bottom flask. PBS (10.5 mL) was gently added, and the organic phase was subsequently removed by rotary evaporation (40 °C, 40 rpm and 0.1 bar for 90 min) (Büchi R-124 rotavapor equipped with Büchi vacuum pump V-500 and Büchi B-480 water bath, Büchi Labortechnik AG, Flawil, Switzerland). All liposomal dispersions were prepared employing buffer 300 (74 mM, Table 1) or, alternatively, buffer 65 (15 mM, Table 1). LUV dispersions were spontaneously formed after the removal of organic phase. In order to reduce liposomal sizes, the dispersions were extruded at room temperature (23–25 °C) through polycarbonate membrane filters (lowest pore-size of 400 nm, Nuclepore Track-Etched Membranes, Whatman International Ltd., Buckinghamshire, UK). To load the fluorescent markers in liposomes, calcein was dissolved in PBS (obtaining a calcein solution with a concentration of 2 mM) prior to the lipid film reconstitution. Differently, for the preparation of rhodamine-LUVs, the marker was dissolved in chloroform together with lipid (rhodamine-lipid molar ratio approx. 1:1).

2.4. Size and surface potential analysis

Photon correlation spectroscopy (PCS) (Nicomp Submicron Particle Sizer 370, PSS Nicomp Particle Sizing Systems, California, USA) was employed for the determination of LUV size distributions after the extrusion. Samples were diluted to a count rate of 250–350 kHz at room temperature (23–25 °C), and each sample was measured in three parallels. Size changes of LUVs induced by tonicity perturbations were measured on the Zetasizer Nano Zen 2600 (Malvern, Worcestershire, UK). Samples were diluted 1/100 (v/v) with PBS 300 (or alternatively PBS 65) previous analysis and LUV dispersions were filtered to remove eventual impurities. Experiments were performed over a period of 70 min at 25 °C in duplicates (n = 2), and each sample was measured in three parallels. The ζ-potential (ZP) of LUVs was measured on the Zetasizer Nano Zen 2600 (Malvern). The respective dispersions were diluted 1/20 (v/v) with filtrated