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Application of the Vulnerability Estimator for Spring Protection Areas (VESPA index) in mountain quaternary aquifers / Banzato, C., Vigna, B., Galleani, L., LO RUSSO, S.. - ELETTRONICO. - (2011). (GeoHydro2011 - CANQUA-IAH Quebec City (CA) 28-31 August 2011).

Availability:
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14 September 2026

Eurodrying'2019
Torino, Italy, July, 10-12 2019

FREEZE-THAWING OF LIPOSOMES: INVESTIGATION OF CRYO-PROTECTORS FOR FREEZE-DRYING

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Abstract
Liposomes are phospholipid membranes widely used for drug delivery in biomedical applications. They can retain, protect and deliver macromolecules to target tissues and then control the release of their cargoes. To exploit this function, the liposomal membrane integrity is crucial to avoid undesired leakage of cargoes. In this work, the influence of low temperature storing conditions on the stability of liposomes is investigated for further delivery applications.

Keywords*: Liposomes, freeze-thawing, freeze-drying*

1. Introduction

After the first description of liposomes more than fifty years ago (Bangham *et al.* 1964), in the last decades, they started acquiring a striking success for their drug delivery ability of both lipophilic and hydrophilic molecules (Sercombe *et al.* 2015), and their biocompatibility (Bozzuto *et al.* 2015). Both natural and synthetic phospholipids can be combined to tune the physical and chemical properties, i.e. dimensions, charge and surface properties (Malam *et al.* 2009), and the liposomal surface can be also functionalized with different molecules (Torchilin 2005, Allen *et al.* 2013). Liposomes can be used for the treatment of a wide range of pathologies and many of them have been already approved by the Food and Drug Administration (FDA), for example: Doxil, DepoCyt, Vyexos and Abraxane (Anselmo *et al.* 2016). Thus, it is crucial to employ a storage method that does not affect the liposomes integrity, preventing the leakage of their cargo. Lyophilization is one of this preservation methods, but attention must be paid to the design of the formulation to make it sufficiently robust to freezing and drying stresses. In this work, the role of various cryo-protectors is investigated with respect to their ability to preserve the morpho-functional parameters of liposomes. Attention was focused on the first step of the lyophilization process, i.e. freezing. More specifically, two freezing methodologies were investigated, quench in liquid nitrogen and shelf-ramped freezing.

2. Material and method

The lipids used in this work, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), alone or in combination with 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG), were all purchased from Avanti Polar Lipids.
Four different formulations of liposomes were analysed: 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), DOPC-DOTAP (70:30 %w/w), DOPC-DSPE-PEG (95:5 %w/w) and DOPC-DOTAP-DSPE-PEG (65:30:5 %w/w), all labelled with Bodipy-FL-DHPE. All the formulations were prepared using the protocol reported by (Dumontel *et al.* 2017) and rehydrated in aqueous solution, i.e. water and physiologic solution (0,9% w NaCl) (50:50 %v/v). To obtain small and unilamellar vesicles, the liposomal formulations were bath sonicated at 40 kHz for 20 minutes and then extruded with 0.2 μm syringe filters. Dimension and surface charge of liposomes were characterized with Dynamic Light Scattering (DLS) and Z-Potential measurements, while their shape evaluated through wide-field fluorescence microscopy images by labelling them with 1% w/w of Bodipy-FL® (Thermofischer Scientific, excitation wavelength at 488 nm). Further experiments of freezing and thawing were carried out only on DOPC-DOTAP and DOPC-DOTAP-DSPE-PEG because of their strong positive Z- potential.

The first experiment aimed to compare the effect of slow or fast freezing and thawing rates on liposomes' integrity. In the slow freezing-slow thawing (SFST) experiment, samples had undergone seven cycles of freezing and thawing, with a cooling/heating rate of 1°C/min between 10°C and -40 °C. In the fast freezing-fast thawing (FFFT) experiment, the samples were dipped in liquid nitrogen for 5 minutes and then thawed at room temperature for 20 minutes for seven times. The same experiments were repeated employing different cryoprotectants: six different excipients (5%w/w), such as cellobiose, glucose, lactose, mannitol, sucrose and trehalose and a surfactant (0,01%w/w), Tween80, added to the aqueous solution before the rehydration. All the previously described samples were analysed with DLS, Z-potential and fluorescence microscopy.

3. Results and discussion

The average size of the liposomes was about 100 nm; however, DOPC and DOPC-DSPE-PEG showed an almost neutral Z-Potential, 0.68 and -0.87 mV respectively, while DOPC-DOTAP and DOPC-DOTAP-DSPE-PEG a strong positive charge, 31.9 and 12.9 mV respectively. Since the Z-Potential is related to the surface charge, a high Z-Potential is preferred in liposomal solutions to avoid the coalescence of vesicles. Therefore, further experiments were carried out only on DOPC-DOTAP and DOPC-DOTAP-DSPE-PEG suspensions.
The comparison of SFST and FFFT of liposomes showed similar behaviour, both SFST and FFFT damaged the lipid membranes by creating small fragments or by fusing in bigger vesicles.
These experiments were repeated by adding six excipients, Tween80 or a combination of each excipient and the surfactant to the solutions. The DLS results of SFST for DOPC-DOTAP-DSPE-PEG showed that the addition of excipients provided a protective effect on the lipidic membranes in almost all the formulations, except in the case of mannitol. On the contrary, the addition of Tween80 did not prevent the breakage of liposomes' membranes, indeed it probably interferes in the formation of new aggregates, so the solution contains mainly fragments of phospholipids.

4. Conclusions

Liposomes are widely used for the delivery of drugs and macromolecules thanks to their tunable encapsulation ability. Therefore, their preservation over time without damages and drugs leakage is essential. High standard low temperature conservation of the constructs is suitable to protect the encapsulated compound activity, avoiding affecting liposomes morphology and physiology. This storage condition can be associated with some sugar-based excipients that successfully protect the liposomal membrane from breakage. The present work is a starting point for further studies on the stability of synthetic or natural-produced phospholipid vesicles, loaded or not with macromolecules, and on their preservation by freeze-drying.

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