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Incorporation of peptides in phospholipid aggregates using ultrasound

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ABSTRACT

This study presents the highlights of ultrasonic effects on peptides incorporated on phospholipid aggregates (liposomes). These liposomes or vesicles are known as transport agents in skin drug delivery and for hair treatment. They might be a good model to deliver larger peptides into hair to restore fibre strength after hair coloration, modelling, permanent wave and/or straightening. The preparation of liposomes 1,2-dipalmitoyl-*sn*-glycerol-3-phosphocholine (DPPC) with peptides (LLLLK LLLLK LLLLK LLLLK; LLLLL LCLCL LLKAK AK) was made by the thin film hydration method. The LUVs (uni-lamellar vesicles) were obtained by sonication, applying different experimental conditions, such as depth (mm) and power intensity (%). Photon-correlation spectroscopy (PCS) and electronic microscopy (EM) results confirmed that the incorporation of these peptides, with different sequence of amino acids, presented differences on the diameter, zeta-potential of membrane surface and shape of liposomes. The liposomes that included peptide LLLLK LLLLK LLLLK LLLLK present an increased in zeta-potential values after using ultrasound and an "amorphous" aspect. Conversely, the liposomes that incorporated the peptide LLLLL LCLCL LLKAK AK presented a define shape (rod shape) and the potential surface of liposome did not change significantly by the use of ultrasound.

1. Introduction

Liposomes or vesicles have been the subject of numerous studies, because of their importance as models for more complex biological membranes. Their potential use as microencapsulators for drug delivery and their applications in cosmetics was also studied [1–7].

Several types of liposomes can be distinguished, depending on the preparation method, such as multi-, oligo- and uni-lamellar vesicles, containing several, few or only one bilayer shell, respectively [1,8–10]. Nevertheless, all liposomes are basically lipid bilayer containers in which several substances could be entrapped or at least anchored into their structure [8].

Due to their unique physical and chemical properties, such as their stability in solution for long periods of time with no significant changes in size or structure [2], and their ability to incorporate lipophilic, amphiphilic and/or hydrophilic compounds [3], liposomes can be used in a wide range of applications.

MLVs (multi-lamellar vesicles) have high encapsulation efficiency, but also great variations in the vesicle size, size distribution and lamellarity. The use of mechanical treatments such as: repetitive freezing/thawing cycles, extrusion through polycarbonate membranes, dehydration/rehydration cycles, microfluidation or sonication can transform the MLVs suspension into LUVs (large uni-lamellar vesicles) and SUVs (small uni-lamellar vesicles). In addition to a stand alone treatment, ultrasound can be applied to the other methods to increase efficiency in the formation of hydrated lipid vesicles of the smallest size.

Ultrasound has recently been applied to obtain stable nano-suspensions which have emerged as a promising strategy for an efficiency delivery of hydrophobic drugs, because of their versatile features such as very small particle size. The ability to produce the nano-particles of desired size with great precision (narrow size distribution and small variation) is the key factor of producing the nano-suspensions [11,12]. The extreme conditions generated within the collapsing cavitational bubbles have been used for the size reduction of the material to the nano-scale. Nano-particle synthesis techniques include sonochemical processing, cavitation processing, and high-energy ball milling. In sonochemistry, an acoustic cavitation process can generate a localized hot zone with an extremely high temperature gradient and pressure. Such sudden changes in the temperature and pressure assist the destruction of the sonochemical precursor and the formation of nano-particles [12].

Use of the cavitation for the formation of nano-particles has been reported by Suslick et al. [13] and Gedanken [14]. Symmetric collapse of a cavitation bubble results in hot spots of nearly 5000 K within the bulk solution [15] and high velocity shock waves travelling through the solution. Collapse in proximity to surfaces can result in deformation of the bubble, manifesting as asymmetric collapse. This behaviour causes micro-streaming and high velocity

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shock waves within the bulk solution in the direction of the surface [16].

Myriad applications of ultrasound in medicine and industry have been developed; however, the details of ultrasound-induced damage to biomolecules, especially proteins, remain poorly characterized [17]. Such characterization is difficult, owing to the potentially complex mechanisms of sonication. These may include the formation of liquid–gas interfaces, local heating effects, sheer and tensile stresses and reactions occurring with generated free radicals [18,19].

Loomis and Wood first reported the damaging effects of ultrasound radiation on biological systems in 1927 [20]. Many applications of ultrasound in common use today may alter protein structures [19]. For example, sonication is used to prepare proteinaceous microspheres of human serum albumin [21]. These are widely used as ultrasound contrast agents, and are being investigated as possible gene transfer vehicles [22]. Sonication is also employed in procedures to encapsulate therapeutic proteins such as asparaginase, insulin, and erythropoietin biodegradable poly (D,L-lactide-co-glycolide) microspheres for controlled release *in vivo* [23–25]. Although the use of ultrasound in the formation of liposomes is not new, a detailed description of the specific methodology is still lacking.

This study describes the sonication conditions to obtain systems of lipid vesicles with low polydispersity and lamellarity and also how these ultrasound conditions can interfere with the physical and chemical properties of liposomes when two different synthesized peptides were incorporated.

First, the liposomes, were characterized without peptides, using different conditions of ultrasound and then the influence of different peptides in the lipid bilayer was studied.

In this work we investigated the size changes as well as the polydispersity and the zeta-potential using the photon-correlation spectroscopy. The morphology, as a function of the ultrasound power and the different depths (from the base of the vessel), were examined using scanning electron microscopy (SEM).

2. Materials and methods

2.1. Materials

2.1.1. Reagents

The 1,2-dipalmitoyl-*sn*-glycerol-3-phosphocholine (DPPC) was purchased from Sigma Chemicals and used as supplied. Two synthesized peptides with approximately 20 amino acids were: C-term: LLLLL LCLCL LLKAK AK and C-term: LLLLK LLLLK LLLLK LLLLK, where L, C, K and A is the one-letter code for the amino acids leucine, cysteine, lysine and alanine. The peptides were covalently linked by the N-terminal to a fluorescent dye, (5(6)-carboxytetramethyl-rhodamine, succinimidyl ester), i.e., 5(6)-TAMRA, with spectral properties of Abs_{max} = 544 nm and Em_{max} = 572 nm, to facilitate the analysis of peptide penetration. The peptides structures were synthesized by JPT Peptide Technologies GmbH (Berlin, Germany). The peptides were supplied as a lyophilized material. They were analysed by HPLC and MS, and their purity was over 70% (HPLC, 220 nm, C18, linear gradient). All the other chemicals were from Sigma–Aldrich with pro analysis grade.

2.2. Methods

2.2.1. Liposomes preparation and peptides incorporation

Liposomes were prepared by the film hydration method as detailed by Ferreira et al. [26]. According to this method, a known amount of DPPC was dissolved in chloroform. The organic solvent was evaporated under a nitrogen stream with residual traces of solvent removed by a further evaporation for a minimum of 3 h. The resulting dried lipid film was dispersed by the addition of phosphate buffer (0.1 M) at pH 7.4. These mixtures were then vortexed above their phase transition temperature (41.4 °C) to produce MLVs which were then sonicated at 54 °C to produce LUVs. Preparation of liposomes with peptides followed a similar procedure with the lipid solution in chloroform and the peptides dis-

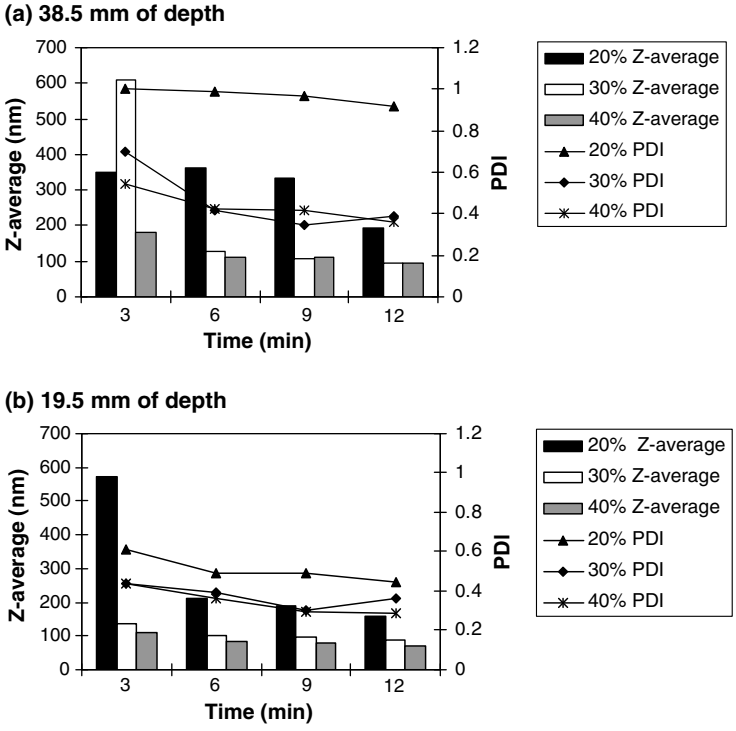

Fig. 1. Effect of sonication on liposomes (1500×10^{-6} M, pH 7.4) using different depths: (a) 38.5 mm, and (b) 19.5 mm), applying different amplitudes (20%, 30%, and 40%) after 3, 6, 9, and 12 min of sonication at 54 °C.