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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-

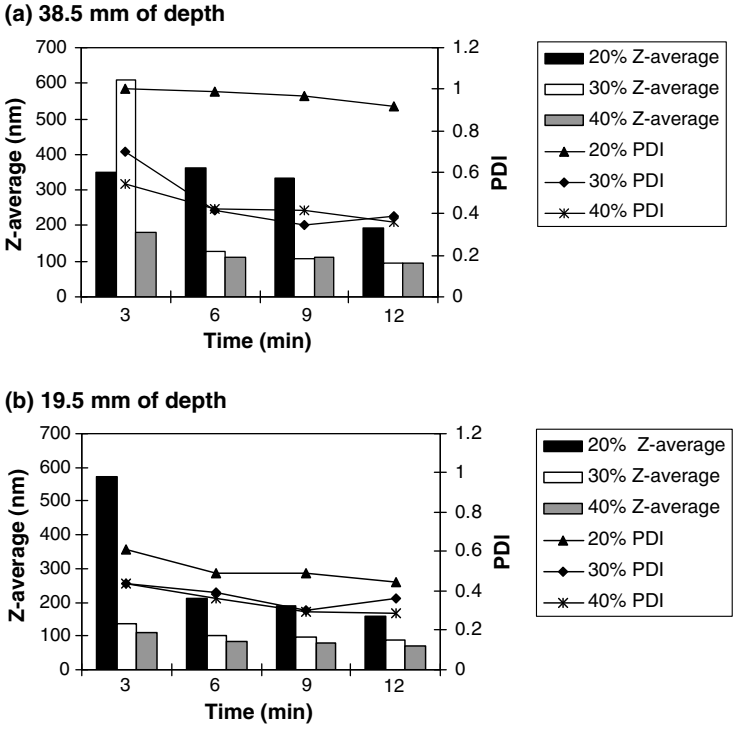

Fig. 1. Effect of sonication on liposomes (1500×10^{-6} M, pH 7.4) using different depths: (a) 38.5 mm, and (b) 19.5 mm), applying different amplitudes (20%, 30%, and 40%) after 3, 6, 9, and 12 min of sonication at 54 °C.

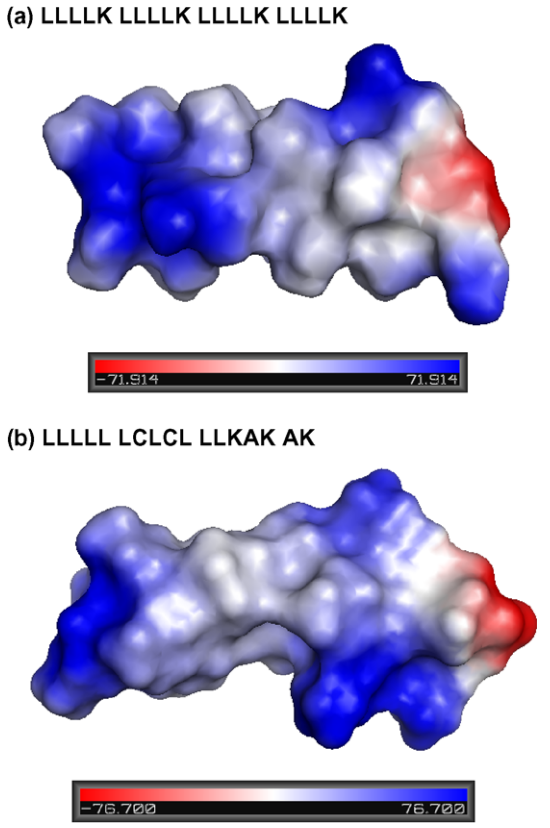

Fig. 2. Surface charge analysis for the C-term and N-term peptides (a: LLLLK LLLLK LLLLK LLLLK, b: LLLLL LCLCL LLKAK AK), attained by PyMol v0.99. Red denotes the negatively charged C-terminus while blue denotes the positively charged side chains. The scale represents the charge potential. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

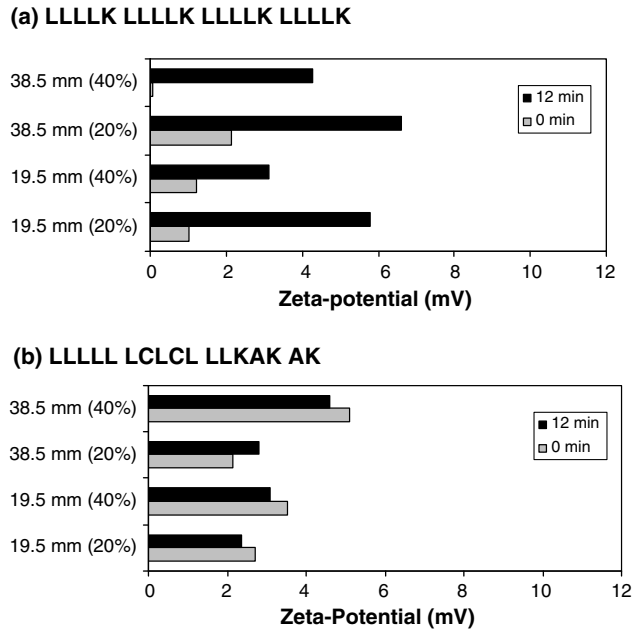

Fig. 4. Zeta-potential (mV) values for peptides (5×10^{-6} M; a: LLLLK LLLLK LLLLK LLLLK; b: LLLLL LCLCL LLKAK AK) entrapped on liposome (1500×10^{-6} M, pH 7.4) using different depths (19.5 and 38.5 mm) and amplitudes (20% and 40%) after 0, 3, 6, 9 and 12 min of sonication at 54 °C.

solved in pure ethanol and dried together under nitrogen. All solutions were thermo-stated at 54 °C.

The experimental set up used was composed of a probe type ultrasound source (20 kHz Sonics & Materials Vibracell CV 33) fitted with a 3 mm diameter titanium micro-tip. Power delivery was controlled as percentage amplitude. The reaction vessel was an open glass cell (diameter 19 mm and height 75 mm), which con-

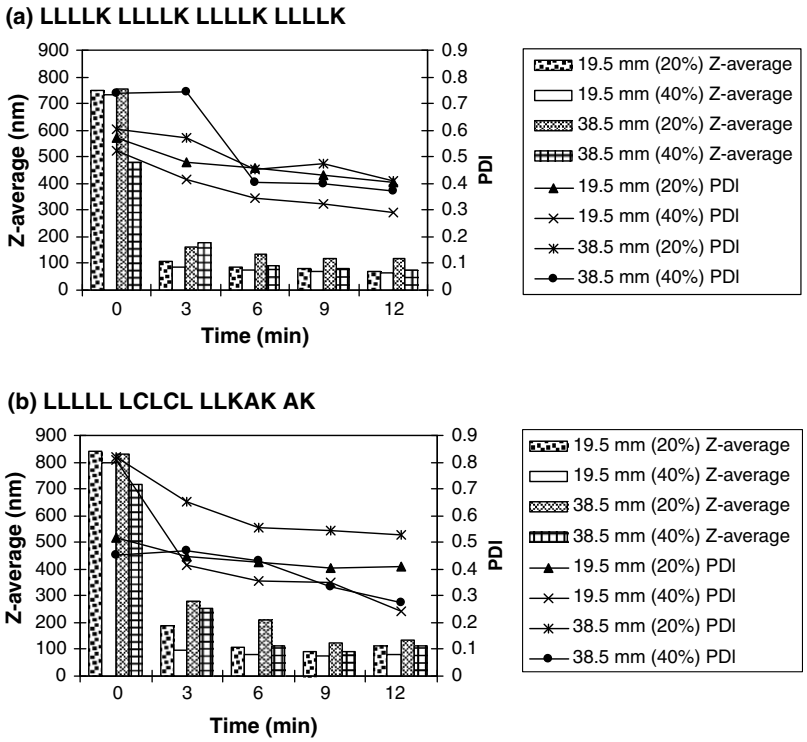

Fig. 3. Z-average (nm) and PDI values for peptides (5×10^{-6} M; a: LLLLK LLLLK LLLLK LLLLK, b: LLLLL LCLCL LLKAK AK) inserted on liposome (1500×10^{-6} M, pH 7.4) using different depths (19.5 and 38.5 mm) and amplitudes (20% and 40%) after 0, 3, 6, 9 and 12 min of sonication at 54 °C.

tained 16 mL of sample solution. The sonochemical reactor temperature was controlled via a thermo-stated water bath with a heat exchanger placed within a thermo jacket cell; this gave a steady operating temperature of 54 °C (±1 °C). Sonication was carried out with a total treatment of 12 min monitored in 3 min increments. A pulsed duty cycle of 8 s on, 2 s off was used for all the experiments.

2.2.2. Photon-correlation spectroscopy (PCS) and zeta-potential determinations

The zeta-potential, the size distribution and the polydispersity of liposomes, with and without incorporated peptides, were determined at pH 7.4 (phosphate buffer) and 50.0 °C via dynamic light scattering (DLS) analysis (Malvern zetasizer NS).

2.2.3. Electronic microscopy (EM) determinations

The morphology of the liposomes with peptides was determined using scanning electron microscopy (LEICA S360). The sample preparation included the drying and covering with a gold layer.

2.2.4. Peptide surface charge analysis

The peptide surface charge analysis was obtained by using the PyMOL v0.99 [27].

3. Results and discussion

Prior to testing of the liposome behaviour to the sonication, the reactor was categorized via an adaptation of the previously published method [28]. This identified the minima and maxima hydroxyl radical activity points as occurring at 38.5 mm (nodal-point $\lambda/2$) and 19.5 mm (anti-nodal point $\lambda/4$) positions, respectively. Subsequently, ultrasonic treatment of the liposomes with and without peptides was carried out at amplitudes of 20%, 30%, and 40% and using the depths, measured from the base of the vessel, of 38.5 mm and 19.5 mm. First, liposome dispersions without peptides were sonicated at the specified powers and depths. An analysis of size distributions of liposomes before applying ultrasound (MLVs) was performed and it was observed that these MLVs presented a large size (≈2400 nm) with a higher polydispersity (≈0.910). This result was attributed to the polydispersity of MLVs population presented in the sample. Fig. 1 shows the size (nm) and the polydispersity (PDI) that were obtained after sonication. Initial data from the zeta sizer gave a high Z-average with a high PDI during the first minutes. After the treatment with ultrasound there was a decrease in the PDI with a rapid drop in the Z-average. Physical size and PDI decreased with higher power intensity (40%), since with this power it is possible to achieve more rapid mixing of solution. The highest value of PDI obtained for the lowest sonication amplitude (20%), is associated with a wide distribution of lip-

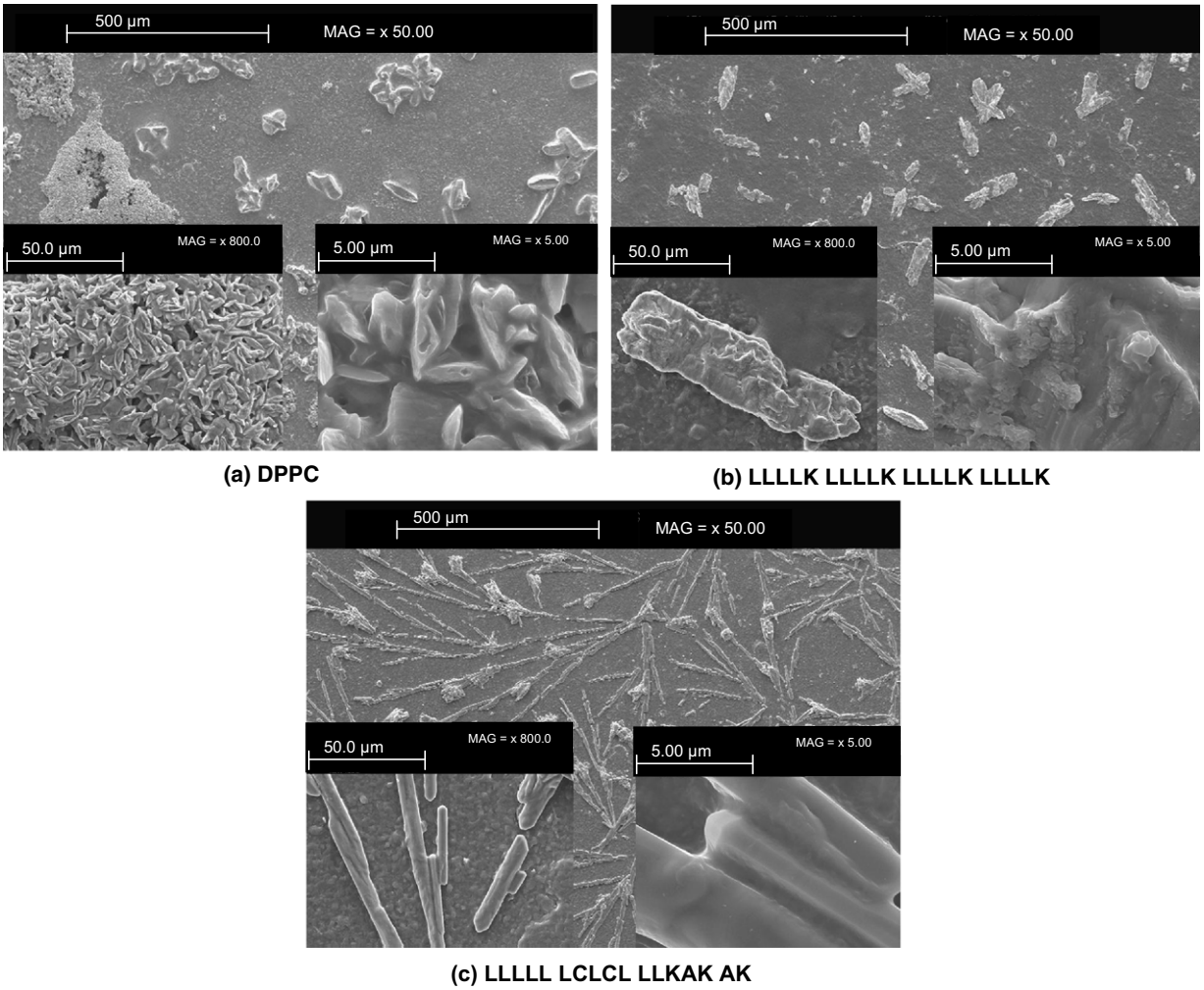

Fig. 5. SEM microphotographs after 12 min of sonication at 19.5 mm of depth and 40% of amplitude (the samples were dried and filtered after ultrasound treatment), using different magnifications (×50; ×800; ×5000).

osomes size. This fact seems to indicate that this amplitude is not enough to decrease homogeneously the size of particles. A drop in the physical size and PDI at 19.5 mm was observed. The production of ·OH radicals is notably higher since at this depth the cavitation phenomenon is more pronounced (anti-nodal point) and the greater extent of streaming from the ultrasound source promotes a decrease in the size of liposomes.

The determinations of liposomes zeta-potential (mV) were made before and after sonication. After the measurements, it was verified that the potential surface of liposomes did not change significantly by the use of ultrasound (≈4 and ≈3 mV, before and after ultrasound, respectively).

Hydrolysis of phospholipids is promoted presumably by free radicals in the cavitation bubbles during the preparation of the sonicated phospholipids suspension in water. Thus, during the sonication procedure, temperature should be controlled otherwise oxidation and hydrolysis reactions are favoured [29,30]. However, it is difficult to guarantee a minimum lipid oxidation level by control of temperature, due to ultrasonic irradiation of water develops hydroxyl free radicals and hydrogen peroxide, which also contributes for lipid oxidation [29]. In order to minimise the oxidation, sonication was carried out in time intervals of 3 min. According to Kruus et al. [31], the formation of free radicals is not a major problem when low frequencies (≈20 kHz) and short sonication times (≈20 min) are used. High temperatures accelerate phosphocholine hydrolysis; however according to Rabinovich-Guilatt et al. [32] a temperature of 50 °C over 24 h induces only 1.6% of phosphocholine hydrolysis. In spite of this, the temperature was controlled during all the experiment using a thermo-stated bath. Therefore, in our working conditions this hydrolysis should be negligible. This is very important as the principle goal is the entrapment of peptides in liposomes and the protection of these peptides against the free radicals produced by sonication.

After the characterization of liposomes, the incorporation of two different peptides on the LUVs was studied. The peptides were formulated together with a lipid, DPPC, which was added to attain a peptide formulation compatible with a water environment. Due to the large size of the Leucine side chain, the synthesized peptides tend to acquire an alpha helix structure in water. The interaction with phospholipids could further stabilize the alpha helix structure [33,34]. However, the exact structure of these peptides in water is yet to be confirmed.

The peptide sequences were visualized by a molecular modelling program to identify the major differences in there structure (Fig. 2). The molecular modelling program allows for creating the structure based on the amino acids sequence, which differs in the position of the charged group (K) in the sequence of peptides. Fig. 2 shows the structures of C-term and N-term peptides in vacuum. Besides illustrating the amphiphatic nature of the helix, it also shows a much narrower spatial distribution of the positively

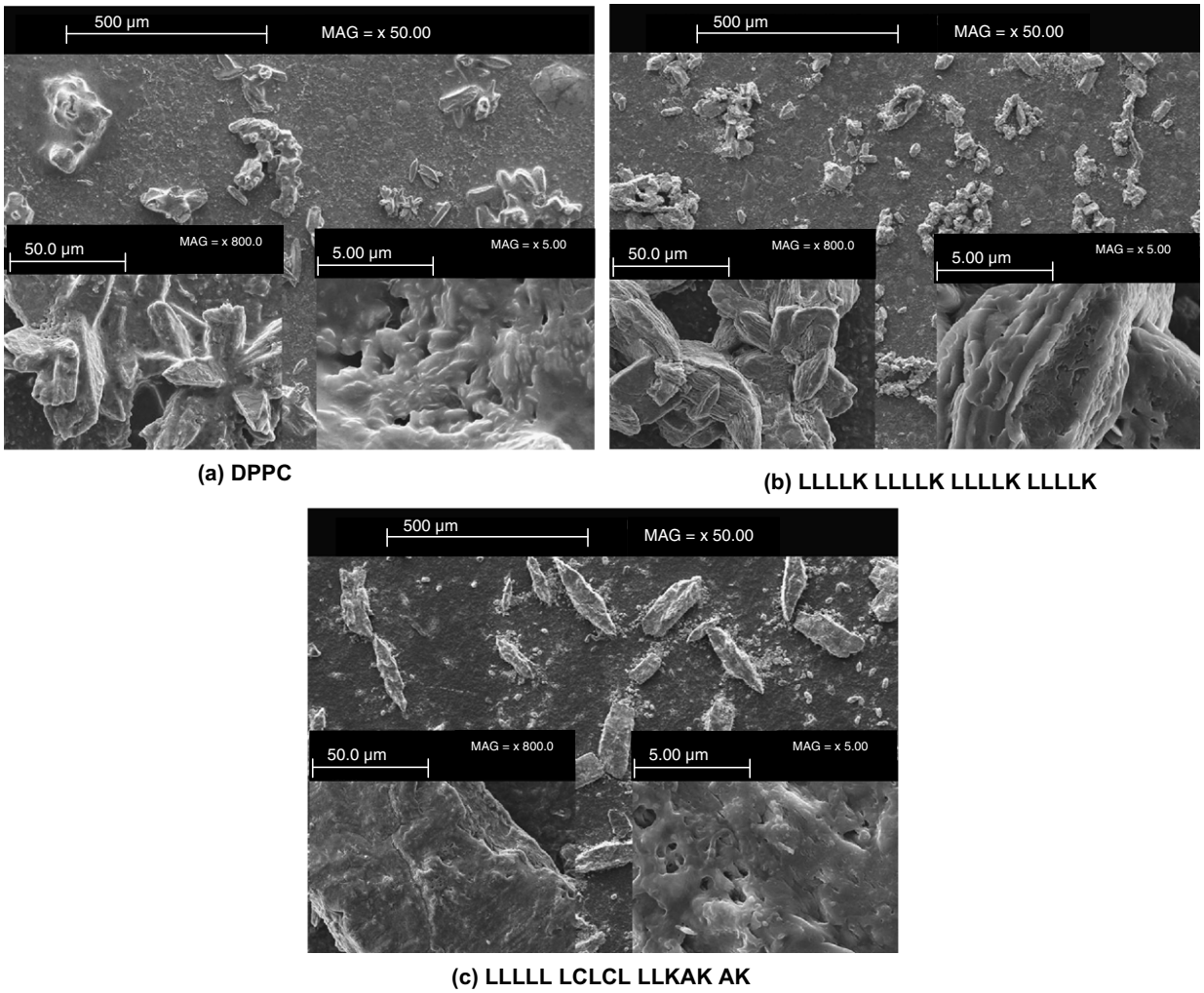

Fig. 6. SEM microphotographs after 12 min of sonication at 38.5 mm of depth and 40% of amplitude (the sample were dried and filtered after ultrasound treatment), using different magnifications (×50; ×800; ×5000).

charged side chains in the peptides. These peptides tend to be therefore both amphipathic and cationic. Amphipathicity increases their affinity for biological membranes, while the positive charge increases their specificity toward negatively charged membranes [33,34].

It was previously described that different depths and amplitudes have different effects on liposomes. For this reason the extent of this influence was investigated when the peptides were incorporated on liposomes. The peptides inserted on liposomes were sonicated at the minimum amplitude (20%) and the maximum amplitude (40%). Fig. 3 shows the influence of power at 19.5 mm and 38.5 mm positions, on the LUVs size (nm) and PDl. Lower sizes were obtained with 19.5 mm of depth, most likely due to the higher production of hydroxyl radicals that are related with higher effects of the cavitation phenomenon. The use of the higher amplitude (40%) promoted size decreases in both peptide formulations. The higher power is associated with greater mixing; promoting a more homogeneous solution and it is possible to obtain particles with lower size and with lower polydispersity.

Fig. 4 shows the influence of sonication on membrane surface of liposomes with the peptides. The lysine amino acids (indicated in bold) of the peptide LLLL**K** LLLL**K** LLLL**K** LLLL**K** are positively charged, and hence they can stay on the polar surface of liposomes resulting in an increased positive charge and consequently an increase in the zeta-potential values. Conversely, for the other peptide (LLLLL LCLCL LL**K**A**K** A**K**), the values of zeta-potential do not change significantly, here the lysine amino acids are at the extremity of the fragment, allowing the positioning of the other amino acids (cysteine, alanine and leucine) to the inside of the liposome as they are more hydrophobic. The zeta-potential values for the maximum of amplitude (40%) were very similar to the results with minimum of amplitude (20%).

Samples of each peptide were analysed by scanning electronic microscopy (SEM), after treatment with ultrasound at 19.5 and 38.5 mm of depth and 40% amplitude. The photographs were taken at different magnifications of ×50, ×800, and ×5000. Fig. 5 shows that peptide LLLLK LLLLK LLLLK LLLLK has an "amorphous" aspect, possibly due to the positively charged lysine amino acid (K) distributed in the sequence of peptide. The peptide with the amino acid cysteine (LLLLL LCLCL LLKAK AK) and with the positive charge on the C-terminal presented a defined rod shape. This suggests that the peptide can have their hydrophilic part (positive charge) oriented towards the polar part of the lipid bilayers while their hydrophobic segment (not charged) is in the upper part and between lipophilic tails.

Fig. 6 shows differences between these two peptides that were analyzed, but these differences are not so pronounced when the 38.5 mm of depth was used. This fact can be related with the effect of cavitation that is more prominent at 19.5 mm due to an increased production of hydroxyl radicals (anti-nodal point).

4. Conclusions

This study shows the three principal factors of ultrasound that could influence these ranges of sizes and zeta-potential: depth, amplitude and duration of treatment. The size and PDl decreased with an increase of amplitude (40 %) as the higher power exerts greater shear forces within the solution. The greater extent of streaming from the ultrasound source promotes greater mixing of the solution and consequently more homogeneity. At 19.5 mm a drop in the physical size and PDl was observed. At this depth the rate of radical production is higher, further promoting the decrease in liposomes size. The sonication can promote the entrapment of these two peptides, with different sequences of amino acids, on liposomes. At 19.5 mm of depth it was observed a decreased in size of liposomes that incorporated the peptides, which increase there surface area that could be in direct contact with the fibres. In order to continue this study, it is our intent to see how ultrasound can aid the penetration of these peptides incorporated in phospholipids onto fibres (migration results).

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