Incorporation of peptides in liposomes using ultrasound

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Introduction

Liposomes are vesicular structures consisting of an aqueous volume enclosed by one, large unilamellar liposomes (LUVs), or more, multilamellar liposomes (MLVs), phospholipid bilayers. Due to their unique physical and chemical properties, their stability in solution for long periods of time with no significant changes in size or structure (Woodle 1995), and their ability to incorporate lipophilic, amphipatic and/or hydrophilic compounds (Sharma 1997), liposomes can be used in a wide range of applications. The most widespread application of liposomes is as carriers of functional substances and drugs to be delivered to hydrophobic surfaces. This work investigated the interaction between two model peptides structures and a model liposome using sonication, with control of different experimental conditions, such as solution depth relative to wavelength (mm) and power amplitude (%).

Materials and methods

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 to the amino acids Leucine, Cysteine, Lysine and Alanine. The peptides structures were synthesized by JPT Peptide Technologies GmbH (Berlin, Germany). All the peptides were covalently linked by the N-terminal to a fluorescent dye, (5(6)-carboxytetramethyl-rhodamine, succinimidyl ester), with spectral properties of Abs_{max}=544 nm and Em_{max}=572 nm, to facilitate the analysis of peptide penetration. All other chemicals used were of analytical grade.

Liposomes preparation and peptides incorporation

Liposomes were prepared by the film hydration method as detailed by Ferreira et al (2005). 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 three hours. 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 ºC) to produce MLVs. MLV suspensions were sonicated at 54 ºC to produce LUVs. Preparation of liposomes with peptides followed a similar procedure with the lipid solution in chloroform and the peptides dissolved in pure ethanol and dried together under nitrogen. All solutions were thermo-stated at 54 ºC.

Sonication of the liposome solutions

The experimental set up used was composed of a 20 kHz RF generator driving a piezoelectric transducer fitted with a titanium probe, diameter 3 mm, (Sonics & Materials, USA). Power delivery was controlled as percentage amplitude. The reaction vessel was an open glass cell (diameter 19 mm and height 75 mm), which contained 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 ºC (±1 ºC). Sonication was carried out with a total treatment of 12 min monitored in 3 minute increments. All the experiments were made using a pulsed duty cycle of 8 sec on, 2 sec off.

Photon-correlation spectroscopy (PCS) determinations

Zeta-potential values and size distribution of liposomes, with and without incorporated peptides, were determined at pH 7.4 (phosphate buffer) and 50.0 ºC via Dynamic Light Scattering (DLS) analysis (Malvern zetasizer NS). All the determinations were made in terms of volume.

Electronic microscopy (EM) determinations

The morphology of the liposomes with and without peptides was determined using a scanning electron microscopy (LEICA S360). The sample preparation included the drying and covering with a gold layer.

Results and discussion

Prior to testing of the liposome behavior to the sonication, the reactor was categorized via an adaptation of the previously published method, Little et al (2007). This identified the minima and maxima hydroxyl radical activity points as occurring at 38.5 mm (nodal-point λ/2) and 19.5 mm (anti-nodal point λ/4) positions, respectively. Subsequently, ultrasonic treatment of the liposome with and without peptides was carried out at amplitudes of 20 %, 30 % and 40 % and using the depths of 38.5 mm and 19.5 mm (from the base of the vessel). 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 (≈ 2358 nm) with a higher polydispersity (≈0.910). This result was attributed to the variation of MLVs presented in the sample. Figures 1 and 2 show the size (nm) and the polydispersity (PDl) that were obtained after sonication. Initial data from the zetasizer gave a high Z-average with a high PDl during the first minutes. After the treatment with ultrasound there was a decrease in the PDI with a rapid drop in the Z-average. The initial size and PDl value were considerably higher before start the irradiation, attributed to the presence of multiple large sized particles of liposome that are rapidly dispersed with ultrasound. Physical size and PDl decreased with a higher power intensity (40 %), since with this power it is possible to achieve more rapid mixing of solution. A drop in the physical size and PDl at 19.5 mm was observed. The production of ·OH radicals is notably higher at this depth.

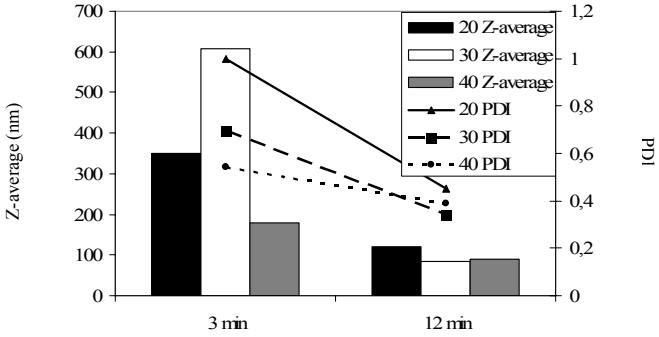

Figure 1: Sonication at 38,5 mm of distance.

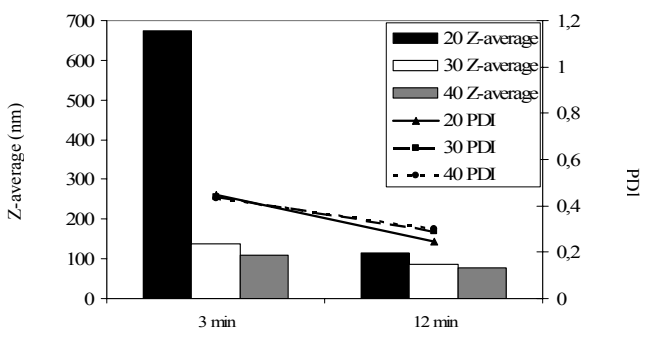

Figure 2: Sonication at 19,5 mm of distance.

The determinations of 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 (≈ 3.90 and ≈ 3.24 mV, before and after ultrasound respectively).

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 %). Figures 3 and 4 show the influence of power at 19.5 and 38.5 positions, on the LUV size (nm) and PDl. Lower sizes were obtained with 19.5 mm of depth, most likely due to the higher production of hydroxyl radicals. 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 obtain particles with lower size and with lower polydispersity.

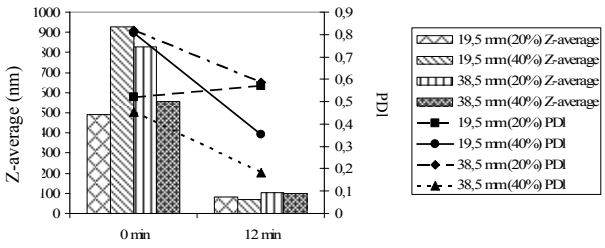

Figure 3: Z-average (nm) and PDl values for peptide LLLLK LLLLK LLLLK LLLLK.

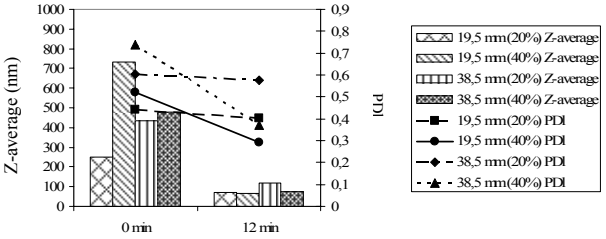

Figure 4: Z-average (nm) and PDl values for peptide LLLLL LCLCL LLKAK AK.

Figures 5 and 6 show the influence of sonication on membrane surface of liposomes with the peptides. The amino acids Lysine (bold) of 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, the amino acids Lysine of peptide LLLLL LCLCL LL**K**A**K** A**K** 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 once 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 %).

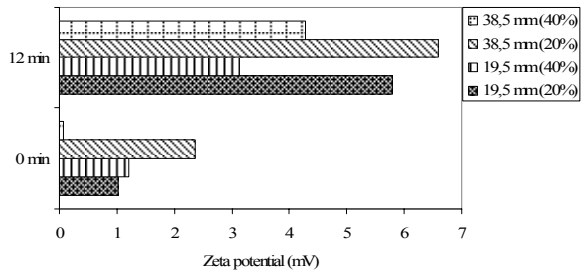

Figure 5: Zeta potential (mV) values for peptide LLLLK LLLLK LLLLK LLLLK.

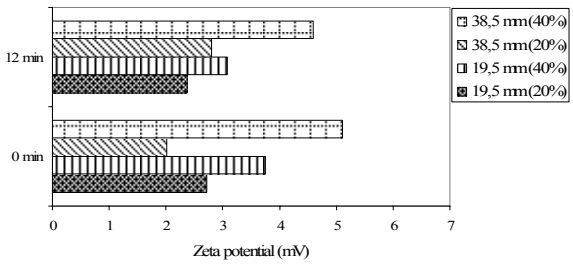

Figure 6: Zeta potential (mV) values for peptide LLLLL LCLCL LLKAK AK.

Samples of each peptide were analysed by scanning electronic microscopy (SEM), after treatment with ultrasound at 19.5 mm of depth and 40 % amplitude. The photographs were taken at different magnifications of x50, x800, and x5000. Figures 7 and 8 show that peptide LLLLK LLLLK LLLLK LLLLK has an "amorphous" aspect, possibly due to the positive charge of 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 present defined rod shape, attributed to their more hydrophobic nature, allowing them a higher penetration on the lipid bilayer.

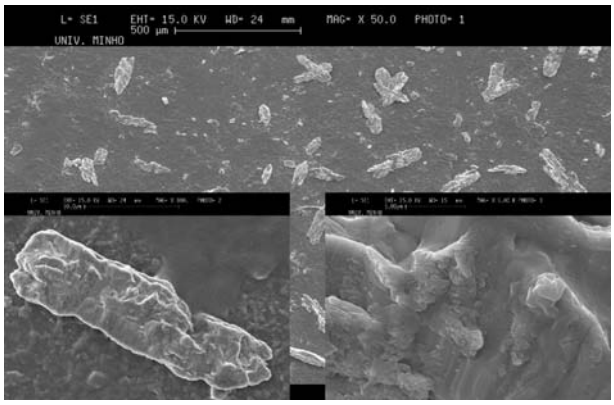

Figure7: SEM microphotographs LLLLK LLLLK LLLLK LLLLK after sonication.

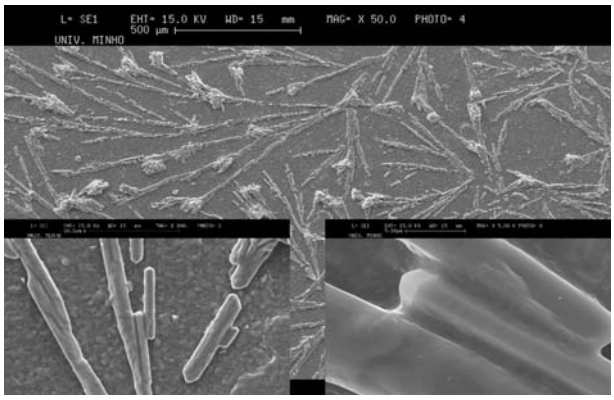

Figure 8: SEM microphotographs LLLLL LCLCL LLKAK AK after sonication.

Conclusions

This study shows the two principal factors of ultrasound that could influence these ranges of sizes and zeta potential: depth and amplitude. 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 higher 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. In order to continue this study, it is our intent to see how these peptides incorporated in liposomes could penetrate on fibres (migration results), using ultrasound.

References

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