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Ultrasonic Surface Acoustic Wave platform for targeted pulmonary delivery of nano drug vehicles

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***Abstract*—This work shows a new method that employs surface acoustic waves (SAW) to form liposomes and simultaneously create a narrow distribution of inhalable aerosols that falls within the ideal respirable range size. The main advantage of using SAW is that, in one step, we can control the aerosols droplet size distribution and simultaneously create drug carriers of biological and active compounds, which are often degraded in alternative nebulisation methods. To corroborate the effectiveness of the proposed SAW nebulisation platform, nucleic acids were nebulised and deliver to lung cancer cells.**

Keywords—Surface acoustic waves, pulmonary drug delivery, liposomes formation, gene therapy platform.

I. Introduction

Nebulisation still represents one of the simplest and most effective methods for delivering drugs to the human lungs [1]. However, there are some technological issues that still hinder the achievement of the optimal aerosols' size distribution for an effective drug delivery. In this regard, clinical studies have shown that efficacious aerosols' size distribution should fall between 1 and 5 μm [2]. Nonetheless, currently available devices, including ultrasonic nebulisers [3], fail to control the aerosols' size distribution, resulting in high drug wastage and potential off-target effects (including toxicity). Recently, we have introduced a new technique [4] based on the ultrasonic control of capillary waves at the surface of the liquid to be aerosolized that enables a precise control of the droplet size distribution, within the clinically desired range. Moreover, in order to have an effective delivery, drug carriers such as liposomes are often adopted to transport different compounds to the cells [5, 6]. However, current methods for producing liposomes require multiple sequential steps [7] that are time consuming and increase the instability of the liposomes because of their storage conditions [8]. Alternative solutions where pre-formed liposomes were nebulised with jet and ultrasonic nebuliser have been explored [9], [10]; resulting in fragmentation of vesicles and consequently losses of the encapsulated drug.

Here we report a new process based on the interactions of ultrasonic surface waves with solutions of drugs and lipids that allows both the generation and nebulisation of liposome suspensions in one step performed on a single platform, without a prior formulation of the drug carrier. This technology promises to revolutionise gene therapies for lungs' diseases.

II. Material and Methods

A. SAW Nebuliser Microfabrication

Surface acoustic waves (SAWs) were generated at 9.6 MHz on a 1 mm thick 128° Y-cut X-propagating Lithium Niobate (LiNbO3) piezoelectric wafer (produced by standard photolithography) and were coupled into a micro-structured plate with an array of cavities having a diameter of 200 μm, as described in [4]. The array of cavities was obtained by using a dry etched silicon wafer with a thickness of 270 μm. A Peltier (RS electronics, UK) was used to control the temperature of the setup and avoid rupture of the piezoelectric crystal because of heating effects. An input power of 5 to 8 Watt was used to activate the nebulisation process. A collection chamber to accumulate the nebulised solution was designed and manufactured by using a laser cutter. The size of nanocarriers (i.e. liposomes) were measured via dynamic light scattering (DLS, Nano90, Malvern Panalytical).

B. Phospholipids solution

Multilamellar vesicles (MLVs) were generated by hydration of a lipid cake made of the cationic phospholipid N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTAP) and Cholesterol in a ratio of 4:1 and a lipid/nucleic acid ratio of 6:1, as per previously published methods. The complexes, which were formed by electrostatic interaction, were incubated for at least 15 min and then nebulised through the microstructured plates. DNA luciferase plasmid (gWiz, Aldevron, USA), Silencer GAPDH siRNA Human and Silencer select negative control siRNA (Invitrogen, Thermo Fisher Scientific UK) were added separately to the lipid suspension as a biological model for gene therapy. The DNA Luciferase activity was measured using a plate reader to test the

luminescence (BioTek Synergy HT, USA). The siRNA knock-down activity was measured both by SAWs direct transfection into A549 lung cancer cells and by standard manual pipetting technique. A549 cells were chosen because they are widely used as a type II pulmonary epithelial cell model for drug metabolism and as a transfection host [11]. A western blot was carried out after freezing and thawing the cells three times and performing a Bradford assay to determine the concentration of protein in each sample. Cell extracts containing 20 µg of protein were loaded into a 10% SDS polyacrylamide gel, and electrophoresis was run at 120 Volts for 120 min. The solutions were then transferred onto a nitrocellulose membrane and blocked by a 5% non-fat dry milk for 2 h. The membrane was washed twice with TBST (Tris-buffered saline with Tween 20) and once with TBS, for 10 min each time, and then incubated overnight with primary antibodies specific to the protein of interest at 4°C on a shaker. The membrane was then incubated with anti β-actin antibody and incubated with horseradish peroxide-conjugated secondary antibody after washing steps. The antibody was finally detected using the ECL western blotting detection reagents. GAPDH expression was evaluated by densitometry using ImageJ software (Version 1.45S), and the results are reported as the mean of three independent repeats. DLS measurements were performed to measure liposome size distribution before and after nebulisation. The nebulised sample was collected in a petri dish and washed with DI water. Cryo transmission electron microscopy (TEM) was used to confirm the liposomes' structures in terms of lamellarity, indicating the number of bilayers in a vesicle (Fig. 2).

III. RESULTS

We first measured the size of the vesicles generated by SAW actuation (through the array of cavities) by using DLS.

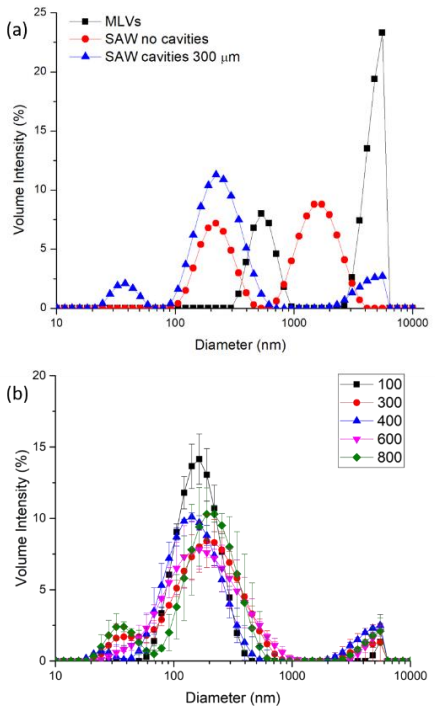

Figure 1. Size distribution of liposomes measured by DLS. (a) Comparison between size distributions of MLVs (black), SAW-formed liposomes without (red) and with cavities (blue). (b) Comparison between size distributions of SAW-formed liposomes for cavities diameter changing from 100 to 800 µm; no significant change can be observed.

TABLE I.

Cavities Diameter (D)	SAW- formed liposomes size		
	Number of size distribution peak	*Aerosol size peak (µm)*	*Vesicle size peak (µm)*
D>800 (µm)	2	100: 1-20	2.5; 0.1-0.2
D<800 (µm)	1	1-20	0.1-0.2

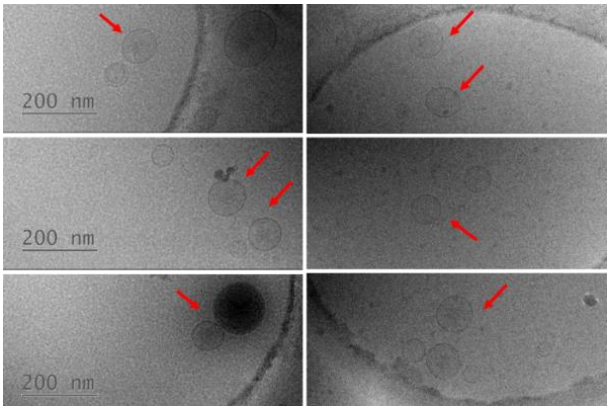

Figure 2. Cryo-TEM images of SAW-formed liposomes. The red arrows indicate unilamellar structure of the liposomes.

The results show that MLVs (Fig. 1a, black line) were broken down to smaller vesicles (Fig. 1a, red line) when nebulised with SAW. Additionally, the use of the array of cavities created a further reduction in the vesicles' size (Fig. 1a, blue line), indicating an effective reduction in size of MLVs in unilamellar liposomes, obtained when combining SAWs and the array of cavities. Interestingly, by varying the size of the cavities in a range of 100 µm to 800 µm there were no significant changes in the vesicles' size distributions (Fig. 1b); all showing an average value ranging between 100-200 nm.

Experimentally we noticed that there is a critical size of the aerosol for the encapsulation of small liposomes (data not reported), which is indirectly controlled by the cavity size [4]. Nebulisation of unconfined sessile droplets and the use of silicon chips with cavity sizes above 800 µm generate a bimodal size distribution of aerosol droplets (with two peaks, one at 1-20 µm and the other around 100 µm) and two size populations of liposomes, with sizes of 100-200 nm and 1-2 µm (Table 1, Fig. 1a, red line). Whereas, the use of smaller cavities (below 800 µm) resulted in formation of droplets below 20 µm and produce a high population of liposomes in the range of 100 to 200 nm. One might speculate that the critical droplet size for encapsulation of liposomes is in range of 20 µm; however further studies are required to fully corroborate this.

Figure (2) shows a cryo-TEM image of SAW-formed liposomes where single bilayer structures are highlighted by red arrows. SAW-formed liposomes are made without prior manipulation of the solution (i.e. via direct nebulisation of hydrated lipid vesicles); their size of 100-200 nm and

unilamellarity make them ideal drug carriers for pulmonary drug delivery [12].

Finally, in order to prove the efficiency of the SAW nebulisation platform to form liposomes to be used as carriers of nucleic acids for transmembrane delivery into cells, transfection of green fluorescent labelled siRNA was carried out in A549 lung cancer cells, both by using a standard manual pipetting technique (Fig. 3a) and SAW transfection (Fig. 3b). Figure (4b) confirms that SAWs nebulization is forming unilamellar vesicles during the nebulization; whereas, by using the standard transfection method (Fig. 4a) MLVs do not break down into LUVs of 100-200 nm and therefore the siRNA cannot easily cross the cell membrane. These preliminary results confirm that our platform, previously shown to control droplet size, holds great potential for on-chip ultrasonic preparation and delivery of advanced gene therapies.

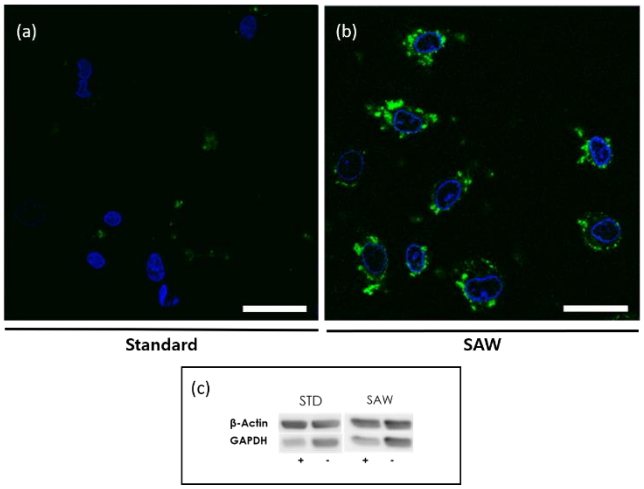

Figure 3. Confocal live images of A549 cell transfection using (a) standard pipetting method and (b) SAW nebulisation transfection of green fluorescent labelled siRNA. (c) Western blot of GAPDH siRNA, lower band intensities indicate a knockdown of the protein expression. Standard pipetting method (STD) resulted comparable with SAW transfection methods as pre-formed liposomes were used in the STD method. Scale bars 40 µm.

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