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## Towards artificial photosynthesis on the lipid bilayer of liposomes

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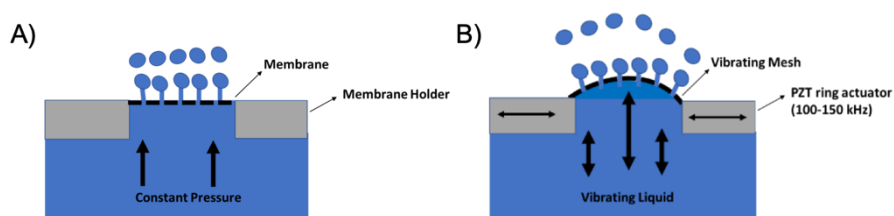
### **Degradation of lipid-based drug delivery formulations during nebulisation**

*Encapsulating pharmaceuticals in protective lipid-based nanoparticles, and nebulising them towards the target area in the body offers a range of clinical advantages. However, the process of nebulisation might possibly damage sensitive nanoparticle structures, such as liposomes, resulting in loss of active pharmaceutical ingredients. We compare this loss for two types of lung inhalation devices: high-frequency piezo-actuated vibrating mesh nebulisers and non-actuated continuous jet nebulisers. We find that vibrating mesh nebulisers cause model liposomes to release more than ten times as much encapsulated material as the continuous jet nebulisers, because the energies involved in nebulisation are much larger. This result highlights the importance of applying a mild nebulisation technology when administering shear-sensitive drug formulations such as lipid nanoparticle-based drugs to the lungs.*

## 5.1 Introduction

The nebulisation route has some advantages over other administration routes for active pharmaceutical ingredients that specifically target the lung area. The intended therapeutic action in the lung can be triggered faster, unpleasant intravenous injections can be prevented, and also a smaller drug quantity is required, as the drug is delivered directly to its intended site of action. This mode of administration typically results in lower systemic side-effects in comparison to oral or intravenous administration. Side-effects can be further suppressed by encapsulating the drug inside liposomes or lipid nanoparticles (LNPs).<sup>1,2</sup> Indeed, entrapping drugs in 'conventional' liposomes has proven advantages such as targeted drug delivery to specific tissues, and prevention against drug degradation.<sup>1</sup> Such protection is appealing for drug and vaccine makers,<sup>3,4</sup> for example in the case of RNA drugs, which are vulnerable to RNase degradation.<sup>3,5</sup> In addition, the use of LNPs facilitates cellular uptake of the drug with a high efficacy.<sup>2</sup> LNPs can be seen as a new generation of liposomes, specifically formulated for an efficient delivery of various active pharmaceutical ingredients, and are characterised by having a smaller internal aqueous content than liposomes.<sup>1</sup> However, in the process of converting the lipid based nanoparticle drug formulations into aerosol droplets (nebulisation), the nanoparticles may get damaged due to shear degradation, resulting in loss of the originally entrapped active pharmaceutical ingredients, in particular in case of hydrophilic materials.<sup>6</sup> This is due to a high shear stress being exerted on the nanoparticles, leading to breakage. The development of lipid-based nanoparticle carriers for inhalation is therefore focused on increasing the strength and rigidity of the nanoparticles with the aim of reducing the detrimental effect of shear stress on nanoparticle stability and maximising its deposition rate and efficacy of the formulation in the 'deep lung'. An example of a lipid based nanoparticle drug formulation success is the recently FDA approved nebulisable liposome formulation of the antibiotic Amikacin, the success of which is attributed to the combined development of a shear-stress resistant nanoparticle formulation of cholesterol-enriched dipalmitoyl-phosphatidylcholine (DPPC-CH) with a liposome size around 300 nm and a PARI eFlow vibrating mesh

nebulisation device.<sup>7</sup> In this communication we investigate a novel facile and fast nebulisation method with a continuous jet atomisation device to nebulise formulations at a throughput well over 1 mL/min with a minimum amount of shear stress enabling a large window to formulate lipid based nanoparticle drug carriers (Figure 5.1).



**Figure 5.1.** Methods of nebulizing used in this study. (A) Continuous atomisation. The nanoparticle formulation is pressed at a constant pressure through a rigid membrane enabling Rayleigh breakup. (B) Vibrating mesh technology. A flexible mesh mounted on a piezo ring actuator is stretching and vibrating. These vibrations are also present in the nanoparticle formulation and are dissipated by heat generation of the liquid.

## 5.2 Materials and methods

**Preparation of liposome formulations.** The osmolarity of aqueous solutions was measured on a Micro-Osmometer Autocal Type 13 from Roebing. Calcein was obtained from Carl Roth and used as received. 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and cholesterol (CH) were purchased from Avanti Polar Lipids and sodium N-(carboxymethylpolyethylene glycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanol-amine (NaDSPE-PEG2K) from Lipoid. All were stored as solids at  $-20^{\circ}\text{C}$ . Liposomes were prepared as follows.<sup>8,9</sup> The lipids were dissolved in chloroform at the desired ratio (DPPC 100, DPPC-CH 50:50, and DPPC-NaDSPE-PEG2K 100:1) in a pressure resistant glass tube. Chloroform was evaporated by rotary evaporation and the resulting lipid film was dried in vacuum overnight to remove residual solvent. The film was then hydrated with a  $\text{NaH}_2\text{PO}_4$  buffer (1 mL, 0.1 M, pH = 7.7,  $p = 661$  mOsm) containing calcein (70 mM), followed by 5 freeze-thaw cycles between liquid  $\text{N}_2$  and a  $50^{\circ}\text{C}$  water bath. Subsequently, the vesicles were extruded 11 times with an Avanti Polar Lipids mini-extruder through a 100

nm polycarbonate membrane at 55 °C. After extrusion, the liposomes were separated from the non-encapsulated calcein using a SEC column (GE Healthcare cartridge) equilibrated with NaH<sub>2</sub>PO<sub>4</sub> buffer (0.1 M, pH = 7.7, p = 663 mOsm, osmolarity adjusted by adding NaCl). The orange/brown non-fluorescence band containing the liposomes was obtained until free calcein eluted, as visualised by UV light. These liposome stock solutions (final bulk lipid concentration 5 mM, assuming no losses) were analysed the same day with dynamic light scattering (DLS) and calcein luminescence. DLS was performed at 25 °C on a Zetasizer Nano-S from Malvern operating at 632.8 nm with a scattering angle of 173°.

**Calcein leakage testing.** To a 1 mL cuvette was added 0.04 mL liposome stock solution and an isotonic 0.96 mL NaH<sub>2</sub>PO<sub>4</sub> buffer (0.1 M, pH = 7.7, p = 663 mOsm). A luminescence measurement was carried out on a Horiba Aqualog spectrometer at RT using 495 nm as excitation wavelength. The emission intensity of calcein was recorded at 519 nm. The maximum luminescence intensity of calcein at 519 nm of the sample was determined by addition of TritonX100 (10 mM, 5 µL, 16 days equilibration time), which resulted in the destruction of the liposomes and subsequent release of all calcein into the bulk aqueous solution. The percentage of release of encapsulated calcein (%) was calculated by dividing the fluorescence intensity at a given time, by the final, maximum fluorescence intensity obtained after TritonX100 addition.

**Nebulisers.** The nebulisers used in this study are shown in Figure 5.2 and all produce drop size distributions (2-6 µm) suitable for inhalation. Nebuliser A ([www.pocketair.com.tw](http://www.pocketair.com.tw)) has a flow rate of 0.4 mL/min, nebuliser B ([www.pari.com](http://www.pari.com)) of 0.5 mL/min and nebuliser C ([www.medspray.com](http://www.medspray.com)) of 1.5 mL/min. Nebuliser A and nebuliser B were filled with 2 mL of liposome formulation and Nebuliser C with 1 mL of liposome formulation. 1 mL of the formulation was nebulised. The nebulised formulation was collected by spraying into a 50 mL centrifuge tube. The degree of calcein leakage inside the collected fluid was measured as described above. The 1 mL liposome formulation that remained in the reservoir of nebuliser A and B at the end of each nebulisation experiment was also analysed for calcein release.

Nebulisation was undertaken within 2 h of preparation of the liposome formulation.



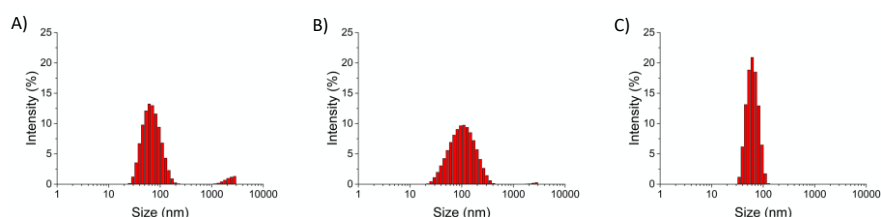
**Figure 5.2.** Nebulisers used in this study. (A) Pocket air, vibrating mesh nebuliser, (B) PARI eFlow, vibrating mesh nebuliser, and (C) Continuous jet nebuliser operated with a syringe.

**Estimating the energy dissipation of the actuated nebulisers.** To estimate the dissipation of the actuated mesh nebulisers water was sprayed for 1 min and the temperature increase of the mesh and reservoir was measured using a Flir C3 infrared camera. The measured temperature increase per second was multiplied with the heat capacity of water and divided by the flow rate to obtain a value for the so-called energy density (in J/g): the amount of energy added to the sprayed product.

### 5.3 Results and Discussion

To ensure that the results apply to different lipid formulations, we prepared three different types of liposomes, i.e. DPPC, DPPC-CH (50:50), and DPPC-NaDSPE-PEG2K (100:1), encapsulating calcein in their inner aqueous compartment as a model of a negatively charged drug. Calcein can be used for testing membrane leakage<sup>10</sup>; it is a self-quenching fluorophore that shows low fluorescence at high concentration inside the liposome (70 mM), but increased fluorescence at lower concentration, for example when it leaks outside the liposome into the bulk. Destroying the liposome membrane by adding the surfactant TritonX100 afforded a maximum fluorescence intensity  $F_{\max}$ , which allowed to quantify the relative fraction of drug released before full membrane disruption,  $X$  (in %), by dividing the fluorescence intensity  $F$  (before or after nebulisation) by  $F_{\max}$ . Figure 5.3 depicts the hydrodynamic

size distributions of the calcein-encapsulating DPPC, DPPC-CH, and DPPC-NaDSPE-PEG2K liposome formulations in phosphate buffer 1 h after preparation, as determined by DLS. Significant aggregation of the pure DPPC (Figure 5.3A) and DPPC-CH formulations (Figure 5.3B) was observed, whereas the 1% PEGylated DPPC formulation (stealth liposomes) did not aggregate at all, as characterised by a low polydispersity index (PDI < 0.1, Figure 5.3C). The characterisation of these size distributions is given in Table 5.1 with the volume average size ( $Z_{ave}$ ) and the PDI defined based on the square of the relative standard deviation.



**Figure 5.3.** Hydrodynamic size distributions of calcein-encapsulated liposome formulations (A) DPPC, (B) DPPC-CH 1:1, and (C) DPPC-NaDSPE-PEG2K 100:1.

**Table 5.1.** Characterisation of the liposomes by DLS.

Liposome formulation	$Z_{ave}$ (nm)	PDI
DPPC	136	0.23
DPPC-CH	181	0.24
DPPC-NaDSPE-PEG2K	119	0.05

In a second step, the relative calcein leakage of each formulation was quantified by measuring the fluorescence intensity  $F$  at the maximum emission (519 nm) of calcein, either directly after preparation, 1 h after preparation, or after nebulisation, and dividing it by the maximum emission intensity  $F_{max}$  when 100% of calcein was released by TritonX100-induced destruction of the liposome membrane. While storage of the liposomes for 1 h did not lead to measurable calcein leakage (variation < 0.1%), all liposome formulations released calcein after nebulisation (Table 5.2). However, clear differences in calcein leakage were observed, depending on the type of nebuliser. The actuated mesh nebulisers caused a lot of calcein leakage

outside the liposome (13 – 16% with the pocket air and 32 – 37% with Pari eFlow), while the continuous jet nebuliser caused a negligible drug leakage (2.3 – 4.6% with Medspray). These results confirmed that it is the nebulisation procedure that induced drug release outside the liposome, and not thermal leakage of the membrane. Secondly, they highlight the difficulty of nebulising drug-encapsulating liposomes for example for delivery to the lungs: the energy used to generate the droplets locally tears the lipid membrane, thus leading to unwanted drug release before the liposomes have reached their target. Another potential issue is the heat generated in the reservoir by the vibrating mesh nebuliser, which as observed here may also lead to up to 24% of calcein leakage. Thermal effects on membrane leakage have been reviewed recently.<sup>11</sup>

**Table 5.2.** Calcein release after nebulisation.

Experiment <sup>a</sup>	Liposome formulation		
	DPPC	DPPC-CH	DPPC-NaDSPE-PEG2K
Pocket air mist	15 ± 1%	13 ± 1%	16 ± 3%
Pocket air reservoir	14 ± 1%	13 ± 3%	4.8 ± 0.9%
Pari eFlow mist	37 ± 2%	32 ± 1%	34 ± 9%
Pari eFlow reservoir	24 ± 1%	23 ± <1%	6.6 ± 2.1%
Medspray mist	4.6 ± 0.7%	2.3 ± 0.3%	2.6 ± 2.6%

<sup>a</sup> all experiments were performed in duplicate.

In order to explain the high leakage observed during nebulisation using mesh nebulisers we determined the energy density dissipated in the solution during nebulisation. The energy density is a parameter that is commonly used to compare emulsification effects.<sup>12</sup> For the non-actuated mesh nebuliser, the energy density is proportional to the applied pressure, which is estimated to be 20 bar giving an energy density of 2 J/g. For the actuated vibrating mesh nebulisers, we calculated the energy density to be in the order of 30 – 60 J/g based on the temperature rise ( $\approx 2$  °C per 30 seconds for the Pocket air and  $\approx 3 - 4$  °C per 30 seconds for the Pari eFlow) of the liquid in the reservoir after nebulisation. In the actuated-mesh nebulisers droplets are produced by the action of high frequency pressure waves with a

frequency in the range of 100 – 150 kHz. On the other hand the use of high frequency ultrasound waves is also a well-known technique to break or disrupt liposomes.<sup>13</sup> It has been reported that the decrease in liposome size is proportional to the energy density.<sup>14</sup> Using a frequency in the order of 100 kHz it was found that the average liposome size decreased with about ten percent at an energy density of 50 J/g. This makes it conceivable that pressure waves used in the actuated-mesh nebulisers cause the liposome membrane to deform and potentially disrupt, leading to a substantial loss of encapsulated calcein. Interestingly this assumption is now experimentally verified, as indeed a substantial leakage of calcein was found in the reservoir of the vibrating mesh nebulisers after nebulisation (5 – 14% with the pocket air and 7 – 24% with the Pari eFlow, see Table 5.2). In the syringe ‘reservoir’ of the Rayleigh jet nebuliser no leakage of calcein was found after nebulisation.

**Liposomes and mechanical rigidity.** DPPC liposomes are characterised by hydrogen saturated acyl chains, a high gel-to-liquid phase transition temperature (41.4 °C), and hence a high packing density at room temperature. Such characteristics lead to a gel-like, mechanically rigid, and stress-insensitive liposomes. Indeed, in absence of nebulisation, none of the formulation used here leaked significant amounts of calcein within 1 h. It is generally accepted that adding cholesterol (CH) make lipid membranes based on saturated acyl chains (e.g. DPPC) more fluid and lipid packing tighter, resulting in less leaky membranes. The addition of PEGylated phospholipids has been reported to weaken liposomes slightly, because PEGylated phospholipids have a higher exchange rate with the aqueous phase.<sup>15</sup> In absence of nebulisation, none of these additives led to significant changes of the membrane leakage, as pure DPPC membranes were found already very tight at room temperature (< 0.1% leakage within 1 h). Upon “hard” nebulisation using the vibrating mesh nebuliser, adding cholesterol to the DPPC formulation had minimal effects on reducing calcein leakage. Addition of PEGylated lipids did increase the amount of calcein release, especially when passing the pores of the vibrating mesh, considering that the PEGylated lipids remained well preserved within the reservoirs of the vibrating mesh nebulisers during nebulisation. This effect is reminiscent from

the effect of air bubbles on the leakage of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) liposomes, which was increased in presence of PEGylated lipids in the membrane.<sup>16</sup> Upon “soft” nebulisation using the Rayleigh jet nebuliser, both additives slightly reduced calcein release, which remained much lower than when nebulisation was performed using the vibrating mesh nebulisers.

## 5.4 Conclusion

In conclusion, we have put forward an efficacious Rayleigh jet inhalation technology that causes less unwanted leakage of encapsulated drugs outside the liposomes during nebulisation, and this is regardless of the liposome composition. This nebulising technology therefore enables nebulising liposomes with a minimal escape of hydrophilic or large encapsulated drug molecules, such as DNA, RNA, proteins, peptides, antibodies, etc. while offering improved freedom in the formulation of lipid drug delivery systems.

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