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TAILORING THE SYNTHESIS OF MONODISPERSE PEG-STABILIZED LIPOSOMES VIA MICROFLUIDIC DEVICES

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ABSTRACT – *Microfluidics has emerged as a valuable tool for the synthesis of micro- and nanostructures, through exploiting the manipulation of small amounts of fluids in micrometric platforms. Hydrodynamic focusing microfluidic devices have been widely investigated for the synthesis of liposomes towards varied nanomedicine applications. Here we describe the synthesis of stealth liposomes, conjugated with 1% of poly(ethylene glycol) (PEG)-lipid, through microfluidic devices based on hydrodynamic focusing and increasing ionic strength by using phosphate-buffered saline (PBS). We were able to synthesize stealth liposomes of 130 nm in size, with low polydispersity index (PDI≤0.2) and positive zeta potential. When compared to the use of aqueous side streams, the proposed process with high ionic strength revealed to be an easy, feasible and reproducible strategy for the synthesis of monodisperse stealth liposomes with monomodal size distribution, for further drug and gene delivery applications.*

1. INTRODUCTION

Formed through spontaneous self-assembly in aqueous environments, liposomes are vesicular systems with an aqueous core composed by one or more phospholipid bilayers (Kesharwani; Gajbhiye; Jain, 2012). Formulated with a positively charged lipid, cationic liposomes (CL) have been widely investigated as non-viral vectors for gene delivery applications due to the ability of condensing and protecting nucleic acids against enzymatic degradation. Additionally, CL are able to efficiently interact with anionic cell membranes to deliver therapeutic genetic materials. Poly(ethylene glycol) (PEG) is one of the most applied surface ligands to form stealth liposomes. This polymer provides a protective surface shield, leading to improved liposome steric stability and extended systemic circulation time (Suk *et al.*, 2016).

Microfluidic platforms have been widely explored for liposome synthesis, especially the ones based on hydrodynamic flow focusing (Balbino *et al.*, 2013). Microfluidics is an interdisciplinary technological tool that allows for investigations under unique hydrodynamic properties, including laminar flow regime, thus, enabling the continuous production of uniform products (Lu *et al.*, 2014).

This work reports the synthesis of stealth liposomes, conjugated with 1% of PEG, in microfluidic devices based on hydrodynamic focusing. We investigated the effects of the increasing ionic strength of the side streams on the formation of stealth liposomes, through the use of phosphate-buffered saline (PBS), by evaluating their resultant physicochemical characteristics. We experimentally demonstrate that high ionic strength side streams represent a relevant microfluidic process parameter for the formation of monodisperse stealth liposomes with adequate features for future applications in drug and gene delivery.

2. MATERIAL AND METHODS

Stealth liposomes were composed of egg phosphatidylcholine (EPC), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) conjugated with PEG2000 (50/25/24/1% molar).

The microfluidic devices based on hydrodynamic flow focusing were made of polydimethylsiloxane (PDMS) by soft lithography, and sealed with glass slides through O_2 plasma surface treatment. The devices had dimensions of 140 µm width and 50 µm height. The stealth liposomes microfluidic synthesis was evaluated using a central stream composed by the lipid dispersion (1% DSPE-PEG) being hydrodynamically focused by side streams composed of ultrapure water or phosphate-buffered saline (PBS), under the flow rate ratio (FRR) of 7.3. We evaluated the effects of PBS under the inlet concentration of 50 mM.

The physicochemical characteristics of the produced stealth liposomes were measured by dynamic light scattering (DLS) technique, in terms of hydrodynamic diameter, polydispersity index (PDI) and zeta potential, using a Zetasizer Nano ZS (Malvern Instruments Ltd.).

3. RESULTS AND DISCUSSION

We used PDMS/glass hydrodynamic flow focusing devices to produce cationic liposomes (CL), following the proportions of 2:1:1 molar for EPC:DOPE:DOTAP, respectively, with side streams of ultrapure water, as previously reported by our research group (Balbino *et al.*, 2013). These CL were used as model nanostructures in order to investigate the effects of the DSPE-PEG insertion on the resultant physicochemical characteristics of the synthesized stealth liposomes.

Table 1 – Cationic and stealth liposomes (1% DSPE-PEG) produced via microfluidics with side streams composed by water.

Sample	Mean Hydrodynamic Diameter (nm ± SD)	Polydispersity Index (PDI ± SD)	Zeta Potential (mV ± SD)
Cationic Liposomes	136 ± 11	0.17 ± 0.03	63.2 ± 5.9
Stealth Liposomes	69 ± 12	0.47 ± 0.15	28.4 ± 2.7

SD: represents the standard deviation of independent triplicates.

Table 1 shows that the CL formed in this study presented around 140 nm in size, low PDI of 0.17 and positive zeta potential of +63 mV. It is worth mentioning that these advantageous features highlight the potentiality of this microfluidic system for synthesis of CL to be applied as non-viral vectors for gene delivery.

However, when the same microfluidic system using side streams of ultrapure water was employed for synthesis of stealth liposomes, containing 1% of DSPE-PEG$_{2000}$ lipid, the resultant self-aggregates presented high PDI of 0.47 (Table 1) and multimodal size distributions (Figure 1a). Additionally, the number-weighted size distribution (Figure 1b) showed the prevalence of aggregates of around 15 nm in size, which may be attributed to the formation of micelles. Due to the laminar flow regime, the PEG chains have the tendency to be rapidly hydrated as they get in contact with the side streams of water, increasing the intermolecular interactions between the PEG chains and leading to micelles formation. In addition, the high hydrophilicity of the PEG chains may induce their interaction with the hydrophilic glass surface, which may also justify the formation of lipidic microaggregates observed by optical microscopy (Figure 1c). Hence, this microfluidic system did not allow for an appropriate incorporation of the DSPE-PEG lipid within the stealth liposome structure.

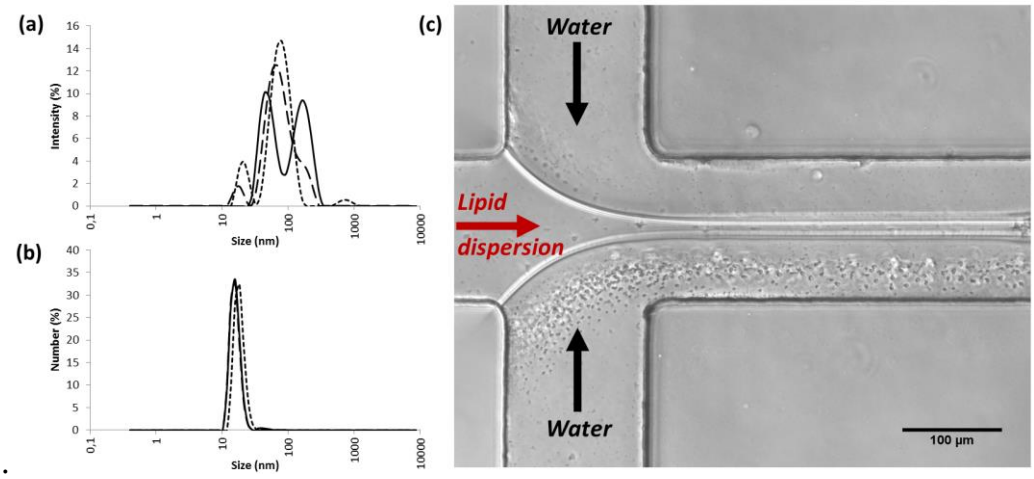

Figure 1 – (a) Intensity- and (b) Number-weighted size distributions for stealth liposomes (1% of DSPE-PEG$_{2000}$) formed by applying side streams composed of ultrapure water. The profiles represent independent triplicates. (c) Microscopic image of the microchannel after 10 min of processing showing lipid retention.

In this way, we hypothesized that by increasing the ionic strength of the surrounding solutions, it would be possible to shield the hydrophilic glass surface and favor the hydrophobic interactions of the lipids apolar chains. This alternative would decrease PEG intermolecular interactions with the surface and enable a better DSPE-PEG lipid incorporation to the liposome structure. For this purpose, we applied 50 mM-PBS side streams to evaluate the microfluidic synthesis of PEG-stabilized liposomes.

As shown in Figure 2, this microfluidic process strategy enabled the synthesis of monodisperse stealth liposomes (Figure 2a-b) of approximately 130 nm in size, PDI of 0.14 and +10 mV of zeta potential. Furthermore, we were able to eliminate the formation of PEG micelles, as shown by the number-weighted size distribution in Figure 2b, and we

significantly diminished the lipid retention on the microchannel glass surface (Figure 2c). These results corroborate our initial hypotheses, elucidating that the ionic strength act not only on the glass surface shielding, but also on the bilayer-oriented lipid aggregation.

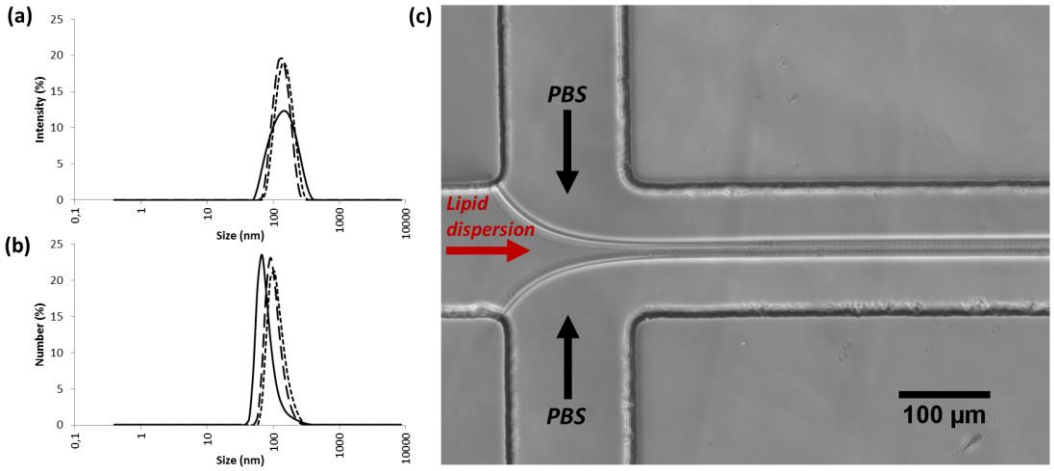

Figure 2 – (a) Intensity- and (b) Number-weighted size distributions for stealth liposomes (1% of DSPE-PEG_{2000}) formed by applying side streams composed of 50 mM Phosphate-buffered saline (PBS). The profiles represent independent triplicates. (c) Microscopic image of the microchannel after 10 min of processing.

In view of this, the proposed approach applying high ionic strength side streams represent an easy, feasible and reproducible strategy to overcome micelle formation and undesirable lipid retention on the microchannel walls, enabling a homogeneous synthesis of stealth liposomes with an efficient PEG surface functionalization.

4. CONCLUSIONS

In this work, we have demonstrated the potential of using microfluidic approaches based on hydrodynamic focusing with high ionic strength for the synthesis of stealth liposomes with low polydispersity, monomodal size distribution and positive zeta potential. These interesting features enable the application of these PEG-stabilized nanoaggregates in varied research fields, especially towards drug and gene delivery applications.

5. REFERENCES

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