SINGLE- AND MULTI-STEP SYNTHESIS OF STEALTH LIPOSOMES THROUGH HYDRODYNAMIC FLOW FOCUSING MICROFLUIDIC TECHNIQUE

Gabriel Perli, Amanda da Costa e Silva de Noronha Pessoa, Lucimara Gaziola de la Torre

perligabriel@gmail.com; amandanoronha@feq.unicamp.br; latorre@feq.unicamp.br

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 flow focusing microfluidic devices have been widely investigated for the synthesis of liposomes towards varied nanomedicine applications. Here we describe the microfluidic synthesis of stealth liposomes, conjugated with 1% of poly(ethylene glycol) (PEG), through two different approaches: (1) single-step synthesis of stealth liposomes and (2) post insertion of PEG in pre-formed cationic liposomes in a multi-step process. By using phosphate-buffered saline (PBS) as side streams, both techniques were able to synthesize stealth liposomes with low polydispersity index (PDI≤0.2) and positive zeta potential. Nevertheless, the single-step technique revealed to be the most suitable strategy for the synthesis of stealth liposomes with interesting features for further drug and gene delivery applications.*

Key-Words*: stealth liposomes; microfluidics; hydrodynamic flow focusing.*

Introduction: Liposomes are vesicular systems composed by a phospholipid bilayer with an aqueous core, formed through spontaneous self-assembly in aqueous environments [1], mimicking cellular membranes. Cationic liposomes, whose formulation contains a positively charged lipid, are widely investigated as non-viral vectors for gene delivery applications due to the ability of condensing and protecting nucleic acids against enzymatic degradation, as well as efficiently interacting with cell membranes to deliver therapeutic genetic materials [2]. Poly(ethylene glycol) (PEG) is one of the most applied surface ligands to form stealth liposomes, which provides a protective surface shield, leading to improved liposome steric stability and extended systemic circulation time [3]. Microfluidic devices based on hydrodynamic flow focusing have been widely explored for liposomes synthesis [4]. Microfluidics is a versatile technological tool that can provide controlled and tunable process conditions through developing unique hydrodynamic properties, including laminar flow regime, enabling the production of uniform products [5]. This work aims to investigate the synthesis of stealth liposomes, conjugated with PEG, in two distinct microfluidic approaches: (1) single-step and (2) multi-step system, based on lipid-PEG post insertion in pre-formed cationic liposomes. We assessed the effects of PEG insertion by comparing their resultant physicochemical characteristics, in terms of hydrodynamic diameter, polydispersity index (PDI) and zeta potential.

Experimental: The stealth liposomes were composed of egg phosphatidylcholine (EPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) conjugated with PEG_{2000} (50/24/25/1% molar). Hydrodynamic flow focusing microfluidic devices were made of polydimethylsiloxane (PDMS) and glass, with dimensions of 140 µm width and 50 µm height. We assessed the microfluidic synthesis of stealth liposomes applying two approaches (Fig. 1): (a) single-step, with side streams composed by phosphate-buffered saline (PBS 5X) focusing the lipids dispersion including DSPE-PEG; and (b) multi-step, through DSPE-PEG post insertion in pre-formed cationic liposomes. We assessed the physicochemical characteristics of the synthesized stealth liposomes by dynamic light scattering (DLS)

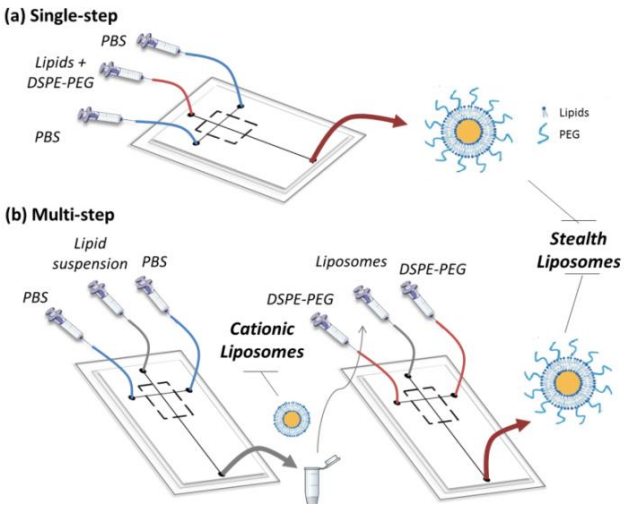

Fig. 1. Schematic diagram of single-step (a) and Multi-step (b) synthesis of stealth liposomes.

measurements using Zetasizer NanoZS (Malvern Instruments). The synthesis was carried out maintaining the total flow rate of 124.2 µL/min and flow rate ratio (FRR) of 7.3.

Results and discussion: Cationic liposomes (CL) were produced in PDMS/glass hydrodynamic flow focusing devices as described in Fig. 1b, following the proportions of 50:25:25% molar for EPC:DOPE:DOTAP, respectively, as previously reported [4]. These CL were used as model nanostructures in order to evaluate the effects of the DSPE-PEG insertion on the resultant physicochemical characteristics of the synthesized stealth liposomes. As shown in Fig. 2, the CL presented around 150 nm, low PDI of 0.16 and positive zeta potential of +29.5 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.

The stealth liposomes synthesized by both strategies presented lower sizes and lower zeta potential, when compared to the pre-formed CL. These results may be justified by the hydration of the PEG molecules which shields the liposomes surface, leading to lower surface charge density values and reduced liposome electrophoretic mobility. In this way, the lower zeta potential values suggest an efficient DSPE-PEG insertion on the CL for both studied microfluidic approaches.

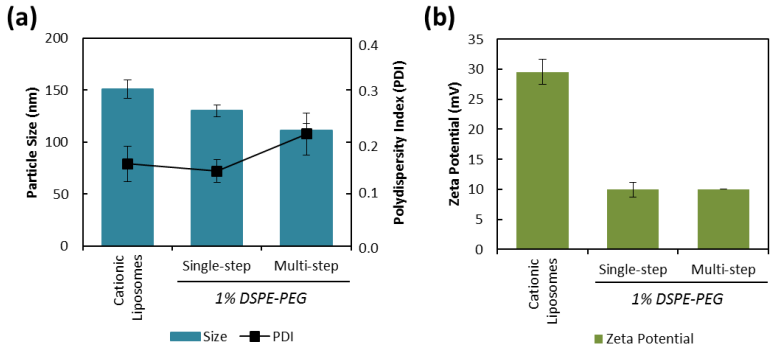

Fig. 2. Intensity-weighted hydrodynamic mean diameter, polydispersity index (PDI) (a) and zeta potential (b) of the synthesized liposomes.

Although the multi-step process was also able to produce stealth liposomes with low polydispersity (Fig. 2a), the size distributions (intensity-weighted) obtained by DLS measurements (Fig. 3b) showed the presence of two distinct nanostructure populations, resulting in an average particle size of 110 nm. On the other hand, this DLS analysis demonstrated that the single-step microfluidic strategy enabled the synthesis of single monodisperse populations (Fig. 3a), demonstrating that the DSPE-PEG was appropriately incorporated into the liposome structure.

Besides the minimized process steps involved, the operational variability is significantly reduced when applying the single-step technique [5]. As a matter of fact, the multi-step technique comprises human factors, such as operator variability between process steps, and the manipulation of the pre-formed CL, possibly affecting their original characteristics and stability.

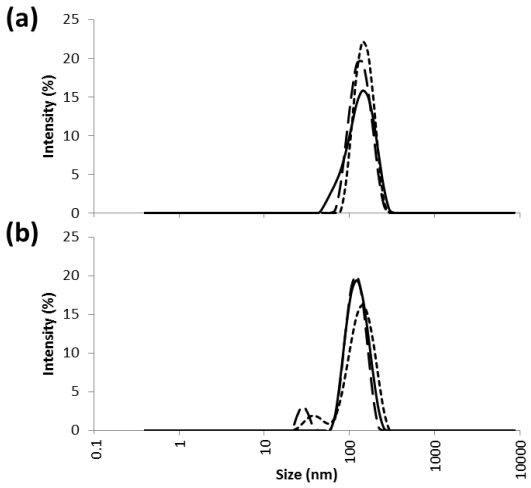

Fig. 3. Size distributions for stealth liposomes (1% DSPE-PEG) synthesized by single-step (a) and multi-step (b) processes. The lines represent independent triplicates.

Conclusion: We have demonstrated the potential of using two different microfluidic approaches based on hydrodynamic flow focusing microfluidic devices for the synthesis of stealth liposomes with interesting features, such as low polydispersity and positive zeta potential, to be applied in varied research fields, especially towards drug and gene delivery applications. The single-step technique showed to be particularly useful for stealth liposomes synthesis because of the advantages of using a continuous process with reduced number of steps and minimized operational variability.

References: [1] P. Kesharwani, *et al*. Biomaterials **33**, 7138 (2012).
[2] J. H. Felgner, *et al*. The Journal of Biological Chemistry **269**, 2550 (1994).
[3] J. S. Suk, *et al*. Advanced Drug Delivery Reviews **99**, 28 (2016).
[4] T. A. Balbino, *et al*. Chemical Engineering Journal **226**, 423 (2013).
[5] M. Lu, *et al*. ACS Nano **8**, 332 (2014).