Microfluidic Synthesis of Nano-Liposomal Anesthetics

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ABSTRACT

Topical anesthesia has the ability to provide painless application of pain management medications for minor procedures such as venipuncture, particularly in the pediatric population. Liposomes have been proposed as a highly advantageous nanocarrier for dermal transport of drugs. Unlike conventional liposome preparation methods, which yield polydisperse populations of liposomes which are too large to passively transverse dermal layers (typically >80 nm), the recently developed microfluidic method for liposome synthesis provides an approach for producing nearly-monodisperse vesicles of tunable size which can achieve a range of size not achievable by traditional methods (approximately 40 nm and below). In this study, we have utilized a previously-demonstrated microfluidic method for continuous-flow synthesis of small, nearly-monodisperse liposomes and demonstrated size-dependent passive uptake into porcine dermal tissue.

Keywords: nanoparticle, liposome, microfluidics, drug delivery

1 INTRODUCTION

The ability to perform local anesthesia without the use of needles has been a long pursued goal, with specific implications for the pediatric population where the ability to provide an enhanced mechanism for procedure-related pain relief is pertinent.[1] It has been suggested that liposomes have great promise as vehicles for transdermal delivery of therapeutic agents due to their ability to improve transport across the stratum corneum (SC), which presents the principal barrier to drug penetration through the skin, since the SC itself consists primarily of a lipid/protein matrix.[2] Although liposomes offer high potential for increasing skin penetration and drug tolerability, these anticipated benefits have not yet been demonstrated, with early results indicating that traditional liposomes do not traverse the SC in substantial quantities.[3] A recent review of the field reported that a number of studies have investigated the dermal transport of conventional liposomes ranging from 60 nm to micrometers in size and have revealed little evidence for transport of intact liposomes through the SC.[4] Conversely, it was recently reported that 20-30 nm diameter lipid dendrimers are readily transported through the SC.[5] Similar to these preliminary studies focusing on SC transport, smaller 40 nm particles have been observed to transport through the dermis via other routes such as porous tissues surrounding hair follicles, while larger particles were excluded.[6]

Despite encompassing various advantageous qualities, liposomes remain limited in their ability to deliver topical agents due to the techniques which are typically employed for preparation. Current liposome synthesis methods are based on bulk scale processes and yield polydisperse populations of vesicles which are both large (typically >80 nm) and polydisperse, with typical populations exhibiting high variance in size and with distributions skewed toward larger diameters. The accumulating evidence indicating that nanoparticles within the range of 20-40 nm can successfully permeate the primary dermal barrier together with the benefits of liposomal drug delivery necessitate the ability to produce smaller, more narrowly distributed populations of liposomes than contemporary methods can currently achieve.

An alternative approach for liposome synthesis which is based on microfluidic technology has recently been demonstrated. [7–9] This method provides the ability to produce nearly-monodisperse vesicles which can realize

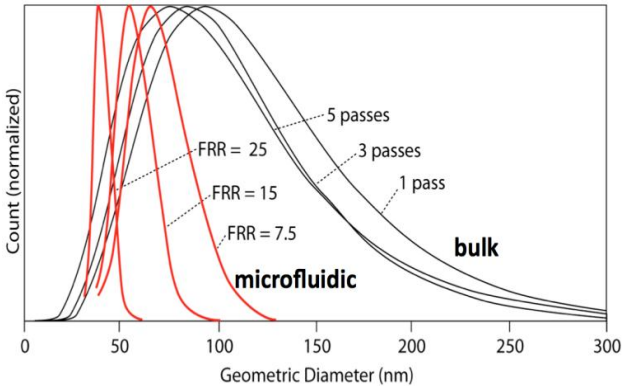

Figure 1: Comparison of liposomes made via microfluidic flow-focusing at different flow rate ratios (FRRs) and traditional bulk homogenization following multiple filtration steps. The microfluidic method enables production of liposomes of tunable size which exhibit narrower size distributions plus lower size limitations than traditional methods.

a range of sizes not achievable by traditional methods (approximately 40 nm and below). This technique presents the ability to produce unilamellar lipid vesicles within the requisite size range to passively traverse dermal tissue. Liposomes produced via the microfluidic technique are of tunable size and exhibit exceptionally low levels of polydispersity compared to conventional, bulk-scale methods (Fig. 1).

Microfluidic formation of nanoscale liposomes involves hydrodynamic focusing of a solvated lipid stream by a sheath of aqueous buffer (Fig. 2),[10] leading to rapid, systematic self-assembly of liposomes with average diameters as small as 30-40 nm. This technique is a unique implementation of the alcohol-injection method for liposome synthesis within a microfluidic network, utilizing hydrodynamic focusing for controlled diffusive mixing of chemical species at the nanoscale to enable precise self-assembly of lipids into nearly-monodisperse populations of liposomes whose sizes may be dynamically varied by simply altering the flow conditions within the microchannels.

In this study, the microfluidic technique of continuous-flow liposome production has been utilized to generate small, nearly-monodisperse lipid vesicles within the size range of interest for dermal penetration to demonstrate size-dependent passive uptake of liposomes into porcine dermal tissue.

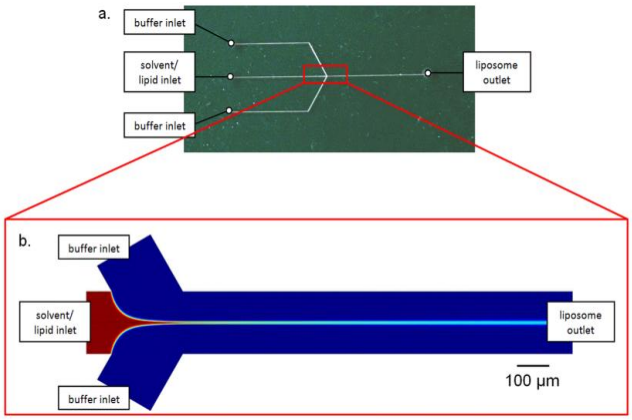

Figure 2: Depiction of the microfluidic process of liposomes production. a) Liposome synthesis chip made using a PDMS mold from an SU-8 master, and (b) numerical simulation of hydrodynamic focusing in the device in which a center stream of ethanol and solvated lipid is sheathed by two oblique streams of aqueous buffer. As lipids slowly diffuse from their solvated state into the aqueous buffer, they systematically self-assemble into vesicles.

2 METHODS

2.1 Microfluidic Device Fabrication

Microfluidic devices made of polydimethylsiloxane (PDMS) and glass were fabricated using soft lithographic methods. Briefly, SU-8 (negative photoresist) was spin coated onto a 4" silicon wafer, patterned via ultraviolet light against a photomask bearing the designs for the desired fluidic channels (50 µm wide, 300 µm tall). The patterned silicon wafer is then developed and used as a mold for PDMS. Once set, the PDMS is removed from the mold and exposed with glass to oxygen plasma. The final device is created by pressing the two pieces, forming a permanent bond. The PDMS-glass microfluidic devices were used to form small, nearly monodisperse liposomes using the method demonstrated previously.[7–9]

2.2 Lipid Mixture and Hydration Buffer Preparation

Dimyristoylphosphatidylcholine (DMPC), cholesterol, and dihexadecyl phosphate (DCP) were mixed in chloroform in molar ratio 7:2:1 then placed in a vacuum desiccator for at least 24 hours to allow complete solvent removal. The dried lipid mixtures were then redissolved in anhydrous ethanol containing 1 wt % of a lipophilic membrane dye (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiI-C_{18}; DiI) for a total lipid concentration of 20 mM. A 10 mM phosphate buffered saline (PBS) solution at pH 7.4 was used as a hydration buffer. All fluids (solvent and buffer) were filtered through 0.22 µm filters before being introduced to the microfluidic device.

2.3 Microfluidic Liposome Synthesis and Characterization

Liposomes were prepared by injecting the lipid-solvent mixture between two buffer inputs into the PDMS glass microfluidic device (Fig. 2). The flow rate ratio (FRR), which is defined as the volumetric flow rate of buffer to that of the solvent, was set to FRR 4 and FRR 50 for two populations of liposomes above and below the size range expected to passively traverse the dermal layer. Linear flow velocity of the total flow for all FRRs was kept constant (0.125 m/s) for a volumetric flow rate of 112 µL/min. The microfluidic device was operated on a hot plate at 50 °C throughout the entire procedure to facilitate the formation of small vesicles.[11] The liposome populations were characterized for size using a Malvern Zetasizer Nano ZSP.

The vesicles produced contained DiI in their bilayer to assist in fluorescence imaging, which helped assess the depth of penetration. To remove any remaining dye not incorporated into the liposomes during the formation process, the samples were purified via size exclusion chromatography on Sephadex G-25 (PD-10) columns that were equilibrated with PBS.

2.4 In Vivo Application of Liposomes to Porcine Tissue

Porcine tissue was used to evaluate passive transdermal diffusion of the microfluidic-synthesized liposomes due to its morphological and functional similarities to human skin.[12] A Yorkshire piglet (4 weeks, 5 kg) which was being sacrificed for another study was used for these experiments. While the piglet was alive but anesthetized, 50 µL aliquots from each liposome population (41 nm and 255 nm) were applied to different locations on the inside of the ear. After 15 min, the ears containing liposomal dye were removed and immediately placed in a freezer in covered petri dishes.

2.5 Cryosectioning and Fluorescence Imaging

Subsequent to liposome exposure, the removed ears were sliced (using a fresh razor) perpendicularly to the skin surface and directly through the sections where liposome-containing solutions were applied. The tissue samples were then embedded into cryo-OCT media and placed in a freezer (-80 °C). Once frozen, the embedded tissues were mounted in a cryostat microtome (HM550 series, Richard Allen Scientific) for slicing. Starting from the initial coarse cut, a few hundred microns of tissue was sliced off and discarded to ensure the sections used for imaging were distant from the blade-cut region. After the initial segment was removed, smaller sections (30 µm) were sliced and placed onto gelatin-treated glass slides. The sections were immediately imaged using an inverted epifluorescence microscope (Nikon TE-2000 S). Bright field and fluorescence images at a 528-553 nm range excitation wavelength (green filter) were taken and overlaid to assess and validate the depth of liposome preparation into the dermal tissues.

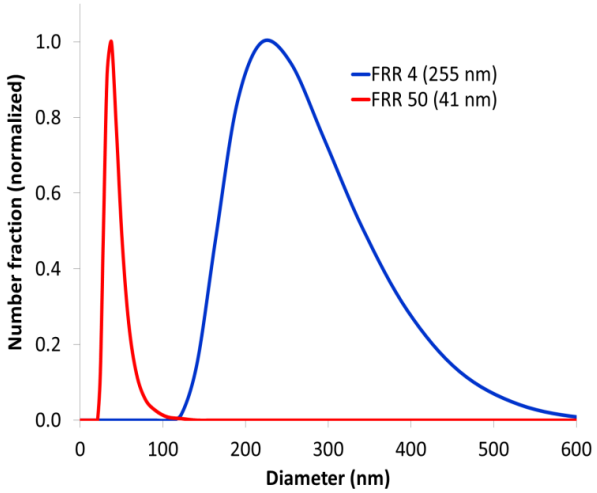

Figure 3: Volume-weighted distributions of populations of liposomes produced using the microfluidic technique. Liposomes with diameters both above and below (FRR 4 and FRR 50, respectively) and below (FRR 50) the size range expected to passively traverse the dermal layer.

3 RESULTS

3.1 Microfluidic Liposome Synthesis

Continuous-flow microfluidic production of liposomes provided populations of vesicles near the size range of particles which have been observed to transport through porous tissues (Figure 3). The liposomes which were on the order of size necessary to traverse skin layers (41 nm) were formed with remarkably with low levels polydispersity. Larger liposomes (255 nm) of identical composition were also formed for comparison.

3.2 Transdermal Liposome Penetration

Cryomicrotoming and fluorescence microscopy of the exposed tissue samples revealed passive uptake of dye-laden, smaller liposomes (41 nm) into the porcine tissue as well as exclusion of liposomes with identical composition but larger diameters (255 nm) (Fig. 4). The bright field/fluorescence overlay images of the porcine skin exposed to larger 255 nm and smaller 41 nm liposomes (Fig. 4 (a) and Fig. 4 (b), respectively) show the similar morphology of the porcine dermis across samples yet an improved penetration of the smaller liposomes through the dermal layers. The single-channel fluorescence images of the larger 255 nm and smaller 41 nm liposomes (Fig. 4 (c)

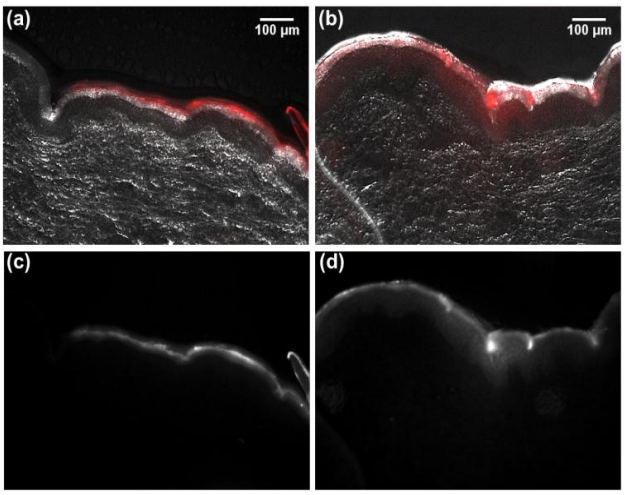

Figure 4: Bright field/fluorescence overlay images (a,b) and single-channel (c,d) fluorescence images of porcine tissue exposed to liposomes encapsulating lipophilic (DiI, red). (a,c) In the case of large liposomes (255 nm) the lipophilic dye does not penetrate deep into the tissue, while (b,d) smaller liposomes (41 nm) appear to transport intact across the stratum corneum, in contrast to prior reports using polydisperse liposome samples.

and Fig. 4 (d), respectively) further divulge the increased permeation depth of the smaller liposomes through the tissue.

Due to the capabilities of the microfluidic technique for liposome synthesis, the small, nearly-monodisperse 41 nm liposomes realized in this study are on the size scale of intracellular junctions and other sites which present openings in the skin, which is a likely explanation for their ability to passively enter the SC and diffuse through the skin layers while the larger 255 nm liposomes simply remain on the top layer of the skin. This is in contrast to other studies, in which larger, more polydisperse liposomes do not penetrate the SC.

4 CONCLUSION

The results presented here validate, for the first time, the ability of the microfluidic technique to produce liposomes of small yet distinct sizes which may be passively incorporated into dermal tissue due to their diameters being within the size range of the pores of the skin. The ability to produce liposomes within a size limit that can traverse the dermal layer without the need for additional treatment enables the use of liposomes for transdermal drug delivery applications with results unattainable by liposomes prepared via contemporary synthesis methods. The microfluidic technique enables the production of novel formulations which will quickly penetrate through the skin layers promoting fast onset anesthesia while also providing long duration and improved safety profiles. These encouraging findings can be used to plan subsequent studies in which microfluidic liposome synthesis may be exploited to produce populations of liposomes with small incremental size differences to optimize formulations for dermal uptake.

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