PHARMACY-ON-A-CHIP: MICROFLUIDIC SYNTHESIS OF PEGYLATED AND FOLATE RECEPTOR-TARGETED LIPOSOMES FOR DRUG DELIVERY

Renee R. Hood[1], Abhay Andar[2], Donna M. Omiatek[3], Wyatt N. Vreeland[3], Peter W. Swaan[2], and Don L. DeVoe[1]

[1]University of Maryland, College Park, USA, [2]University of Maryland, Baltimore, USA, [3]National Institute of Standards and Technology, USA

ABSTRACT

This study demonstrates the extension of a formerly established microfluidic hydrodynamic focusing technique for the production of small, nearly monodisperse liposomes toward the continuous-flow synthesis of PEG- and PEG-folate- modified liposomes for drug delivery applications via controlled laminar flow in thermoplastic microfluidic devices. These liposomes have been used to investigate the relationships between liposome size and cellular uptake mechanisms that go beyond previous studies which have focused on large, polydisperse liposome populations formed by traditional bulk methods. Our findings offer a low cost, rapid method for synthesizing functionalized liposomes and render the previously demonstrated microfluidic technology more practical for medical applications.

KEYWORDS

Liposome, Vesicle, Nanoparticle, Microchannel.

INTRODUCTION

Liposomes are unilamellar lipid vesicles that are well-suited as drug delivery vehicles. Liposomes are highly biocompatible and exhibit numerous advantages over traditional, unencapsulated drugs such as enhanced pharmacokinetics, improved efficacy, and decreased toxicity. Site-specific drug delivery and reduced bloodstream clearance rates may be achieved by appending a steric barrier of poly(ethylene glycol) (PEG) and tissue-specific targeting ligands to the exterior of the liposomes. Standard liposome preparation methods are on the bulk-scale, requiring multiple steps to achieve populations of vesicles with a limited size range and high polydispersity, with additional steps required to attach PEG or targeting moieties. The accepted size range for nanoparticles in drug delivery applications is 10 nm -100 nm in order to avoid renal filtration and passively target tumors by percolating through their characteristically leaky neovasculature. [1] Current methods of production based on bulk-scale synthesis offer limited ability to produce monodisperse liposomes within this size range, while the microfluidic method provides significantly smaller and more uniform liposomes (Fig. 1), with the additional advantage of elimination of post-processing steps for further size modification. This concept has the potential to provide on-demand liposomal drug production, a concept we term "pharmacy-on-a-chip".

The formerly demonstrated technique for synthesis of nearly-monodisperse unilamellar liposomes of tunable size via microfluidic hydrodynamic flow focusing of miscible streams of an aqueous buffer and lipids dissolved in an organic solvent [4] was extended in this study to demonstrate the formation of PEGylated and folate-targeted liposomes. This paper demonstrates the ability to synthesize populations of tumor-targeted vesicles in one simple one-step process using a disposable PDMS/glass device (Fig. 2). Significantly less expensive and much simpler fabrication methods were employed for device production, eradicating the costly, unnecessary MEMS-based fabrication technologies used for previous silicon wafer/glass devices. Folate was chosen as a targeting ligand in this work due to the multitude of epithelial cancers and inflammatory diseases in which significant overexpression of the folate receptor is a defining characteristic. [3] In addition, folate is well-suited as a targeting ligand for nanoparticle conjugation due to its affordability, accessibility, and small molecular weight in comparison to the overall size of nanoparticles.

In this paper, we also demonstrate an application for microfluidic-directed formation of liposomes toward the realization of tumor-targeted "stealth" liposomes for drug delivery in a disposable PDMS/glass device by reporting the outcome of *in vitro* studies designed to illuminate the relationships between liposome size and cellular uptake mechanisms, going beyond previous studies which have focused on large, polydisperse liposome populations formed by traditional bulk methods. [5]

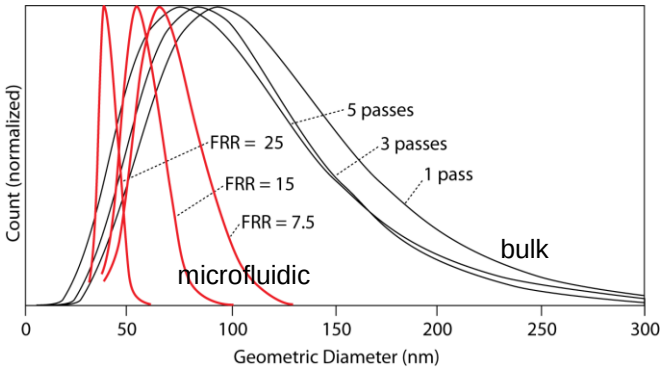

Figure 1. Comparison of polydisperse liposomes produced by traditional methods [7] *and significantly smaller and nearly monodisperse liposomes formed in a microfluidic system at different flow rate ratios (FRRs* [4]*).*

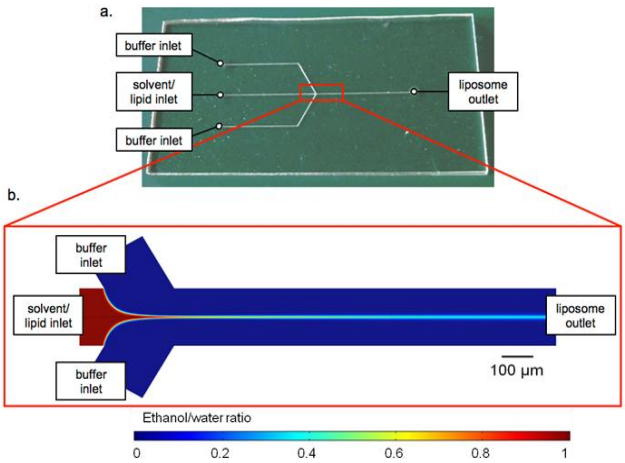

Figure 2. (a) Liposome synthesis chip made using a PDMS mold from an SU-8 master, and (b) numerical simulation of hydrodynamic focusing in the device.

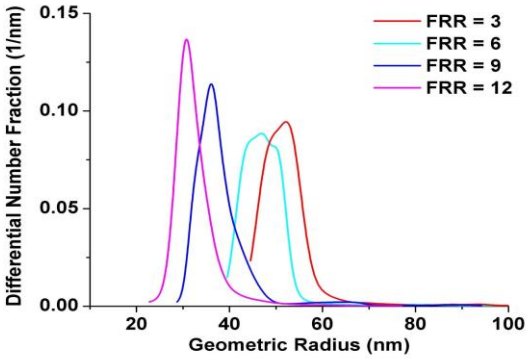

Figure 3. Geometric radius from AF[4] in line with MALLS/QELS. Different flow rate ratios (FRR) of buffer to lipid in solvent create monodisperse populations of liposomes of tunable size.

EXPERIMENTAL SECTION

Microfluidic devices were fabricated using soft lithography techniques. Briefly, raised microchannel patterns were made in SU-8 substrates which were subsequently used as master molds for creating microchannels in PDMS. The PDMS pieces are bonded to glass via microwave-oven-generated plasma oxygen plasma. [2] These devices were used to form PEGylated liposomes and folate-targeted liposomes by including PEG-conjugated or PEG-folate-conjugated lipids during liposome synthesis with phosphate buffered saline (PBS) as a hydration buffer. The resulting liposomes were characterized using asymmetric flow field-flow fractionation (AF^4) in-line with QELLS and MALLS for particle size analysis (Fig. 3), which verified the formation of nearly-monodisperse liposomes which incorporated PEG-modified lipids of tunable size with no presence of large aggregates (data not shown). AF^4 in-line with UV/Vis spectroscopy confirmed the effective integration of both PEG-lipids and folate-PEG-lipids by comparing absorption values of the fractionated samples to the theoretical values calculated from the initial lipid solution (Fig. 4).

Although the microchannel geometries, aspect ratios, precision, and surface properties all varied significantly from the previously used silicon/glass chips, the PDMS/glass devices were successful in producing size-tunable liposome populations with similarly low polydispersity as previous work, [4] albeit with slightly different relationships between flow rate ratio and average liposome diameter. The PDMS/glass device also demonstrated effective incorporation of large, PEG- and PEG-folate- modified lipids into the liposomes (Fig. 4), which is notable due to the large molecular weight of the functionalized lipids compared to the native lipids. Post characterization, the liposomes were used for novel size-dependent cellular uptake studies.

While numerous studies have investigated the effect of particle size on cellular uptake mechanisms *in vitro*, most have focused on particles larger than 100 nm, particularly liposomes which tend to be polydisperse and larger in size. [6] The present study enables, for the first time, the detailed exploration of size-dependent cellular uptake mechanisms for nearly monodisperse liposomes ideal for biomedical applications, featuring liposomes in the range of 10 nm -100 nm [1] in addition to larger liposomes in the scope commonly explored for *in vitro* studies which investigate liposome uptake. Caco-2 cells (human-derived colon carcinoma cells) were incubated with microfluidic-synthesized PEGylated liposomes ranging from 50 nm-236 nm in diameter and containing Dil-C_{18} lipophilic dye to facilitate fluorescence imaging and flow cytometry studies. Intracellular uptake and localization were investigated using confocal fluorescence microscopy (Fig. 5), which revealed a general increase in the colocalization of liposomes with early endosomal regions in the cell with decreasing size. Intracellular uptake was also investigated using flow cytometry (Fig. 6), which verified a significant increase in intracellular uptake with decreasing liposome size. Both *in vitro* cellular uptake studies resulted in the conclusion that a decrease in liposome size corresponds to a considerable increase in uptake, demonstrating that the microfluidic synthesis technique offers unique benefits for liposomal drug formulations. In addition to these studies which describe detailed cell uptake data as a

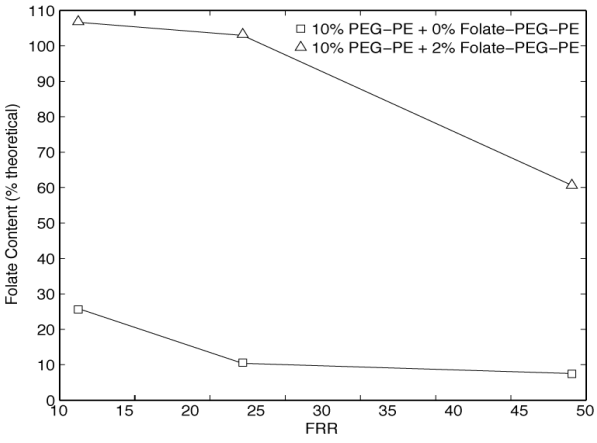

Figure 4. UV-vis absorption data in line with AF[4] from PEGylated non-folate (10% PEG-PE + 0% folate-PEG-PE), and folate (10% PEG-PE + 2% folate-PEG-PE) liposome samples, normalized to control (0% PEG-PE + 0% folate-PEG-PE) liposomes.

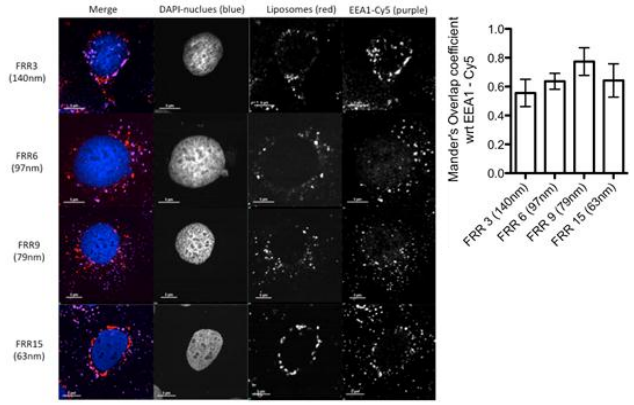

Figure 5. Fluorescence confocal micrograph revealing endosome colocalization for different liposome sizes (EEA1 marker, Caco-2 cells, 60 min incubation).

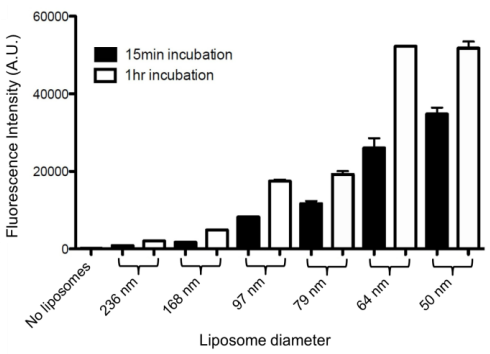

Figure 6. Flow cytometry results for Caco-2 cells incubated with liposomes containing Dil-C_{18} at 15 min and 60 min intervals.

function of liposome size and surface functionalization, ongoing studies using various inhibitors to elucidate cell uptake mechanisms as a function of liposome size are underway. Due to the small and nearly monodisperse liposomes produced by the microfluidic technique, these studies provide a unique insight into the field of nanomedicine.

CONCLUSION

This study demonstrates the ability of a disposable PDMS/glass device to create nanoscale lipid vesicles functionalized for targeted drug delivery with distinct sizes and narrow distributions. The technique has enabled unique *in vitro* cellular uptake studies to be performed, with the potential to identify the most favorable size liposome for drug delivery applications targeting specific uptake pathways. Future efforts will be directed toward the continuation of cell uptake studies to probe the behavior of different sized liposomes in multiple cell types and with different lipid formulations or surface chemistries, animal models for pharmacokinetic analysis, and ongoing evolution of the microfluidic device to create novel features for further liposome manipulation and formation of unique liposome populations to create a fully functional "pharmacy-on-a-chip" for on-demand liposomal drug production.

REFERENCES

[1] J. Wang, J. D. Byrne, M. E. Napier, and J. M. DeSimone, "More Effective Nanomedicines through Particle Design," *Small*, vol. 7, pp. 1919-1931, Jul. 2011.

[2] B. T. Ginn, O. Steinbock, and F. Distillers, "Polymer Surface Modification Using Microwave-Oven-Generated Plasma," pp. 8117-8118, 2003.

[3] W. Xia and P. S. Low, "Folate-Targeted Therapies for Cancer," *Journal of Medicinal Chemistry*, vol. 53, no. 19, pp. 6811-6824, 2010.

[4] A. Jahn, W. N. Vreeland, D. L. DeVoe, L. E. Locascio, and M. Gaitan, "Microfluidic directed formation of liposomes of controlled size," *Langmuir: The ACS Journal of Surfaces and Colloids*, vol. 23, no. 11, pp. 6289-6293, May 2007.

[5] S. Ramachandran, A. P. Quist, S. Kumar, and R. Lal, "Cisplatin Nanoliposomes for Cancer Therapy: AFM and Fluorescence Imaging of Cisplatin Encapsulation, Stability, Cellular Uptake, and Toxicity," *Langmuir*, vol. 22, pp. 8156-8162, Sep. 2006.

[6] H. Hillaireau and P. Couvreur, "Nanocarriers' entry into the cell: relevance to drug delivery," *Cellular and Molecular Life Sciences: CMLS*, vol. 66, no. 17, pp. 2873-2896, Sep. 2009.

[7] L. A. Maguire, H. Zhang, and P. A. Shamlou, "Preparation of small unilamellar vesicles (SUV) and biophysical characterization of their complexes with poly-l-lysine-condensed plasmid DNA," *Biotechnology and Applied Biochemistry*, vol. 37, no. Pt 1, pp. 73-81, 2003.

CONTACT

Don L. DeVoe, tel: +1-301-405-8125, ddev@umd.edu