Controlled Encapsulation of a Hydrophilic Drug Simulant in Nano-Liposomes Using Continuous Flow Microfluidics

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ABSTRACT

A new method to tailor the size and size distribution of nanometer scale liposomes and control loading of liposomes with a model drug in a continuous-flow microfluidic design is presented. Size and size dispersion are determined with tandem Asymmetric Flow Field Flow Fractionation (AF^4) and Multi-Angle Laser Light Scattering (MALLS). Fluorescence Correlation Spectroscopy (FCS) combined with Fluorescence Cumulant Analyis (FCA) allow for determining the number of encapsulated molecules [1]. Results show that this system allows for control of loading efficiency as well as minimization of encapsulant consumption.

Keywords: liposomes, encapsulation, fluorescence spectroscopy, hydrodynamic focusing, microfluidics

1. INTRODUCTION

Liposomes are a promising example of nanoparticles for a wide variety of medical applications including quantized reagent packets for the delivery of drugs, therapeutic agents and proteins [2-4], the encapsulation of contrast agents for enhanced magnetic resonance imaging [5], and model systems for the study of biological membranes. Traditional liposome preparation methods are mostly conducted through mixing of bulk phases, leading to inhomogeneous chemical and/or mechanical conditions during the formation process, hence producing liposomes that are often polydisperse in size and lamellarity. Traditional methods are often accompanied with additional process steps such as membrane extrusion or sonication to yield the desired homogenous liposome populations. A previously reported microfluidic method to produce homogeneous liposome populations omitting size-altering post processing steps is applied to determine its feasibility for controlled encapsulation of a hydrophilic drug simulant [6,7].

A stream of lipids dissolved in isopropyl alcohol is hydrodynamically focused and sheathed between two oblique aqueous buffer streams in a microfluidic channel as shown in Figure 1. The laminar flow in the microfluidic channel enables controlled diffusive mixing at the liquid interfaces causing the lipids to be insoluble and self-assemble into vesicles. While in traditional bulk mixing techniques (e.g., test tubes, macroscale fluidic containers, and mixers), individual fluid elements experience uncontrolled mass transfer profiles and mechanical stresses. Microfluidics enable precise and reproducible control of the flow conditions and hence reproducible fluidic mixing on the micrometer length scale. This reproducibility allows for facile adjustment of the lipid self-assembly and the resultant liposome size and size dispersion. Using this technique, the liposome size is tunable over a mean diameter of 50 nm to 150 nm by adjusting the aqueous to solvent volumetric flow rate ratio.

For drug delivery applications, the encapsulation efficiency needs to be controlled as well; further, it is desirable to minimize the waste of the encapsulant not incorporated into the interior aqueous space of the liposome. In contrast to conventional mixing techniques, where liposomes are formed in a bulk aqueous solution containing a homogenous concentration of the water-soluble encapsulant, the microfluidic method allows confining the encapsulant to the immediate vicinity where lipids self-assemble into liposomes and concomitant encapsulation is expected to occur. Here we report on the ability to control the loading efficiency, which is the concentration of molecules encapsulated in liposomes with respect to a starting concentration, by adjusting the flow parameters in the microfluidic channel network.

2. EXPERIMENTAL SECTION[1]

2.1 Device Fabrication

Microfluidic channels were fabricated in a silicon wafer as previously described [7]. Briefly, a microchannel network was transferred with photolithographic techniques onto a silicon wafer (76.2 mm diameter, 305 µm to 355 µm thick, Nova Electronics Materials, Inc., Carollton, TX), which was subsequently etched with deep reactive ion etching and sealed by anodic bonding to a borosilicate glass

[1] Certain commercial materials and equipment are identified in order to adequately specify the experimental procedures. In no case does such identification imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the items identified are necessarily the best available for the purpose.

wafer. The resulting channels have a rectangular cross section with a depth of 100 µm and a width of 42 µm or 64 µm. Commercially available Nanoports (F-124S, Upchurch Scientific, Oak Harbor, WA) were bonded to the back side of the silicon wafer. Polyetherketone (PEEK) tubing connected the Nanoports to a syringe. All fluidic reagents were injected through a 0.2 µm syringe filter (Anatop, Whatman, NJ) by syringe pumps (model PHD 2000, Harvard Apparatus Inc., Holliston, MA).

2.2 Preparation of Lipid Mixture and Model Drug

Dimyristoylphosphatidylcholine (DMPC), cholesterol (both Avanti Polar Lipids Inc., Alabaster, AL), and dihexadecyl phosphate (DCP) (Sigma Aldrich) in a molar ratio of 5:4:1 were dissolved in chloroform (Mallinckrodt Baker Inc., Phillipsburg, NJ). The chloroform solvent was evaporated under a stream of nitrogen to form a dry lipid film on the bottom of a scintillation vial. The vials were subsequently stored in a desiccator for at least 24 h to ensure complete solvent evaporation. The dried lipid mixture was redissolved in 4 mL dry isopropyl alcohol (IPA) at a 5 mmol/L concentration of total lipid. Phosphate buffered saline (PBS) solution (10 mmol/L phosphate, 2.7 mmol/L potassium chloride, 130 mmol/L sodium chloride, 3 mmol/L sodium azide, pH 7.4) was used as a hydration buffer. Sulforhodamine B (SRB) dye was dissolved at 0.5 mmol/L concentration in phosphate buffered saline.

2.3 Liposome Preparation

Nanoscale liposomes were prepared with a flow focusing technique described earlier [6,7]. Briefly, a lipid mixture dissolved in IPA is injected into the left center channel of the microfluidic network shown in Figure 1. SRB dye dissolved in PBS is injected into the two inner side channels and PBS without SRB dye into the two outermost side channels all intersecting with the center channel. Liposome size and size distribution studies were performed at a constant volumetric flow rate (VFR) of 100 µL/min and an aqueous to solvent flow rate ratio ($FRR_{A/S}$) varying from 14 to 39, defined as the ratio of the overall buffer VFR_A, Q_A, to IPA VFR_S, Q_S. The VFRs of PBS in the inner and outer side channels were identical. Sulforhodamine B was present only in the PBS of the inner side channels at a concentration of 0.5 mmol/L. Encapsulation studies were performed with a constant $FRR_{A/S}$ of 35 and a total VFR of 200 µL/min. The total SRB dye content in the system was varied between 5 v/v % to 40 v/v % of the total liquid volume by adjusting the VFRs of the outer and the inner side channels, respectively. A volume of 500 µL of the liposome effluent was collected from the outlet channels of the microfluidic network and subsequently filtered through a polyacrylamide column with a MWCO of 6 kDa to remove non-encapsulated SRB.

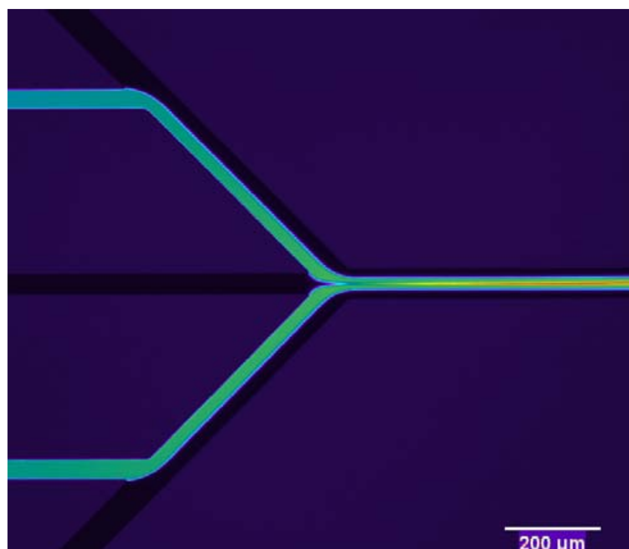

Figure 1: False color confocal fluorescent micrograph of the microchannel network showing the fluorescent intensity of 0.5 mmol/L SRB in PBS injected into the left two inner side channels. PBS without SRB dye is injected into the left two outer side channels, and lipid mixture is injected into the left center channel.

2.4 Light Scattering and Asymmetric Flow Field Flow Fractionation (AF4)

High resolution separation of the liposome population according to their hydrodynamic radius and subsequent size distribution analysis was performed using AF^4 with multiangle laser light scattering (MALLS) and quasi-elastic light scattering (QELS) (model DAWN EOS and QELS, Wyatt Technology, Santa Barbara, CA) as previously described [7]. The liposome radii of the eluted fractions were monitored using MALLS and a QELS detector with data processing using vendor supplied software (ASTRA, Wyatt Technology, Santa Barbara, CA).

2.5 Fluorescent Spectroscopy

Fluorescence measurements were performed on an inverted microscope operating *via* confocal detection as described earlier [1]. A frequency-doubled Nd:YAG laser operating in continuous wave mode at 532 nm was sent through the back aperture of a water-immersion objective to excite SRB dye molecules. Liposome and free dye samples were prepared by transferring 100 µL of solution into a well formed in a microscope slide. An avalanche photodiode detects the photons emitted from the SRB dye. The detected photons create transistor-transistor logic (TTL) pulses that are recorded with a data acquisition card and subsequently analyzed to create a photon counting histogram and fluorescence autocorrelation for 10 second time intervals. For each sample, we collect 20 to 25 ten-second intervals of data in order to perform ensemble

averaging and to extract standard deviations for each average. All results are reported with standard error bars at the 95 % confidence interval. The free dye brightness, Q_{SRB}, is calibrated with 7 to 10 separate measurements of approximately 1 nmol/L SRB in PBS. The autocorrelation of each liposome sample is used to extract the average number of background dye molecules, N_d, contained within the laser excitation volume. It is assumed that a liposome containing n SRB molecules will be n times brighter than a free SRB molecule as long as the concentrations are within the non-self quenching regime of SRB. For an ensemble of liposomes, each having an integer number of SRB molecules, the first two cumulants of the fluorescent signal are given by

$$\langle P \rangle = N_d Q_{SRB} + N_{lip} Q_{SRB} \langle n \rangle \quad \text{and} \qquad (1)$$

$$\langle \Delta P^2 \rangle - \langle P \rangle = N_d Q_{SRB}^2 + N_{lip} Q_{SRB}^2 \langle n^2 \rangle \quad , \qquad (2)$$

where $\langle n \rangle$ is the average number of molecules contained within a liposome, $\langle P \rangle$ is the average number of photons detected per unit time, Q_{SRB} is the free dye molecular brightness measured in the number of detected photons per molecule per unit time, N_{lip} is the average number of liposomes regardless of how many molecules they contain within the laser excitation volume, and $\Delta P = P - \langle P \rangle$. Equations (1) and (2) can be manipulated to arrive at the following expression,

$$J = \frac{\langle n^2 \rangle}{\langle n \rangle} = \frac{1}{Q_{SRB}} \frac{\langle \Delta P^2 \rangle - \langle P \rangle - N_d Q_{SRB}{}^2}{\langle P \rangle - N_d Q_{SRB}} \quad . \qquad (3)$$

In this work we assume that all liposomes in a sample are equally bright so that $\langle n^2 \rangle = \langle n \rangle^2$ and therefore J is equal to the average number of molecules contained within the liposomes. A more realistic approach assumes a non-zero variance for the distribution of encapsulated molecules, but this is beyond the scope of this work.

3 Results and discussion

Hydrodynamic focusing allows for fast and controlled mixing in a microfluidic format with the benefit of reduced sample consumption. The flow conditions in the micro channels are laminar with Reynolds numbers Re of about 20, which allows for mixing based entirely on molecular diffusion in a direction normal to the liquid flow streamlines. Increasing the $FRR_{A/S}$ reduces the focused lipid solvent stream (center stream) width and thereby the diffusion length for mixing of the lipid solvent stream and the aqueous side streams, thus reducing the distance in the center channel distal from the mixing intersection in order to reach the critical alcohol concentration [7]. At a critical alcohol-to-water ratio the lipid monomers become insoluble and spontaneously self-assemble into closed spherical structures sequestering the surrounding fluid. In this way formed liposomes resemble kinetic equilibrium structures that vary in radii according to the force field and chemical conditions in the microchannel. As shown in Figure 2, it is possible to control the mean geometric diameter from about 68 nm to 34 nm by reducing the $FRR_{A/S}$ from 14 to 39 at a VFR of 100 µL/min.

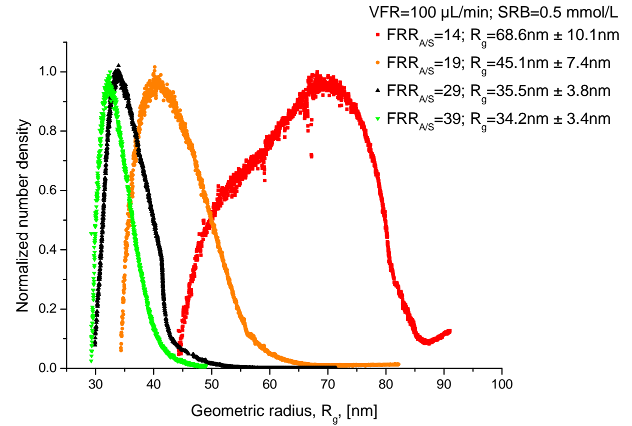

Figure 2: Liposome size and size distribution for different $FRR_{A/S}$ at a constant total VFR of 100 µL/min. Increasing $FRR_{A/S}$ from 14 to 39 results in a decrease of liposome mean geometric radius from about 68 nm ± 10.1 nm to 34 nm ±3.4 nm (one standard deviation).

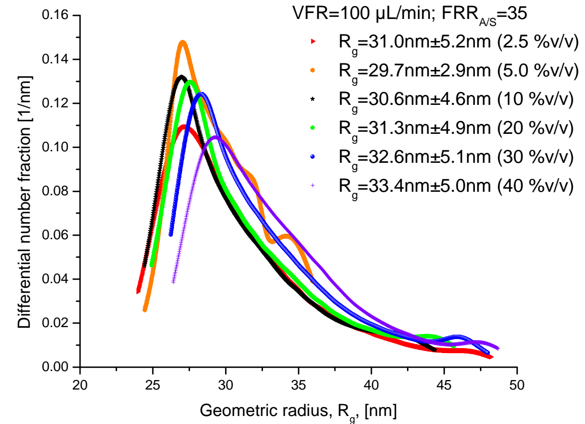

Figure 3: Reproducible liposome size and size distribution at constant VFR of 100 µL/min and $FRR_{A/S}$ of 35 for 6 different volume fractions of SRB in the total sample volume.

Control of the loading efficiency of SRB into liposomes was studied by injecting different volume fractions of SRB (2.5 v/v %, 5 v/v %, 10 v/v %, 20 v/v %, 30 v/v %, 40 v/v %) with a total VFR of 200 µL/min. In

addition to changing the number of molecules loaded into the liposomes, a reduced volume fraction of SRB was determined that allowed encapsulation without adversely affecting the loading efficiency of liposomes. Figure 3 shows the liposome size and size distribution obtained during the encapsulation studies. It further demonstrates the high reproducibility of liposome formation achievable in a microfluidic format.

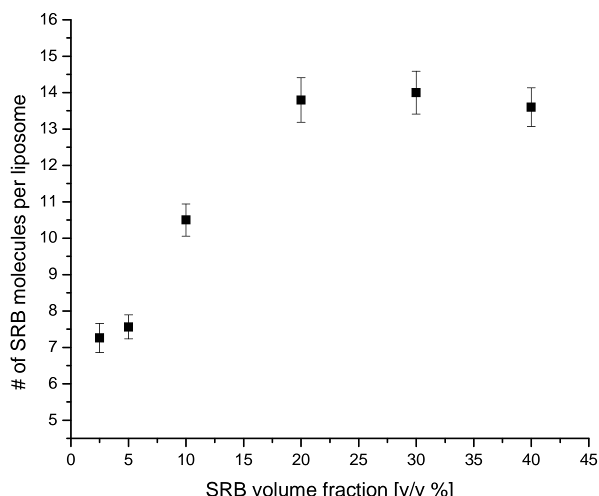

Figure 4: Data shows the number of SRB molecules per liposome along with the 95 % confidence interval. We assume that the average number of encapsulated molecules is equal to the parameter *J*. Reducing the SRB volume fraction reduces the number of encapsulated molecules from about 14 to 7.

Figure 4 shows that the volume fraction of SRB can be reduced significantly before a moderate change in the amount of encapsulated dye is detected. A 40-fold reduction in SRB content reduces the total number of encapsulated SRB molecules by about a factor of 2. In contrast to common batch fabrication methods, microfluidics has the ability to spatially localize the encapsulant to the immediate vicinity where encapsulation is expected and thereby reduce the encapsulant waste substantially without adversely affecting the liposome's loading efficiency. Control over loading efficiency of SRB into liposomes can be achieved below 20 v/v % of SRB of the total sample volume. This allows for tuning the loading efficiency beginning from an initial SRB concentration. The control over loading efficiency is again facilitated by the laminar flow conditions in the microchannel which enables controlled diffusive mixing at the liquid interfaces prior to mixing with the center stream. In macro scale batch processing this can only be achieved by replacing the entire buffer volume. Filling all side channels with PBS containing SRB is representative of common batch processing.

In addition to reduced sample consumption, which is desired from an economical standpoint, the continuous flow microfluidic approach allows the control of the concentration of the substance to be encapsulated from an initial starting concentration *via* controlled diffusive mixing. This enables fine control over the loading efficiency of a drug simulant into liposomes.

4 Conclusion

The formation of liposomes and encapsulation of a hydrophilic drug simulant (SRB) has been demonstrated using a microfluidic technique. It was shown that microfluidics enables reproducible and fine control over liposome size and size distribution, tunable loading efficiency of liposomes, and much reduced encapsulant consumption without adversely affecting the loading efficiency by confining the encapsulant to the region of interest. The simplicity of this liposome formation and drug encapsulation strategy could allow for implementation in point-of-care drug encapsulation, eliminating shelf life limitations of liposome preparation and reducing encapsulant consumption. Fluorescence correlation spectroscopy for encapsulation efficiency measurements has the potential for integration in future lab-on-a-chip applications for online liposome characterization.

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