Preparation of Nanostructured Lipid Drug Delivery Particles Using Microfluidic Mixing

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Abstract: Background: Cubosomes are highly ordered self-assembled lipid particles analogous to liposomes, but with internal liquid crystalline structure. They are receiving interest as stimuli responsive delivery particles, but their preparation typically requires high energy approaches such as sonication which is not favourable in many applications. 

Objective: Here we investigated the impact of microfluidic preparation on particle size distribution and internal structure of cubosomes prepared from two different lipid systems, phytantriol and glyceryl monooleate (GMO).

Methods: The impact of relative flow rates of the aqueous and organic streams, the total flow rate and temperature were investigated in a commercial microfluidic system. The particle size distribution and structure were measured using dynamic light scattering and small angle X-ray scattering respectively.

Results: Phytantriol based particles were robust to different processing conditions, while cubosomes formed using GMO were more sensitive to composition both locally and globally, which reflects their preparation using other techniques.

Conclusion: Thus, in summary microfluidics represents a reproducible and versatile method to prepare complex lipid particle dispersions such as cubosomes.

Keywords: Cubosome, glyceryl monooleate, lipid drug delivery, microfluidics, phytantriol, small angle X-ray scattering, solvent dilution.

1. INTRODUCTION

The interest in lipid-based drug delivery particles is growing as the field moves from passive delivery particles towards responsive materials [1]. Self-assembled lipid systems in particular are thermodynamically stable and provide a platform to enable stimuli responsive behaviour which is useful for drug delivery applications where the on-demand release is desirable. Thermally activated liposomes are in development as one example of such a system, where the lipid self-assembly is responsive to temperature changes at the tumour site to stimulate release of drug [2]. While liposomes have been translated through to the market, their preparation is complex and attempts to standardise and simplify their manufacture have led to developments including microfluidic mixing on a manufacturing scale.

Analogous to liposomes, other nanostructured lipid particles have also shown promise as stimuli responsive systems. Of these systems, dispersed...
cubic phase particles, known as cubosomes, have received interest in applications across a range of therapeutic areas [3, 4]. The high internal lipid content means that in addition to the internal water channels for hydrophilic drugs, there is also a high capacity for dissolving hydrophobic and amphiphilic substances. Adding responsive elements to the particles, such as light [5-7], magnetic [8] and pH responsive components [9, 10] enables control over the nanostructure in response to these stimuli.

As mentioned for liposomes, a major hurdle in their translation is effective, reproducible preparation of stable dispersions of particles. The formation of cubosome dispersions is also compromised by the need to include a colloidal stabiliser - most often this is a block copolymer such as a commercial Pluronic, although other stabilisers such as lipid-based surfactants [11], proteins [12] and custom polymers [13, 14] have also been reported. Previous studies have primarily used sonication or high pressure homogenisation as a means to prepare the particles which are high energy techniques that are problematic for labile lipids or cargo and can be difficult to conduct in a reproducible manner due to the inherent chaotic mixing and shear applied. Lower energy dilution approaches have also been reported, including the enzymatic ‘side-ways’ approach introduced by Fong [15] and Hong [16], in which an emulsion precursor is digested in order to form cubic phase particles. The low energy ‘hydrotrope dilution’ approach reported by Spicer [17], in which a solvent-based solution is diluted into the two-phase cubic phase and excess aqueous solution regime also formed cubosomes, however the particles were relatively polydisperse, and relied on a rotor-stator shear step or hand mixing, both of which would be difficult to control in a reproducible manner suitable for satisfying pharmaceutical regulatory requirements.

Therefore, if cubosomes are to make an impact as novel therapeutic systems, there needs to be increased focus on controlling particle preparation methodologies. The hydrotrope dilution method avoids the influence of high energy processing and is analogous to approaches reported for the preparation of liposomes using microfluidic mixing of a solvent and antisolvent stream [18, 19]. With the aforementioned advances in microfluidic approaches with potential for scale-up in mind, in this study a commercial staggered herringbone microfluidic mixing system was used to understand the influence of formulation composition and processing variables on particle size distribution and internal structure of the putative cubic phase particles. Specifically, the effects of total flow rate, flow rate ratio, lipid to ethanol ratio and operating temperature on dispersion properties were investigated. Particle size distributions were determined using dynamic light scattering and the internal structure was verified using small angle X-ray scattering (SAXS). The results highlight the potential to prepare cubosomes using a commercially available and scaleable microfluidic system.

2. MATERIALS AND METHODS

2.1. Materials

Glyceryl monooleate (GMO, 1,3-dihydroxy-2-propanyl (9Z)-9-octadecenoate; Myverol™ 18-99K) was obtained from Kerry Ingredients, Singapore. Myverol contained approximately 65% GMO and has previously been shown to form bidicontinuous cubic phase is excess water at ambient temperatures. Phytantriol (PHYT; 3,7,11,15-tetramethyl-1,2,3-hexadecane-triol, >95% pure by gas chromatography) was obtained from DSM Nutritional Products Ltd. (Basel, Switzerland) and Pluronic F127 was from Sigma-Aldrich Co. (MO, USA). Water used in this study was purified by a Milli-Q system (0.22 μm membrane filter, Millipore, Sydney, Australia). Absolute ethanol (AR grade) used in the analysis was from Merck KgaA (Darmstadt, Germany).

2.2. Preparation of Solvent and Antisolvent Precursor Solutions

In all experiments, lipid (GMO or phytantriol) was first dissolved in ethanol as the solvent/hydrotrope. The mixture was vortexed and warmed in an oven at 40°C to ensure the lipid was dissolved to form cubic phase particles. The low energy ‘hydrotrope dilution’ approach reported by Spicer [17], in which a solvent-based solution is diluted into the two-phase cubic phase and excess aqueous solution regime also formed cubosomes, however the particles were relatively polydisperse, and relied on a rotor-stator shear step or hand mixing, both of which would be difficult to control in a reproducible manner suitable for satisfying pharmaceutical regulatory requirements.

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2.3. Particle Preparation by Microfluidics

The NanoAssemblr™ (Precision NanoSystems, Vancouver, CA) commercial microfluidics systems was used to prepare the particle dispersions by controlled, bottom-up microfluidic mixing. The NanoAssemblr was fitted with an aluminium chip holder, heating block and heating controller. The standard staggered herringbone mixing commercial chips made from cyclic olefin copolymer (COC) polymer were used as illustrated in Fig. (1). Before each experiment, the microfluidic chip was washed with ethanol and then with water. The lipid-ethanol solution was drawn into a 1-mL Terumo syringe while the aqueous F127 solution was drawn into a 3-mL syringe. Using the NanoAssemblr control software, the total volume collected was set to 1.5-3.0 mL. Start and end waste collection was set to 0.25-0.50 and 0.05-0.10 mL respectively. Total flow rate and flow rate ratio were also adjusted according to experimental requirements. The dispersions were collected in clean plastic 12 mL polypropylene tubes and placed in a vacuum oven at room temperature overnight to remove the ethanol (verified by weight).

2.4. Investigation of Formulation and Processing Variables

2.4.1. Total Flow Rate (TFR)

TFR is the sum of the flow rates in the two streams (solvent/organic and antisolvent/aqueous). This parameter was set in the NanoAssemblr software with other parameters detailed in Table 1.

Table 1. Experimental details and final concentration of components in determining effect of total flow rate (conc.: concentration; temp.: temperature).

<table>
<thead>
<tr>
<th>Variable: Total flow rate (ml/min)</th>
<th>GMO</th>
<th>Phytantriol</th>
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<tbody>
<tr>
<td>Lipid conc. 12, 14, 16, 18, 20, 22, 24</td>
<td>5% (w/v)</td>
<td>12, 16, 20, 24</td>
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<tr>
<td>Ethanol conc. 3.82% (w/v)</td>
<td>3.66% (w/v)</td>
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<tr>
<td>F127 conc. 0.556% (w/v)</td>
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<tr>
<td>Flow rate ratio 1:9 (v/v)</td>
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<tr>
<td>F-127:lipid ratio 1:9 (w/w)</td>
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<td>Temp. Room temperature</td>
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2.4.2. Flow Rate Ratio (FRR)

FRR is the volume ratio of lipid-ethanol stream to aqueous F127 stream. This parameter was also set in the NanoAssemblr software with other parameters in Table 2.

2.4.3. Lipid: Ethanol Ratio

Lipid: ethanol ratio is the weight ratio of lipid in the ethanolic stream. The experimental parameters for exploring this variable are shown in Table 3.

2.4.4. F127: Lipid Ratio

F127: lipid ratio is the final weight ratio of F127 to lipid which is already known to be an important factor in colloidal stability [21]. Other experimental parameters are also shown in Table 4.

2.4.5. Operating Temperature

Operating temperature is the temperature of the heating block adjusted by the heating controller. Products were kept in the desired temperature dur-
ing microfluidic mixing process. After formation, the products were cooled to room temperature. This ensured that the only factor affecting particle size and PDI would be operating temperature. Table 5 summarizes the experimental details.

### 2.4.6. Dynamic Light Scattering

Particle size and polydispersity index (PDI) were determined by dynamic light scattering (DLS) using a Malvern Zetasizer Nano-ZS from Malvern Instruments Ltd. (UK). Before particle size measurement, the dispersions prepared using microfluidic mixing were vortexed and diluted at 1 drop of dispersion (~20 μL) in 1 mL of water immediately prior to measurement. Equilibrium time and temperature of measurement was set as 60 seconds and 25°C, respectively. The number of runs was set to be automatic (usually 11-17 runs) and each run lasted for 10 seconds. Volume-weighted particle size was adopted in this study as it can better reveal the existence of larger particles. To ensure data precision and reproducibility, three measurements of particle size and PDI were obtained, with mean and standard deviation calculated.

### 2.4.7. Synchrotron Small-angle X-ray Scattering

Select samples were studied using SAXS to confirm structural characteristics of the formed particles with each variable. SAXS measurements
were conducted at the SAXS/WAXS facility at the Australian Synchrotron (Melbourne, Australia) [22]. Samples were injected into capillaries (1.5 mm diameter, Charles Supper, Natick, MA) at room temperature (25°C) and placed in the beam. The incident X-rays had a wavelength 0.95 Å (energy = 13 keV) and the detector (Pilatus 2M with active area of 254 × 289 mm² with 172 μm pixels) was positioned 1200 mm from the sample, determined by calibration against silver behenate, which provided a $q$ range of $0.09 < q < 0.99$ Å$^{-1}$. The two-dimensional scattering patterns were acquired over 1 sec and integrated into a one-dimensional scattering function $I(q)$ using the in-house software, Scatterbrain (version 1.15). It should be noted that some minor peak splitting was evident as the diffraction patterns were obtained on a newly installed detector system that had not yet been fully commissioned. Identification of the nanostructures from the scattering function was determined by correlating the relative spacing ratios between the positions of the Bragg peaks to literature [23].

3. RESULTS

3.1. Particle Formation from GMO Precursor Solution

Fig. (2) illustrates the impact of the variables studied on the particle size distribution of the
GMO-based systems. The particle size was generally between 100 and 300 nm with a generally low polydispersity (<0.2) being achieved at certain experimental conditions for each variable measured. Although there were some subtle trends, overall the particle formation was quite insensitive to the five variables studied. A higher TFR and lower temperature favoured smaller particles being formed. A lower lipid:solvent ratio also favoured the formation of smaller particles as might be anticipated. The particle size was less sensitive to the effect of flow rate ratio and the GMO:F127 ratio, but with a minimum in PDI appearing at intermediate ratios of 5-6 in both cases. Overall the data reflect that uniform dispersions were readily obtained using the microfluidics approach with GMO as the core lipid.

Fig. (3). SAXS profiles of GMO dispersions prepared under different conditions of lipid: Pluronic F127 concentration, and different processing temperature. The major cubic phase peaks corresponding to the Im3m ‘primitive-type’ bicontinuous cubic phase structure are indicated.

SAXS was used to probe the particle internal structure under differing preparation conditions and compositions. GMO-based dispersions prepared at close to room temperature (in this case 30°C) with a GMO:Pluronic ratio of 9:1, had particles with an internal structure of the Im3m ‘primitive-type’ bicontinuous cubic phase structure, indicated by the spacing ratios for the main peaks at $\sqrt{2}$, $\sqrt{4}$, $\sqrt{6}$ (Fig. 3, top profile). This is entirely consistent with previous reports for this system [24, 25]. Increasing the processing temperature to 55°C led to the reduced definition of the peaks (middle curve), likely due to degradation of the lipid (although they were still apparent). With a higher Pluronic concentration (bottom curve), additional peaks were evident, likely indicating a different co-existing cubic phase, of the $Pn3m$ “double diamond” type (see peak ratios below for PHYT systems).

3.2. Particle Formation from PHYT Precursor Solution

Overall, the phytantriol systems produced particles that were generally larger and more polydisperse than those prepared from GMO under the same conditions (Fig. 4). As with GMO, increasing the total flow rate had a positive effect on mean particle size (Panel A). The polydispersity index displayed a minimum around 20 mL/min consequently this TFR was selected for other experiments. The lipid:solvent ratio again had a large impact on size, with a greater lipid content leading to larger mean particle size. Operating temperature did not have a significant effect on particle size distribution unlike the GMO-based systems.

The SAXS profiles for the phytantriol-based dispersions displayed peaks reflecting the production of cubosomes (Fig. 5). The bicontinuous cubic phase was a different space group to that of GMO-
Fig. (4). Mean volume-weighted particle size (with line) and polydispersity index (without line) of PHYT-based cubosomes synthesized by microfluidic mixing. The variables were in Panel A. Total flow rate; B. Flow rate ratio; C. PHYT: ethanol ratio; D. F127: PHYT ratio; E. Operating temperature. The positive and negative error bars show standard deviations for three separate measurements.

based dispersions, displaying peaks at spacing ratios of $\sqrt{2}: \sqrt{3}: \sqrt{4}$ which indicates the $Pn\bar{3}m$ ‘double-diamond’ space group. Again, this is consistent with previous studies on phytantriol cubosomes stabilised using F127 [24]. Compared to profiles for the GMO samples in Fig. (3), the changes in processing temperature or Pluronic F127 concentration did not have any significant effect on the internal structure of the particles, with only very minor differences in peak positions being apparent.

4. DISCUSSION

The use of microfluidics for the synthesis of nanoparticles has been increasingly explored over traditional approaches due to the control over par-
Microfluidics operates using smaller volumes of materials which can improve control over the manipulation of sample and yield more reproducible nanoparticles [26]. By manipulating simple device parameters such as increasing the flow rate ratio between the aqueous phase and lipid phase, for example, the particle size of the liposomes produced can be tuned [18]. Additionally, microfluidics also enables high encapsulation efficiencies [19, 27], can improve mixing efficiency, and is a compact and convenient device. Preparation of cubosomes has been reported in custom herringbone systems [28], however their synthesis was yet to be conducted in the NanoAssemblr. In the device fabricated by Kim et al., there was a Y-junction similar to that of the Precision Nanosystems Inc. chip where the solutions converged. The herringbone structure in their device was featured on a short and straight channel that facilitated the mixing of the GMO-based cubosomal system within 0.6 s when operating at 0.05 mL/min which was slower than the NanoAssemblr device (approximately 0.002 s at 20 µL/min). In either devices, cubosomes did not form immediately after discharge from the microfluidic chip and the final mixture was subjected to further evaporation of the precursor solvent which resulted in the gradual formation of the cubic phase [28]. In addition to the difference in mixing times, the device was also fabricated with polydimethylsiloxane (PDMS) which is hydrophobic and has potential for fouling by the hydrophobic lipids, although the same disadvantage is potentially present in using the NanoAssemblr chips as they are composed of cyclic olefin copolymer (COC). However, the advantage of using the COC-based chip is that the process can be made scalable and more reproducible between batches and devices [29, 30].

When comparing the impact of lipid composition on particle formation and stability, GMO-based systems required more benign conditions and lower stabiliser concentrations to form nanoparticles in the NanoAssemblr chip. Deviation from the expected scattering of the Im3m phase (black scattering profile in Fig. 3) in experiments with an increase in temperature or increase in Pluronic concentrations (red and green scattering profiles in Fig. 3, respectively) demonstrated the susceptibility of the packing of the GMO molecules. Chong et al. found previously that an increase in the proportion of Pluronic F127 in a GMO-based cubic system would result in a distortion of the Pn3m geometry towards the Im3m geometry, which was attributed to the greater association of the hydrophobic regions of the stabiliser.
interfering with the phase behaviour [11]. The temperature was also noted to destroy all cubic structure above 47°C [11].

Unlike liposomes, the particle size of cubosomes was affected by the increase in flow rate ratio and total flow rate but trends were consistent for cubosomes between different lipids. Generally, a decrease was observed for liposomes formed in the NanoAssembldr when the proportion of aqueous phase and the flow rate ratio increased and there was no influence from a change in total flow rates [19, 30-32]. On the contrary, the particle size of cubosomes appeared to trend upwards with an increasing flow rate ratio of aqueous phase (Fig. 2B and 4B), save for the lower flow rate ratio in the GMO-based system. An increase in total flow rate induced a corresponding decrease in particle size which could suggest that the decrease in retention time as the mixture progressed through the staggered herringbone structures did not allow sufficient time for the lipids to self-assemble into the more intricate cubic phases and resulted in the formation of smaller particles. Cubosomes generated by Kim et al. also exhibited a decrease in particle size with increasing flow rates [28]. Nonetheless, the trends observed were consistent between both lipids, suggesting that this could be unique to the cubosomes.

CONCLUSION

This work demonstrates that cubosomes could be reproducibly manufactured by mixing a lipid precursor formulation with an anti-solvent using a commercially available microfluidic device. Variables such as temperature and flow rate ratios could be adjusted to manipulate the size of the cubosomes, affording more control than existing production processes which is advantageous for pharmaceutical applications. Overall, the microfluidic approaches present a new avenue in the preparation of cubosome dispersions and offer an opportunity for the development of cubosomes as the next trending drug delivery system.

LIST OF ABBREVIATIONS

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<table>
<thead>
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<tbody>
<tr>
<td>COC</td>
<td>Cyclic Olefin Copolymer</td>
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<tr>
<td>conc.</td>
<td>Concentration</td>
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<tr>
<td>F127</td>
<td>Pluronic F127</td>
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<tr>
<td>FRR</td>
<td>Flow Rate Ratio</td>
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<tr>
<td>GMO</td>
<td>Glyceryl Monooleate</td>
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<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
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<td>PHYT</td>
<td>Phytantriol</td>
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<tr>
<td>SAXS</td>
<td>Small Angle X-ray Scattering</td>
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<tr>
<td>temp.</td>
<td>Temperature</td>
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<td>TFR</td>
<td>Total Flow Rate</td>
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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The raw data that supports the findings of this study are available from the corresponding author, [Dr. BJB] upon reasonable request.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

The SAXS data was acquired on the SAXS/WAXS beamline at the Australian Synchrotron.

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