Layer-by-Layer Polymer Coating on Discrete Particles of Cubic Lyotropic Liquid Crystalline Dispersions (Cubosomes)

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ABSTRACT

Cubic phase lyotropic liquid crystalline colloidal dispersions (cubosomes) were surface-modified with seven polyelectrolyte layers using a layer-by-layer (LbL) approach. The first layer consisted of a copolymer synthesized from methacrylic acid and oleyl methacrylate for enhanced incorporation within the bilayer of the cubic nanostructure. Six additional layers of poly(L-lysine) and poly(methacrylic acid) were then sequentially added, followed by a novel washing procedure to remove polymer aggregates from the soft matter particles. Polymer build-up was monitored via microelectrophoresis, dynamic light scattering, and small angle X-ray scattering. Polymer coated cubosomes were observed with cryo-transmission electron microscopy. A potential application of the modified nanostructured particles presented in this study is to reduce the burst-release effect associated with drug-loaded cubosomes. The effectiveness of this approach was demonstrated through loading and release results from a model hydrophilic small molecule (fluorescein).
Introduction

When bulk, hydrated amphiphiles of inverse bicontinuous lyotropic liquid crystalline cubic phase are dispersed in the presence of a steric stabiliser under high shear, nanostructured particles known as cubosomes may form. These dispersed colloidal particles have a diameter typically between 100 and 300 nm, are mechanically rigid, structurally stable, and can be biodegradable. They can accommodate significant amounts of hydrophilic, amphiphilic, and/or lipophilic molecules due to their large interfacial surface area per unit volume, making them suitable candidates for use as drug delivery vehicles. The external surface of these particles is generally coated with uncharged macromolecules such as poly(ethylene oxide) and poly(propylene oxide) block copolymers which provides sterically induced stability.

Although the bulk cubic phase has shown promise as a controlled drug delivery system, in-vitro release studies from cubic phase lyotropic liquid crystalline colloidal dispersions have frequently indicated that the large surface area of the colloidal dispersions may contribute to an immediate burst release of the entire loaded cargo, particularly for small hydrophilic drugs. As the cubic nanostructured particles become smaller, the surface area increases greatly, and a large proportion of the cargo may release rapidly from the colloidal particles. This burst-release behaviour is typically described as diffusion limited. Although burst release can be desired in some cases, such as for encapsulated flavours or topical wound treatment, the amount of initial release can be unpredictable, and may cause systemic toxicity and/or a reduced lifetime of the delivery device.

The layer-by-layer (LbL) technique can create polymer-coated core templates by depositing alternating polyelectrolytes of positive and negative charge on silica or other substrates. Typically, the template is then removed and a polymer-shell capsule remains, which can be
loaded with therapeutic agents. These particles may exhibit tuneable semi-permeability, high chemical stability, and a functionalisable outer shell wall to create stealth and/or active targeting properties.\textsuperscript{22} However, depending on the system, core removal may lead to mechanical stress, particle aggregation, or leftover residues from the dissolved template.\textsuperscript{19, 23}

By utilising the LbL technique to coat the exterior of discrete cubosomes with polyelectrolytes (Figure 1), the interfacial charge on the surface of the particles may be readily altered, potentially contributing to more desirable interactions between the particles and their external environment.\textsuperscript{24} Additionally, minimising the exposed surface area of the cubosome carriers by coating them with semi-permeable polymer layers may provide a reduction in the initial burst effect, leading to a more controlled drug release pattern.\textsuperscript{25} As an added benefit, the drug-loaded soft cubosome templates do not require dissolution after LbL polymer deposition, thus potentially increasing their stability.

![Figure 1](image.png)

**Figure 1.** Schematic diagram of a discrete cubosome coated with a polymer bilayer to encapsulate the lipid nanostructure. In practice cubosomes can retain internal cubic periodicity while for example being either spherical or cubic in particle shape depending on processing conditions.

In this study, monoolein cubosomes were layered with up to seven polymer layers of poly(methacrylic acid) (PMA) and poly(L-lysine) (PLL). The amphiphilic copolymer,
poly(methacrylic acid-co-oleyl methacrylate) (PMAO),\cite{26} was utilised as a first layer to enhance surface interaction with the cubosomes based on the hydrophobic groups present in the polymer. After polymer layering, a simple yet novel centrifugation technique for removing built up free-floating polymer aggregates was also developed. Polymer coating buildup was monitored via microelectrophoresis (ζ-potential), dynamic light scattering (DLS), and small angle X-ray scattering (SAXS). Coated cubosomes were observed using cryo-transmission electron microscopy (cryo-TEM) and negative stain TEM. Preliminary drug-loading and release properties of the coated cubosomes were examined using fluorescein as a model hydrophilic small molecule in comparison to non-coated cubosomes.
Materials and Methods

Materials

Pluronic F127 (P.F127), 1-oleoyl-rac-glycerol (monoolein, MO), phosphate buffered saline (PBS), poly(L-lysine hydrobromide) (15 - 30 kDa, PLL), and hydrophilic fluorescein NaCl (MWT: 376.28) were purchased from Sigma-Aldrich. Poly(methacrylic acid) (15 kDa, PMA) was purchased from Polysciences. Ultrafiltration centrifuge tubes with MW cutoff pores of 100 kDa were obtained from Pall. Cellulose ester dialysis tubing of 15 kDa was purchased from Spectrum. All aqueous solutions were prepared with ultrapure water (Millipore Milli-Q™, 18.2 MΩ cm⁻¹).

The synthesis of poly(methacrylic acid-co-oleyl methacrylate) (PMAO, Figure 2) by reversible addition–fragmentation chain transfer (RAFT) polymerisation has recently been reported elsewhere.²⁶ The average percentage of oleyl methacrylate (OleoylMA) to methacrylate (MA) was 2 mol% [(MA)₂₁⁷-co-(OleoylMA)₄, PMAO, Mn = 20900 Da, PD = 1.20].
Figure 2. Chemical structure of RAFT synthesized poly(methacrylic acid-co-oleyl methacrylate).

Cubosome Preparation

For polymer layering experiments, single samples of MO cubosomes were created in 1 mL batches in an 1.5 ml centrifuge tube, at a concentration of 25 mg mL\(^{-1}\) lipid to water and 0.2 mol% P.F127. The solution was dispersed using an Ultrasonic Processor (Mixsonix, Inc.) for a program of Amplitude = 30, On = 1 s, Off = 1 s for 2 min, followed by 2 min cooling time. This program was repeated seven times for a total sonication “On” time of 7 min.

Drug Loading

For model drug loading experiments, fluorescein was used at a concentration of 1 mg mL\(^{-1}\) in a 50/50 (v/v) mixture of chloroform/methanol. A 25 mg aliquot of monoolein was measured into a
centrifuge tube, dissolved into 200 µL chloroform and the dissolved model compound was mixed in at measured concentrations. The solvent was evaporated under vacuum, and the final mixtures contained 0, 0.5, 1, or 5 mol% fluorescein. These samples were hydrated with 1 mL of ultrapure water containing 0.2 mol% P.F127 to the lipid, and probe sonicated to disperse into nanoparticles. Samples were analysed with SAXS. Any free drug within the system was not removed prior to drug release experiments.

Samples containing 0.5 mol% fluorescein were chosen for further analysis. Each sample contained a final ratio of 1:16 (v:v) PBS (pH 8.4) to water.

**Excess Polymer Separation from Cubosomes**

For experiments in which polymer aggregates were separated out of the coated cubosome mixture by centrifugation, polymers PMAO, PLL and PMA were dissolved at a concentration of 10 mg mL⁻¹. Since PMAO has limited solubility in water, PBS (pH 8.4) was utilised to aid dissolution of the polymer. The increased pH enhances ionisation of the carboxylic acid group of the MA component, allowing the polymer chains to extend into the solution and dissolve completely.²⁷ PMA and PLL were dissolved in ultrapure water unless otherwise stated. An aliquot of 2.5 µL [1 %(w/w)] or 10 µL [4 %(w/w)] PMAO was added to 100 µL of cubosome solution and mixed for 20 min on a vortex mixer adapted to hold centrifuge tubes. The mixture was then placed in a water sonication bath for 5 minutes to aid in dispersing any flocculated cubosomes. The PMAO layer was followed by sequential polyelectrolyte layers of PLL and PMA, applied in the same manner as PMAO, up to a total of seven polymer layers (Figure 3). Once the layering cycles were complete, the aggregates formed within the coated cubosome solution were separated out of the sample by centrifugation for 10 min at 4000 g. The polymer
aggregates separated to the bottom of the tube and the layered cubosome solution was transferred into a new centrifuge tube.

**Figure 3.** Cubosomes were layered with the precursor polymer layer (PMAO), leaving the cubosomes coated in a layer of polymer with excess polymer dissolved in the surrounding solution (a). The next polymer layer was added, and adhered to the PMAO-modified cubosomes. Some also interacted with free PMAO in solution to form aggregates (b). A third polymer was then added, again adhering to the cubosomes and also to the growing polymer aggregates. The polymer layering was repeated until the desired number of layers was achieved (c). Finally, the solution was centrifuged at low speed (d), resulting in polymer aggregates separating to the bottom of the solution while coated cubosomes remained free-floating.
**Drug Partitioning**

Drug-loaded samples were tested to determine the level of drug partitioning into either the cubic phase or the external aqueous region. Cubosome/drug dispersions (75 µL) were filtered for 2 min at 10,000 g through a 100 kDa ultracentrifuge filter to separate the cubosomes from the aqueous solution. To 20 µL of separated drug solution was added 20 µL blank aqueous solution. This mixture was diluted further by adding 50 % (v/v) ethanol and placed in a UV-transparent glass-bottom 384-well plate (Greiner). Calibration samples were prepared using serial dilutions of fluorescein at 50/50 (v/v) ethanol/aqueous solution ratios. Absorbance scans were compared against the calibration curves to determine the total amount solubilised into the aqueous region, and from this the total drug partitioned into the cubic phase could be extrapolated.

**Drug Release Experiments**

Drug release experiments were carried out in 15 kDa dialysis tubing, which was thoroughly washed with ultrapure water and then boiled for 30 min to remove any extractable impurities from the tubing. At each of 6 time points (24 h, 7 h, 4 h, 1 h, 20 min, 5 min) a 100 µL aliquot of solution was loaded into approximately 4 cm of dialysis tubing, clamped at either end, and suspended into a beaker containing 250 mL of aqueous solution (at a concentration of 1/17 PBS, pH 8.4). The solution in the beaker was agitated via stirring for the duration of the experiment to ensure complete mixing and continuous diffusion through the dialysis tubing. At the end of the experiment, the dialysis membranes were removed from solution and the internal cubosome solution was collected. This solution was diluted by 50 % (v/v) ethanol to ensure that any structured nanoparticulate phases were dissolved so as not to interfere with absorption or fluorescence measurements. Some of the original nondialysed cubosome sample was utilised to
create a serial dilution in 50 % (v/v) ethanol. This serial dilution enabled the capture of the 0 min 
time point \( (A_i, \text{ Equation 1}) \), and also created a benchmark for which to measure the amount of 
drug remaining in the sample at each time point. Dialysis experiments were completed twice, and 
each time point was measured in triplicate, giving six total repeats. Samples for analysis were 
loaded into a Greiner 384 well plate at 20 µL per well and centrifuged briefly in an Atlas 
evaporator (Genevac) to remove any trapped bubbles.

Drug loading and release were quantified using a Flexstation 3 plate reader (Molecular 
Devices), which measured fluorescence and absorbance within each sample. Values were 
background corrected using a blank buffer solution. For fluorescein, absorbance monitoring was 
set to the measured maximum at 495 nm and fluorescence to 538 nm. All measurements taken 
were within the measurement range of the instrument. The amount of drug released was then 
calculated based on Equation 1, and plotted versus time.

\[
L = 100 - C = 100 - \frac{A_T}{A_i} \times 100 \quad \text{Equation 1}
\]

\( L \) Amount of drug released from cubosomes into the release medium (%) 
\( C \) Amount of drug remaining inside the cubosomes (%) 
\( A_i \) Amount of drug initially loaded into cubosomes (µg) 
\( A_T \) Amount of drug inside cubosomes at time = T (µg)
Dynamic Light Scattering

The particle hydrodynamic diameter and polydispersity index (PDI) were analysed with a Zetasizer (Nano ZS, Malvern Instruments, UK). DLS measurement samples contained approximately 10 - 20 µL sample in 1.5 mL ultrapure water (approximately 0.6 - 0.7 mg mL⁻¹ lipid/water). Square, 12 mm disposable polystyrene cuvettes were utilised for measurements. The refractive index was set to 1.467, which is the refractive index of pure monoolein. The temperature was set to 25 °C and allowed to equilibrate for 3 min before 3 successive measurements of 11 averaged runs. The data was then analysed using Dispersion Technology Software V5.03 provided by Malvern Instruments. Diameter results are based on intensity averages.

Microelectrophoresis

The electrokinetic potential (ζ-potential) was measured with a Zetasizer (Nano ZS, Malvern Instruments, UK). Measurements were based on the Smoluchowski equation at 25 °C. Samples were diluted to approximately 20 - 30 µL in 1.5 mL water and placed in a disposable capillary cell. Three measurements were performed for each sample.

Small Angle X-ray Scattering

SAXS was performed at the Australian Synchrotron. 2-D diffraction patterns were recorded on a Dectris–Pilatus 1 M detector of 10 modules. Silver behenate (λ = 58.38 Å) was used as the low-angle X-ray diffraction calibrant for all measurements. Samples were pipetted into a 1.5 mm X-ray glass capillary (Capillary Tube Supplies, UK). Exposures were taken at 25 °C for 2 to 13 s at a wavelength of 1.032 Å. The temperature was controlled to within 0.1 °C using a custom
designed cell capable of holding 36 capillaries. The diffraction data was converted from 2-D to 1-D and analysed using aXcess software developed by Dr. A. Heron, Imperial College, London. Measured lattice parameter spacings are accurate to within 0.1 Å.

**Cryo-Transmission Electron Microscopy**

A custom-built humidity-controlled vitrification system was used to prepare the samples for Cryo-TEM. Humidity was kept close to 80 % for all experiments, and the ambient temperature was 22 °C. After glow-discharging in nitrogen to render the carbon film hydrophilic, a 4 µL aliquot of the nanoparticle dispersion was pipetted onto a 300-mesh copper grid coated with lacy formvar-carbon film (ProSciTech). After 30 s adsorption time, the grid was blotted manually using Whatman 541 filter paper for 3 s. The grid was then plunged into liquid ethane cooled by liquid nitrogen. Frozen grids were stored in liquid nitrogen until required.

The samples were examined using a 626 cryoholder (Gatan) and Tecnai 12 transmission electron microscope (FEI-Company) at an operating voltage of 120 kV. At all times low dose procedures were followed, using an electron dose of 8 - 10 electrons per Å² for all imaging. Images were recorded using a Megaview III CCD camera and AnalySIS camera control software (Olympus) at magnifications between 70 000 × and 110 000 ×.

**Negative Stain Transmission Electron Microscopy**

TEM grids were glow discharge treated in nitrogen for 10 s prior to use. Three microlitres of sample were adsorbed onto carbon-coated 300 mesh copper grids (ProSciTech) for 30 s. Excess sample was wicked off and the grid stained with 4 µl of a 2 % (w/w) aqueous uranyl acetate solution. *Caution: uranyl acetate is toxic as well as radioactive and should be handled with care.*
Investigations were undertaken using an FEI Company Tecnai 12 transmission electron microscope (FEI-Company) at an operating voltage of 120 KV, fitted with a Megaview III CCD camera and AnalySIS camera control software (Olympus).
Results and Discussion

Polymer Separation

During LbL deposition, excess polymer is typically removed before addition of the next polymer layer, otherwise dense free-floating polymer aggregates may form. When silica beads are utilised as substrates, the particles sink to the bottom of the centrifuge tube when spun, and the supernatant can be collected and replaced with a polymer-free solution. Several washes are required between each polymer layer.\textsuperscript{22} Although this is time consuming, it is necessary to ensure complete removal of excess polymer. Near complete separation from the dilution media can be achieved and few particles are lost if care is taken when removing the liquid.\textsuperscript{30}

The system used in this study consisted of neat monoolein with a density of 0.97 g cm\textsuperscript{-3}.\textsuperscript{31} However, the high water content of the cubic phase leads to a density much closer to that of water (1.00 g cm\textsuperscript{-3}). As such, it proved very difficult to centrifuge the cubosomes in such a way that the particles separated completely from solution without lipid aggregation due to the high centrifuge speed and spin time required. However, if no washing step was conducted between layering steps (Figure 3, above), dense, free-floating polymer aggregates formed, which could be separated from the aqueous phase by mild centrifugation conditions that did not cause significant aggregation of the coated cubosomes.

Although monoolein and the steric stabiliser Pluronic F127 do not inherently carry a charge, monoolein cubosomes in water appear to exhibit a negative $\zeta$-potential (Figure 4). One suggested explanation for this phenomenon is that a polarised water layer surrounds the outer surface of the particles, leading to the observed negative charge.\textsuperscript{32} However, an initial polyelectrolyte layer may not adhere to the observed charge on the polarised water layer, which is why it was essential to create a hydrophobically modified precursor copolymer layer, where the hydrophobic moieties
of the copolymer would anchor in the amphiphilic cubic structure and the charged moieties of the copolymer would then contribute to the external surface charge. This has previously proved successful with unilamellar vesicles, with the additional benefit of preventing rearrangement of any charged lipids during polyelectrolyte adhesion.\textsuperscript{33}

The $\zeta$-potential and diameter of the cubosomes was monitored following the addition of each layer, both before and after centrifugation to remove any polymer aggregates. Results indicated similar diameter and $\zeta$-potential values for samples both before and after centrifugation. $\zeta$-potential measurements (Figure 4) showed a clear sawtooth pattern indicative of the layering of oppositely charged polymers. The first PLL layer shifted the $\zeta$-potential to almost 0 before, and slightly positive (+2 mV), after centrifugation. The second and third PLL layers maintained a negative $\zeta$-potential, but the value consistently increased between 11 - 15 mV from that of the previous PMA layer, indicating that there was indeed charge (and polymer) interaction with the surface of the nanoparticles. PLL, which is a random helix at neutral pH, may partially adopt $\beta$-sheet morphology upon adsorption to a negatively charged surface and this structural change reduces the positive charge associated with the polymer.\textsuperscript{34} After centrifugation to remove polymer aggregates, the differences between the $\zeta$-potential of each layer became more pronounced, suggesting that the PMA polymer was adhering more strongly to the nanoparticle surface, and that stability was further increasing.
Figure 4. ζ-potential measurements of monoolein cubosomes both before (black circles) and after (red squares) centrifugation to remove polymer aggregates. Lines are drawn to guide the eye. Cub – cubosomes only; PMAO1 – first layer PMA oleoyl; PMA – PMA layer; PLL – PLL layer.

Dynamic light scattering also indicated similar behaviour with pre- and post-centrifuged samples. The diameter was more stable after centrifugation, indicating the removal of some polydisperse polymer aggregates. The samples up to seven layers maintained a nanoscale size distribution of between 200 - 250 nm and a relatively low polydispersity for most layers (between 0.13 - 0.18), indicating that the polymers were not bridging across the cubic nanoparticles to form a significant number of aggregated cubosome clusters (Figure S1, Supplementary Information).

Table 1 summarises the diameter, polydispersity, and ζ-potential measured at each layer of polymer deposition following removal of polymer aggregates. The first PLL layer after PMAO addition showed a marked increase in polydispersity to 0.37, indicating aggregation. This was
likely due to the near-neutral ζ-potential (-0.6 mV before centrifugation and +2 mV after) causing flocculation of the nanoparticles.\textsuperscript{35} The polydispersity of layers 3 - 7 was lower than the values of the initial PLL layer, indicating that the flocculation caused by the first PLL layer was reversible upon adding further layers, and that the particles became separated again. This reversal of flocculation can be explained by the ζ-potential of layers 3 - 7, which fell within a range for nanoparticle stability.\textsuperscript{35}

As the polymer layers were incrementally added to the cubosomes, no significant change in diameter was observed, as has been the case in previous colloidal LbL studies.\textsuperscript{30} Polymer layers are often very fragile and hydrated and may not provide an even coating to each of the cubosomes. Additionally, the hydrodynamic diameter is not a measure of the actual size of the particles, but of its hydration sphere. Therefore, the change in diameter of the soft matter particles due to polymer layering was difficult to accurately evaluate using dynamic light scattering as a sizing technique.
Table 1. Average diameter, polydispersity, and ζ-potential measured at each layer of polymer deposition on monoolein cubosomes (post centrifugation).

<table>
<thead>
<tr>
<th>Layer</th>
<th>Polymer</th>
<th>Diameter (nm)</th>
<th>ζ-potential (mV)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>257 ± 4</td>
<td>-28 ± 1.5</td>
<td>0.16</td>
</tr>
<tr>
<td>1</td>
<td>PMAO</td>
<td>251 ± 6</td>
<td>-38 ± 0.6</td>
<td>0.13</td>
</tr>
<tr>
<td>2</td>
<td>PLL</td>
<td>217 ± 20</td>
<td>+2 ± 0.2</td>
<td>0.37</td>
</tr>
<tr>
<td>3</td>
<td>PMA</td>
<td>234 ± 17</td>
<td>-28 ± 0.5</td>
<td>0.16</td>
</tr>
<tr>
<td>4</td>
<td>PLL</td>
<td>239 ± 25</td>
<td>-14 ± 0.1</td>
<td>0.17</td>
</tr>
<tr>
<td>5</td>
<td>PMA</td>
<td>201 ± 13</td>
<td>-31 ± 1.0</td>
<td>0.17</td>
</tr>
<tr>
<td>6</td>
<td>PLL</td>
<td>235 ± 26</td>
<td>-20 ± 0.5</td>
<td>0.17</td>
</tr>
<tr>
<td>7</td>
<td>PMA</td>
<td>237 ± 19</td>
<td>-31 ± 0.6</td>
<td>0.18</td>
</tr>
</tbody>
</table>

The lattice parameter and phase behaviour of the cubosomes upon addition of each polymer layer was determined with SAXS. At each layer, the internal structure of the monoolein cubosomes retained their inverse bicontinuous cubic phase primitive (with Im3m space group) lattice. Although this phase is different to that of the bulk phase monoolein it is typically observed in sterically stabilised monoolein cubosomes dispersions. Additionally, the scattering intensity did not vary significantly upon addition of each polymer layer and subsequent aggregate separation, which is indicative that the cubosomes were at approximately the same concentration with limited loss due to the centrifugation process (Figure 5).
Figure 5. 1-D SAXS scattering patterns of monoolein cubosomes with 0, 3, 5 and 7 polymer layers showing Bragg reflections indicative of a Primitive inverse bicontinuous cubic phase with the relevant peaks indexed. Plots have been offset for clarity.

Although the cubosomal structure remained unaltered upon the addition of each layer, the lattice parameter was observed to decrease from 151 Å to 144 Å upon introduction of the oleyl containing PMAO, indicating an increase in bilayer curvature. This is an indication that the PMAO anchors to the structure of the cubosomes, interdigitating into the monoolein bilayer and altering the hydrophobic chains packing with a change in amphiphile tilt. The first PLL-PMA bilayer caused a slight increase in D-spacing of about 2 Å over the two layers. Some loosely associated PMAO may have been removed due to polymer association and transitioned into aggregates in solution. The lattice parameter of the following two PLL-PMA bilayers remained stable, suggesting that the polymer was able to interact with the surface without further removal. Figure S2, Supplementary Information shows a plot of the lattice parameter of each cubosome sample as the layers were increased.
Cryo-TEM images of these monoolein cubosomes were produced for samples at 0, 3, 5 and 7 polymer layers (Figure 6). Fast Fourier transforms of the nanoparticles showed retention of the internal ordered structure up to three polymer layers. By 5 layers (Figure 6.c) vesicles surrounding the coated particles could still be seen; however, the internal structure of the cubosomes was obscured due to the electron dense polymer shell. The initial polymer layers did not necessarily adhere in a single, uniform sheet. By seven layers (Figure 6.d), however, a smooth, uniform surface structure was visualised. This uneven layering is typical of the first several polymer layers in an LbL system, particularly in cases where the polymers exhibit a weaker binding interaction toward each other. Further evidence from negatively stained TEM data from lipidic material entrapped in the layer-by-layer polymer shell is presented in the Supplementary Information (Figure S3).
**Figure 6.** Cryo-TEM images of monoolein cubosomes coated in 0 (a), 3 (b), 5 (c), or 7 (d) polymer layers. Internal structure (insets) can be resolved up until 3 polymer layers via FFT, after which the presence of the coating completely masks the internal structure. Scale bars = 200 nm.
Drug Loading

Fluorescein, which has a low molecular weight and high water solubility, has a high likelihood of exhibiting burst release from drug delivery vehicles due its size relative to the water channels present in the cubic phase material.\textsuperscript{16} This molecule was chosen for testing as a “worst case scenario” model drug, given that higher molecular weight molecules would be more likely to have decreased burst release since they would be slower to diffuse from the external polymer coating. Fluorescein was added to monoolein cubosomes at 0, 0.5, 1 or 5 mol\% prior to dispersion.

The internal structure of the cubosomes in each sample was verified by small angle X-ray scattering and the results are presented in Table 2. The fluorescein-loaded samples retained their primitive structure (P) cubic up to 1 mol\% added drug. Fluorescein exhibited a continual decrease in lattice parameter (corresponding to an increase in bilayer curvature) up to 5 mol\% added drug, where two inverse bicontinuous structures were observed, the primitive lattice (P) and the double diamond phase (D). This shift in phase may be due to the hydrophilic fluorescein molecule interacting with the hydrophilic domain of the cubosomes. For testing of drug release from these cubosome samples, fluorescein was loaded into the monoolein cubosomes at 0.5 mol\%, a concentration well below the point where the model drug precipitates from or affects the internal structure of the particles.
Table 2. Lattice parameter and phase as determined by SAXS upon addition of increasing amounts of fluorescein to monoolein cubosomes (P: primitive inverse bicontinuous cubic phase, D: double diamond inverse bicontinuous cubic phase).

<table>
<thead>
<tr>
<th>Fluorescein Dissolved into Cubosomes (mol%)</th>
<th>Cubic Phase</th>
<th>Lattice Parameter (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>P</td>
<td>149</td>
</tr>
<tr>
<td>0.5</td>
<td>P</td>
<td>133</td>
</tr>
<tr>
<td>1</td>
<td>P</td>
<td>130</td>
</tr>
<tr>
<td>5</td>
<td>P</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>91</td>
</tr>
</tbody>
</table>
Drug Partitioning

It was important to determine the amount of drug which was partitioned into the cubic phase of the self-assembled lipid, and how much was located in the external aqueous solution. Any free drug within the aqueous solution will not exhibit sustained release when placed into an external environment since its movement is in no way restricted. Fluorescein is a hydrophilic small molecule with an octanol-water partition coefficient (Log $K_{o/w}$) of -0.48. The hydrophilic drug can diffuse freely between aqueous domains of the cubic and the aqueous environment in which they are placed. Therefore the drug is equally likely to partition within the water channels of the cubic structure as it was within the external aqueous region. Model drug molecules present in the aqueous phase of the cubosomes rapidly diffuse from the confines of the nanoparticles due to their high surface area. A change in lattice parameter with increasing amount of fluorescein loaded in cubosomes is indicative of at least some of the drug loading within the cubic phase matrix.

It was first determined that the filter did not capture any drug molecules during centrifugation (Table 3). Drug release experiments were then carried out for fluorescein in solution (containing no cubosomes), polymer aggregates plus fluorescein in solution (containing no cubosomes), fluorescein-loaded cubosomes with no polymer layers, and fluorescein-loaded cubosomes with 7 polymer layers. The calculated amount of fluorescein within the external aqueous phase for each sample tested is summarised in Table 3. When polymers were added to fluorescein in solution, most of the drug remained within the solution (89 ± 7 %), but there was a slight amount of partitioning of drug into the polymer aggregates, as hypothesized in previous work with liposomes. Negative charge may exist on a fraction of the ionized carboxylic acid moieties in the fluorescein at pH below 9. This may have caused complexation with the positively charged...
poly(L-lysine) during polymer addition. However, the effect was minimal. Additionally, after the drug loaded cubosomes had been coated with polymer and centrifuged to remove polymer aggregates, the aggregates remained white with no visible sign of fluorescein adhesion or incorporation. For monoolein cubosomes containing no polyelectrolyte, 75 ± 2 % of the hydrophilic fluorescein remained within the aqueous phase, and 25 ± 2 % remained partitioned into the water channels of the cubosomes. However, with no barrier between the water channels and the excess aqueous phase, the drug readily diffused between the cubosomes’ water channels and the external environment. A large decrease in the amount of fluorescein in the aqueous phase was seen with the 7-layered monoolein cubosomes (only 46 ± 2 %), meaning that more than half of the loaded drug had been trapped within the cubic phase nanoparticles and polymer coating due to the layering steps.

### Table 3. Amount of fluorescein dissolved into the external aqueous phase of each sample tested.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Polymer</th>
<th>Drug Dissolved Into</th>
<th>Amount of Drug Loaded Within External Aqueous Phase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>Solution</td>
<td>98 ± 2</td>
</tr>
<tr>
<td>None</td>
<td>7 layers</td>
<td>Solution</td>
<td>89 ± 7</td>
</tr>
<tr>
<td>MO</td>
<td>None</td>
<td>Lipid</td>
<td>75 ± 2</td>
</tr>
<tr>
<td>MO</td>
<td>7 layers</td>
<td>Lipid</td>
<td>46 ± 2</td>
</tr>
</tbody>
</table>
Drug Release

Drug release was monitored over a 24 h period. At each time point, an aliquot of cubosomes was removed from dialysis and tested to determine the amount of drug still residing within the nanoparticles. The combinations of lipid, polymer and drug which were tested are summarised in Table 4. The sample for each time point was placed into an individual beaker at a ratio of 100 µL of sample to 250 mL of external solution. In some experiments, drug was dissolved into the external aqueous solution instead of the lipid to assess drug-polymer or drug-nanoparticle interactions.

Table 4. Summary of samples created for drug release experiments.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Lipid</th>
<th>Polymer</th>
<th>Hydrophilic Molecule</th>
<th>Drug Dissolved Into</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>None</td>
<td>Fluorescein</td>
<td>Solution</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>7 layers</td>
<td>Fluorescein</td>
<td>Solution</td>
</tr>
<tr>
<td>3</td>
<td>MO</td>
<td>None</td>
<td>Fluorescein</td>
<td>Lipid</td>
</tr>
<tr>
<td>4</td>
<td>MO</td>
<td>None</td>
<td>Fluorescein</td>
<td>Solution</td>
</tr>
<tr>
<td>5</td>
<td>MO</td>
<td>7 layers</td>
<td>Fluorescein</td>
<td>Lipid</td>
</tr>
</tbody>
</table>

All samples represented the same aqueous mixture of 1:16 (v:v) PBS (pH 8.6) to water. Each sample also contained an initial concentration of 68.9 µg/mL fluorescein in solution. Controls consisted of (1) fluorescein in solution (containing no cubosomes), (2) polymer aggregates plus fluorescein in solution (containing no cubosomes), (3) fluorescein-loaded cubosomes with no polymer layers, and (4) blank cubosomes with fluorescein solution added after cubosome preparation. Sample (5) was the experimental sample of drug-loaded cubosomes coated in 7
polymer layers. As Figure 7a demonstrates, all controls released their cargo at very similar rates, with each sample depicting a large burst release of between 75 - 85 % of the cargo within the first 20 min, and near 100 % release by 4 h. The fluorescein solution containing no cubosomes (1) released slightly faster than any of the other controls, and had released close to 100 % of the fluorescein by 2 h. Fluorescein-loaded cubosomes (3) and cubosomes with fluorescein added in the aqueous solution (4) exhibit similar release rates. The difference in measured release rate from the fluorescein (1) and the fluorescein with cubosomes (3&4) or with polymers (2) are most likely caused by interaction of the delivery vehicles with the dialysis membrane.
Figure 7. (a) Fluorescein released over 30h from four separate controls: (1) fluorescein in solution (containing no cubosomes); (2) polymer aggregates plus fluorescein in solution (containing no cubosomes); (3) fluorescein-loaded cubosomes with no polymer layers; and (4) blank cubosomes with fluorescein solution added after cubosome preparation. Drug was completely released in all cases by 4 h. (b) Fluorescein released from non-coated cubosomes (black circles, [3]) and 7-layered cubosomes (red squares, [5]) over 30 h.
Once all controls were analysed, a sample of fluorescein-loaded monoolein cubosomes was layered with 7 polymer layers PMAO-(PLL-PMA)$_3$ and a release experiment was performed. The results shown in Figure 7b depicting the 7-layered cubosome sample showed a potential decrease in burst release over the first three hours of release as compared to fluorescein loaded cubosomes with no added layers. Where the control sample had released 70 ± 2 % of the fluorescein in the first 5 min, the layered cubosomes released 54 ± 3 %. By 20 minutes, the control had released 80 ± 2 % of its cargo, while the layered cubosomes had released 57 ± 3 %. By about 4 h, the control sample had released nearly 100 % of its cargo (97 ± 1 %), whereas the coated cubosomes had released 83 ± 1 %. The coated nanoparticles continued to slowly release a further 10 % of fluorescein over the next 26 h. The bound polymer layers on the coated cubosomes, have demonstrated that with further development, they may be used to provide a barrier for hydrophilic molecule release. (Figure 7b)

Fitting the post burst release data of the 7-layered cubosomes to the Higuchi diffusion kinetic model shows that the drug was released via diffusion through the polymer layers following the initial burst (Figure 8). In a Higuchi release system, the percent of drug released is directly proportional to the square root of time, and the slope of the plotted line is related to the solubility of the drug and diffusion coefficient through the matrix. Bulk, non-dispersed cubic phase drug release has been shown to follow Higuchi kinetics, where the release is based on Fick’s law of diffusion of the drug through the pores of the system.\textsuperscript{10, 12}
Figure 8. Higuchi kinetics plot of fluorescein released versus the square root of time shows a linear trend after the initial burst release of fluorescein from 7-layered monoolein cubosomes, indicating diffusion controlled release.

The size of the pores or gaps created between LbL polymer layers, which can be adapted by altering polymer composition, layer number, or layering conditions, are known to modify the diffusion process. The modulation of the release rate within the cubosome colloidal dispersion may be affected by the polymer coating which effectively reduces the mesostructural pore size of the cubosomes, thus physically limiting the fluorescein release. Since fluorescein is a very small hydrophilic molecule, it represents the lower extreme of a loaded therapeutic as per Higuchi’s theory. Should larger and more hydrophobic drug molecules be loaded into the system, a greater effective reduction in release rate would be expected to occur, since the ratio between the pore size in the nanoparticle mesostructure and the molecular size of the drug are likely to be intimately related. An increase in the number of PLL-PMA polymer layers may also induce a similar effect. The polymers may also act as a barrier to diffusion.
Conclusions

Cubosomes are capable of loading a versatile array of additives.\textsuperscript{2} These colloidal dispersions may be able to passively target tumour cells through the enhanced permeability and retention (EPR) effect of tumour blood vessels.\textsuperscript{41, 42} However, a strategy for modulating the surface properties as well as the initial burst-release from cubosomes must be further developed.

In this study, polymer coated cubosomes were prepared via a new simplified LbL coating and centrifugation method in which polymer aggregates were separated from the solution due to their density differential from coated cubosomes. A monoolein cubosome system was achieved which contained a 7-polymer coating on discrete nanoparticles.

A lipophilic PMA was synthesised \textit{via} RAFT-mediated radical copolymerisation by incorporating $\sim$2 mol\% oleyl methacrylate. The lipophilic pendant oleyl groups were able to embed into the cubosome structure and provide anchoring for the PMA precursor polymer, allowing for further polyelectrolyte layering as has successfully been demonstrated for liposomal anchoring with both oleyl-\textsuperscript{26} and cholesteryl-functionalised copolymers.\textsuperscript{43-45} PLL and PMA were chosen due to being biocompatible and functionalisable (e.g. for the addition of stealth and/or targeting moieties).\textsuperscript{27, 46-48} For example, PLL capsules grafted with PEO and biotin have shown excellent antifouling and streptavidin binding properties.\textsuperscript{49}

These cubosomes were then loaded with hydrophilic fluorescein, a small model compound for preliminary \textit{in-vitro} drug delivery studies. The initial burst release of this model drug was decreased for 7-layered cubosomes as compared to non-layered cubosomes. A similar shift to release profiles has been noted in the literature for other drug loaded systems.\textsuperscript{50} Boyd \textit{et. al.} have demonstrated that equilibrium dialysis may be inappropriate for measuring release rates from cubosomes because the release rate from the particles is faster than the diffusion through the
membranes. The current study used standard dialysis membranes with over 400% larger MW cutoff, allowing for the release rate through the pores of the tubing to more readily mimic the release rate from the particles. Additionally, the controls did indeed show that the drug would exhibit a rapid burst effect if the molecules were not entrapped within the cubic nanoparticles. Therefore, the decrease in burst release seen in cubosomes via dialysis is a likely phenomenon when properties of the carrier are physically altered, aiming to reduce burst release by slowing the movement of the drug particles into the external environment.

Although the initial burst release and subsequent release rate of fluorescein was not altered dramatically, this proof-of-concept study demonstrated that polymer multilayers may be used to modulate burst release and help obtain diffusion controlled release from the cubosomes, which can hold water soluble, lipid soluble or amphiphilic molecules within their 3-dimensional nanostructure. By altering variables such as the number of polymer layers, the specific polymers utilised, or the type and size of the loaded drug molecules, it may be possible to further reduce the burst-release and replace it with a slow and stable release rate.
Supporting Information. Dynamic light scattering results, lattice parameters for the lbl coated inverse bicontinuous cubic phases and negatively stained TEM data is to be found in the supplementary information. This material is available free of charge via the Internet at http://pubs.acs.org.

ABBREVIATIONS

PMAO, poly(methacrylic acid-co-oleyl methacrylate); DLS, dynamic light scattering; SAXS, small angle X-ray scattering; TEM, transmission electron microscopy; PMA, poly(methacrylic acid); PLL, poly(L-lysine); PEO, poly(ethylene oxide); LbL, layer-by-layer; RAFT, reversible addition–fragmentation chain transfer; PBS, phosphate buffered saline; P.F127, Pluronic F127; MO, 1-oleoyl-rac-glycerol (monoolein);

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REFERENCES


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