Bottom-Up Cubosome Synthesis Without Organic Solvents

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Abstract

Hypothesis

Bottom-up synthesis of cubosomes is more energetically favourable than top-down approaches. However, bottom-up methods often rely on organic solvents such as ethanol as diluents, and lead to concurrent formation of liposomes. We propose using non-toxic diluents such as honey, glycerol and lactic acid for bottom-up cubosome synthesis.

Experiments

Cubosomes were prepared using solutions of phytantriol in a range of diluents including choline chloride-glycerol, honey, lactic acid, glycerol, and ethanol. These solutions were added dropwise to water containing the stabiliser, poloxamer 407, following an established method of cubosome synthesis.

The resulting structures were characterised using small-angle X-ray scattering, DLS and cryo-TEM.

Findings

Cubosomes were successfully formed using a range of non-toxic diluents. This demonstrates that harmful organic solvents like ethanol are not required, and that the diluents need not be hydrotropes. Furthermore, unlike ethanol, these other diluents allowed formation of cubosomes without concurrent formation of liposomes. Given the huge potential for cubosomes in drug delivery, this new method offers a potentially useful low-cost, low-toxicity synthesis option.

Keywords: Cubosomes, deep eutectic solvent, cubic phase, drug delivery, liquid crystal phase, phytantriol

Abbreviations: choline chloride-glycerol (ChCl-Gly), deep eutectic solvents (DESs)

Introduction

Liquid crystal phases offer an exciting range of tuneable properties that can be sensitive to a number of parameters such as temperature and pH. Ongoing research into amphiphilic liquid crystal phases continues to unearth new properties and applications. The bicontinuous cubic phase is one of the more complex phases, being composed of a bicontinuous lipid-water network with three dimensional symmetry.[1] Details of the different types of cubic phases have been summarized elsewhere.[1]

Cubic phases offer great potential for controlled release of both hydrophilic and hydrophobic components due to the large interfacial area and slow release kinetics.[2-4] However, bulk cubic phases are extremely stiff and solid-like, making them hard to work with and non-viable for certain applications such as injection. Thus, it is often necessary to disperse the cubic phase into cubosomes.[2] These cubosomes can encapsulate drug components or even be partially composed of therapeutically-relevant molecules.[5-9]

Cubosomes are usually composed of either unsaturated monoglycerides (GMO) or phytantriol. Each of these have different benefits and drawbacks, but both are capable of forming bicontinuous cubic phases in water. Phytantriol has greater structural stability but phytantriol cubosomes have been shown to be more toxic than those made from GMO,[10] which must be considered for real-world applications.

In addition to the primary amphiphile, cubosomes require a secondary surfactant to act as a stabilizer.[11] One of the most commonly used surfactants for this purpose is poloxamer 407. This triblock polymer is made of a polypropylene oxide block between two polyethylene oxide blocks and the amphiphilic nature means that the hydrophobic portion can incorporate into the cubosome while the hydrophilic chains extend out into the media, preventing fusion of particles via steric stabilisation.[11, 12]

Traditionally, cubosome synthesis required high-energy dispersion of a bulk cubic phase, for example through ultrasonication or high-pressure homogenisation.[13] Spicer et al.[14] demonstrated a novel bottom-up method of cubosome synthesis. This method involved dissolving monoolein in a hydrotrope (ethanol) and then diluting that with water. Subsequently, a number of studies have used similar methods for hexasome[15] and cubosome synthesis, including in microfluidic systems to improve cubosome uniformity for use in drug delivery formulations.[16] These have utilized different stabilizers, different amphiphiles, and different conditions, but generally ethanol is used as the hydrotrope.[11] The key requirement is to sufficiently dilute or dissolve the lipid to allow the lipid solution to be dispersed rapidly into water, where the lipid is insoluble, and so precipitates into cubosomes during solvent exchange.

However, there are concerns with the use of ethanol for pharmaceutical applications, especially in formulations intended for children and infants.[17-19] There are also restrictions on ethanol in some cultures which must be taken into consideration. Furthermore, the use of ethanol for cubosome synthesis may preclude the encapsulation of some ethanol-sensitive biomolecules due to instability e.g. the denaturation of proteins in the presence of ethanol.[20] Therefore, it is desirable to find an alternative which allows inexpensive production of cubosomes but which avoids the use of this organic solvent.

Deep eutectic solvents (DESs) are mixtures of hydrogen bond donors and hydrogen bond acceptors that have much lower melting points than the individual components.[21, 22] There are

thousands of potential DESs and the flexibility in the constituents means that properties can be fine-tuned. Furthermore, these solvents can be green and non-toxic, made from sustainable and even food-grade components.[21, 23-25]

The research presented here demonstrates that honey, a genuine food product, and lactic acid, which is an accepted food additive, can be used in place of ethanol to create cubosomes. Indeed, honey is recognised to be a natural example of a molecular mixture with a highly depressed melting point, characteristic of deep eutectic solvents.[26, 27] Furthermore, standard deep eutectic solvents such as choline chloride-glycerol were also effective. The low cytotoxicity of species such as glycerol[28] and lactic acid[29] at the concentrations expected in the final cubosomes are already well established in the literature, and that of the deep eutectic solvent choline chloride:glycerol has also been studied[30, 31] demonstrating it has low cytotoxicity to various cell lines. In fact, choline chloride is used as a food additive to increase chicken growth.[32] Honey has long been used as an antibiotic for wound treatment and in medicines,[25] so is compatible for use in making cubosomes. Thus these diluents offer a low-toxicity alternative to ethanol.

Variation of the properties of the solvent offers fine-tuning opportunities for lipid and encapsulant solubility and stability in that solvent, while also potentially allowing design of cubosome properties such as size.

Materials and Methods

To prepare the deep eutectic solvent, choline chloride (Sigma, 98%) was combined with glycerol (Sigma, 99%) in a 1:2 molar ratio and stirred until they formed a homogenous isotropic liquid (ChCl-Gly). Other diluents, ethanol (BDH Chemicals, >99%), lactic acid (aqueous solution) (Fluka, >88%), glycerol, honey (Co-op brand, standard honey), were used as received. The fructose to glucose molar ratio in the honey was determined using quantitative ^{13}C NMR with the honey sample dissolved in D_2O, on a Bruker 400 MHz Avance Neo with an iProbe. The fructose to glucose ratio, calculated using the distinctive ^{13}C peaks above 90 ppm,[33] (see Figure S1 in the SI) was determined to be 1.07:1. The water content of the honey was determined by Karl Fisher titration (using a Metrohm 899 Coulometer) to be 10.2 wt%.

The method for cubosome formation was adapted from Akhlaghi et al.[34] and is illustrated in Figure 1. Briefly, 20 mg of phytantriol (DSM, 97%) was added to 2 ml of either ethanol, lactic acid (aqueous solution), glycerol, honey or ChCl-Gly (i.e. 'the diluents') and stirred for 20 mins at 40 °C. Phytantriol is expected to be chemically stable in the acid solution based on prior studies on persistence of phytantriol cubic phases in acidic gastric fluids.[35] Surface tension measurements of these dispersions was made using drop shape analysis on an FTA1000 B Class instrument (First Ten Angstroms). Measurements were made in triplicate based on an average of fifty images.

Separately, 10mg of poloxamer 407 (Sigma, >99%) was added to 4.5 ml of deionised water (18.2 MΩ·cm) and also mixed for 20 mins at 40 °C. The phytantriol mixture was then added dropwise to the aqueous solution, with continuous stirring at 40 °C. The dispersion was left to stir at 40 °C for a further ten minutes.

In the case of ethanol as the dispersant, one sample was left stirring with the lid off at 40 °C to allow evaporation of the ethanol and also concentration of the cubosomes. The mixture was allowed to evaporate to about 20% of its former volume to form suspensions with a sufficiently high concentration of cubosomes to obtain small angle scattering data. The cubosomes appear to remain stable in the more concentrated suspension with no obvious sedimentation, aggregation or change over the time period assessed.

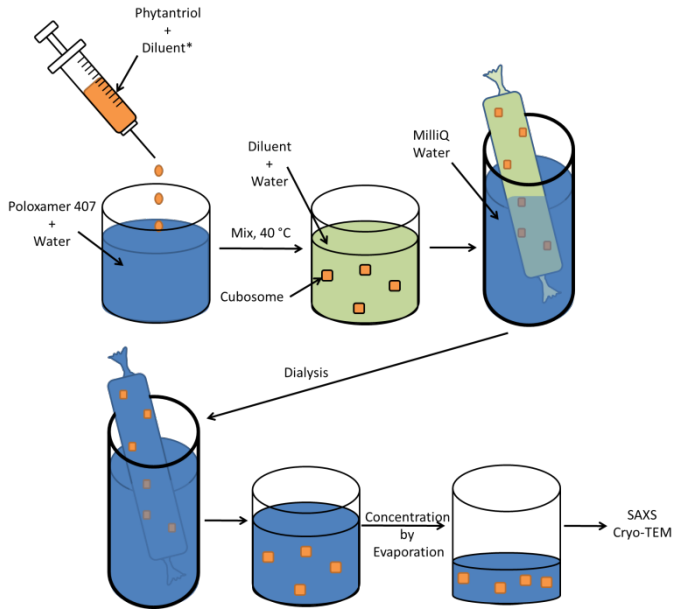

Figure 1. Schematic illustration of the process of cubosome synthesis and subsequent concentration steps required for characterisation. Phytantriol was dissolved in the diluent (ethanol, lactic acid, honey, ChCl-Gly or glycerol) and then added dropwise to a solution of poloxamer 407 in water. After stirring at 40 °C for ten minutes, the mixture was dialysed against MilliQ water or poloxamer 407 solution to remove the diluent. The aqueous dispersion was then heated to force evaporation of water and thus concentrate the cubosomes in the remaining liquid.

For lactic acid, glycerol, honey, ChCl-Gly, and one sample made with ethanol, the mixtures were dialysed (tubing: Fisher, 3500 MWCO) against deionised water for three days with regular replacement of the water. Some white aggregates of cubic phase (confirmed by SAXS) appeared in the tubes in all cases, possibly due to a decrease in the concentration of the poloxamer 407 during dialysis. To test this a second batch of cubosomes were prepared where poloxamer 407 was added to the dialysis water at the same concentration as in the initial suspension, to avoid particle aggregation during the dialysis step. Any visible aggregates were removed prior to the next step. After dialysis, the mixtures were returned to a vial and allowed to evaporate down to about 20% volume in the same way as the sample made using ethanol without dialysis. In all cases, this resulted in a cloudy mixture that became more opaque with evaporation. It should be noted that this time-consuming process of dialysis followed by evaporation was only required to get the cubosomes to a sufficient concentration for observation by small angle X-ray scattering (SAXS). If the diluent is sufficiently non-harmful, and is sufficiently dilute in the synthesis solution to allow cubosome formation, then theoretically the mixture could be used as-is for drug delivery and other applications, without

removal of the diluent, unlike the current process where ethanol must be removed prior to the cubosomes being used as drug delivery agents.

Dynamic light scattering (DLS) and zeta potential measurements were made on a Malvern Zetasizer Nano-ZS at 25 °C. For DLS measurements samples were initially taken following injection of the phytantriol mixture into the poloxomer 407 aqueous solution with and without filtration through a 0.45 μm syringe filter. Particle sizes were also measured after dialysis into pure water or poloxamer 407 aqueous solution, after filtration through a 0.45 μm syringe filter. All measurements were performed in triplicate. Zeta potentials were measured for samples after dialysis against pure water or poloamer 407 aqueous solutions. Samples were placed in a folded capillary electrode cell and were equilibrated at 25 °C for 120 s prior to testing. The data were taken from an average of five measurements from 100 scans each.

Small angle X-ray scattering (SAXS) measurements were performed on an Anton-Paar SAXSPoint 2.0 provided by the Material and Chemical Characterisation Facility (MC^2)[36] equipped with a copper source (Cu K-α, λ=1.542 Å) and a 2D EIGER R series Hybrid Photon Counting (HPC) detector (Dectris, Baden-Daettwil, Switzerland). The sample detector distance was 556.9 mm covering a q range of about 0.008-0.4 $Å^{-1}$. Samples were loaded into 1 mm quartz capillaries. Data was collected for 1800 s, then reduced. Background subtraction was not performed. Peaks were analysed for relative position in order to confirm the presence of the cubic phase, to calculate the lattice parameter and to determine the domain size using the Scherrer equation.[37] The temperature was kept at 25 °C using a Peltier unit (±0.1 °C).

Cryo-TEM was performed at the Wolfson Bioimaging Facility at The University of Bristol on an FEI Tecnai T20 LaB6 TEM with a Gatan 626 cryotransfer holder. Images were collected on an FEI CETA camera. 3 uL of each sample was pipetted onto glow discharged lacey carbon support films and plunge frozen into liquid ethane using a Leica GP plunge freezer. They were subsequently transferred to the Gatan cryotransfer holder and observed under low dose conditions on the T20 instrument.

Results and Discussion

The work presented in this paper demonstrates that diluents other than organic solvents can be used in the bottom-up method of cubosome synthesis. The role of diluents in this method is to solubilise the lipid—phytantriol—to allow easy dispersion into the aqueous media which in turn leads to the formation of discrete cubosomes.

To date, the role of dispersant has largely been carried out using ethanol. Here we present four very different alternatives and characterise the resulting cubosomes. These alternatives provide not only a less hazardous option than organic solvents, but may also allow the encapsulation of new drug types that would otherwise be destroyed or denatured by organic solvents.

As discussed below, the diluent can affect the nanoparticle size, the lattice parameters, and the presence of other structures such as liposomes. Future optimisation work could take advantage of these influences to design nanoparticles for specific applications.

SAXS

Small angle X-ray scattering measurements on phytantriol mixed with the diluents prior to injection into water showed no structural features except for solutions in honey and ChCl-Gly. Figure 2 shows a single sharp peak for honey+phytantriol, and a broader peak in a similar q position for ChCl-Gly+phytantriol. The single peak makes it difficult to say with certainty what phase this represents, however it is possible that a lamellar phase is forming in both cases as the mixtures appear slightly cloudy, consistent with lamellar phase formation, particularly precipitated particles or multilamellar vesicles.[38] The D spacing from these peaks was 37±2 Å for the dispersion in honey and 39±2 Å for the dispersion in ChCl-Gly. These values are slightly larger than that reported for the phytantriol lamellar phase in water (30-33 Å),[39] and this is most likely due to the weaker solvophobic effects in these solvents, allowing for greater bilayer swelling compared to water. Spontaneous formation of a lamellar phase by lipids in DESs has been reported previously and so is not unexpected.[40] The presence of these lamellar phases at this stage does not appear to influence the formation or physical properties of cubosomes formed following injection, as discussed below.

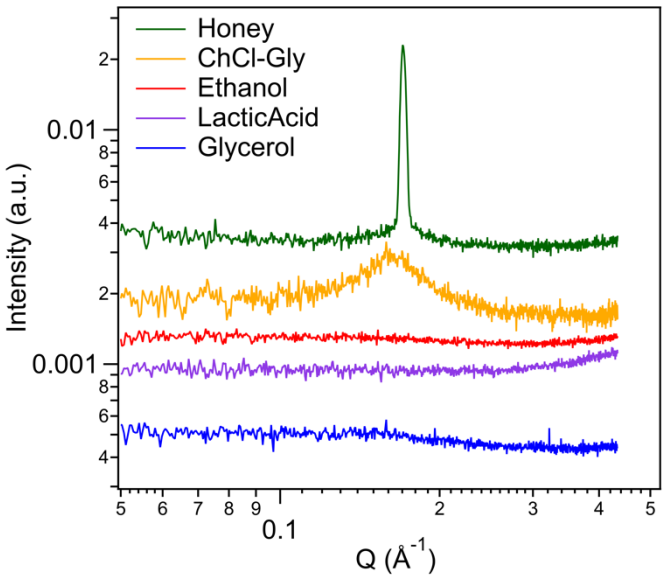

Figure 2. SAXS scattering patterns in the high q range from phytantriol dispersed in a range of diluents. From top to bottom: honey (green), ChCl-Gly (yellow), ethanol (red), lactic acid (purple), glycerol (blue). Offset in the y-axis for clarity.

Following injection of the phytantriol solutions into water and subsequent dialysis and evaporation, SAXS was used to confirm the presence of a cubic phase and to calculate the lattice parameters of the cubosomes. It should be noted that despite the high viscosity of some of the pure diluents (e.g. honey), the viscosity of the final dispersion of cubosomes was low and easily manipulated using a pipette or needle. Figure 3 shows the scattering patterns of concentrated cubosome dispersions made using either ethanol, glycerol, lactic acid, honey, or ChCl-Gly, followed by dialysis. The peak positions are given in Table 1. The sample made from ethanol without dialysis had the most intense peaks. This is attributed to a higher concentration of cubosomes as nothing is lost due to dialysis. However, on a SAXS instrument with greater flux, there is no doubt that more and higher intensity peaks would be visible in all samples.

The peak position in the dispersion made from ethanol moves to higher q following dialysis, due to further removal of ethanol from the structure during this procedure. The scattering pattern of samples made from the other diluents had similar peak positions. The Bragg peaks of all samples had relative positions of $\sqrt{2}$, $\sqrt{3}$, (and then $\sqrt{4}$ in the case of the sample made from ethanol without dialysis) which is consistent with a Pn3m cubic phase.[41] This is the expected phase formed by phytantriol in excess water.[39] Table 1 also shows the lattice parameters for each sample, calculated from the peak positions and the Miller indices of the Pn3m unit cell.[39]

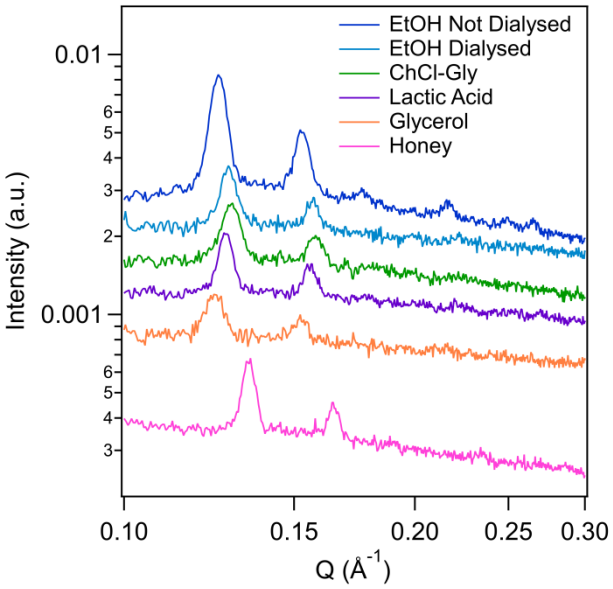

Figure 3. SAXS scattering patterns in the high-q range for concentrated cubosome suspensions in water made using different initial diluents. From top to bottom: ethanol (without dialysis) (dark blue), ethanol (with dialysis) (light blue), ChCl-gly (green), lactic acid (purple), glycerol (orange), honey (pink). Patterns are offset in the y-axis for clarity.

The peak positions are very similar between the different samples, especially considering that some of the peaks are broad, probably due to smaller domain sizes as well as the low concentration resulting in a lack of intensity and high background noise. Across all of the samples, the lattice parameter varied by less than 3 Å, with an average of around 70 Å, except for honey which had a slightly smaller parameter of 66 Å, although, given the error range, this value is not significantly different from the others. Previous work has found lattice parameters of 64 Å,[34] 70 Å[42] up to 74 Å[43] for phytantriol cubosomes with variation depending on the concentration of ethanol used, temperature, and additives. When only water is used to swell a bulk phytantriol phase the lattice parameter in excess water at 22 °C is 63.6 Å.[39] Therefore, the results presented here are in good agreement with literature values. The small decrease in lattice parameter following dialysis of the sample made using ethanol suggests the water channels shrink upon continued exposure to water. This is due to removal of ethanol that would otherwise have stayed associated with the phytantriol (as in the non-dialyzed sample), resulting in greater insolubility of the lipid in water, and thus a decrease in lattice spacing. The fact that the lattice parameters all exceed those of phytantriol swollen in pure water, suggests that some of the dispersion medium may be retained in the cubosomes, although at small concentrations.

In addition to the lattice parameter, the peaks from SAXS can be used to estimate particle size for comparison (assuming the cubosomes are single, perfectly ordered, homogeneous domains),[37] using the Scherrer equation, $L=K\lambda/(B(2\theta)\cos\theta)$, where L is the crystallite size, λ is the X-ray wavelength (here 1.54Å), $B(2\theta)$ is the full width at half maximum of the diffraction peak, and θ is the Bragg angle for the peak. K is a shape factor, here assigned the value of 0.9, which approximates spherical crystallites. Given the assumptions made, the values quoted for domain size are not exact but can be used to compare the samples prepared from different diluents, measured under the same conditions. As shown in Table 1, the crystallite sizes of cubosomes made using glycerol as the diluent were smaller (170 nm) than that of the other samples. Cubosomes made using honey had the largest domain size at 240 nm. The domain sizes scale roughly with the viscosity of the diluent (Table S2), except for the case of honey, where the SAXS pattern in Figure 2 indicates that larger aggregates are already present in solution before addition to water. Since all of these diluents are miscible with water, when the diluent is more viscous, the exchange with water will occur more slowly, which appears to adversely affect cubic phase organisation into extended domains, or alternatively allows smaller particles to form. All of these domain size estimates are similar to the particle sizes measured by TEM, and DLS after dialysis (see below).

Table 1. Peak positions (reported as Q ($Å^{-1}$)), lattice parameters and domain sizes based on SAXS patterns shown in Figure 3. All peak positions have an instrumental error of ±0.003. Domain sizes calculated from the Scherrer equation have errors based on the difference between domain sizes calculated from the first and second diffraction peaks.

Preparation Material	**First Peak Position ($Å^{-1}$)**	**Second Peak Position ($Å^{-1}$)**	**Third Peak Position ($Å^{-1}$)**	**Lattice Parameter (Å)**	**Domain Size (nm)**
Ethanol (Not Dialysed)	0.126	0.153	0.177	70.5±2	210 ± 22
Ethanol (Dialysed)	0.129	0.157	-	69±2	220 ± 9
Glycerol	0.125	0.153	-	71±2	170 ± 5
ChCl-Gly	0.130	0.159	-	68.5±2	180 ± 20
Lactic Acid	0.128	0.157	-	69.5±2	220 ±6
Honey	0.135	0.165	-	66±2	240 ± 8

As shown in Figure S2, the samples made from ethanol (both with and without dialysis), and the sample made with honey had scattering at low-q that was not evident in the other samples. This scattering is from large structures, possibly vesicles. In the case of samples made from ethanol, this was further confirmed by Cryo-TEM, as shown below. For the samples made from ethanol (both with and without dialysis) the large structure at low-q can be fit to a vesicle model with parameters (e.g. radius and shell thickness) that match the cryo-TEM image (see SI). Such fitting is not possible for the scattering curve from the particles prepared from honey and is probably a result of large aggregates, possibly from the honey itself in addition to aggregated phytantriol.

Surface tension measurements of the diluents with phytantriol prior to injection into water revealed little variation, although the ethanol mixture appeared to have a slightly higher surface tension than the others, as shown in Figure 4. Interestingly ethanol is the only one of the solvents which has a higher surface tension on addition of phytantriol, compared to the solvent on its own. It is possible that the increased surface tension of the ethanol mixture changed the process of solvent exchange enough (compared to the other diluents) that it allowed liposome formation, which is not observed for any of the other diluents.

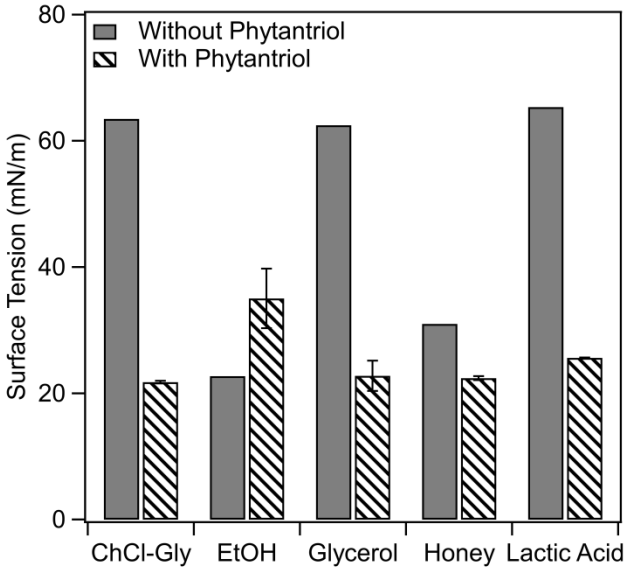

Figure 4. Surface tension measurements of different diluents with and without phytantriol. Error bars are based on standard deviation across triplicate measurements. Values for diluents without phytantriol were taken from the literature.[44-48]

The scattering curves suggest that vesicles are, at least temporarily, a stable conformation for phytantriol when particles are formed using ethanol but that using other diluents such as honey, glycerol, ChCl-Gly, or lactic acid, results in only cubosomes with no evidence of vesicles. This could be extremely useful, e.g. to eliminate unwanted lamellar structures and produce only cubosomes for drug delivery applications.

Dynamic Light Scattering

Dynamic light scattering was used to assess the size of particles made from the differing diluents both before and after the dialysis step. Immediately after preparation, before dialysis, without filtering, the cubosomes made with ethanol as the diluent were relatively homogenous with an average z-average diameter of 360±5 nm with a polydispersity of 0.14. In contrast, cubosomes made with ChCl-Gly, lactic acid, glycerol or honey had polydispersity indexes of 1, 1, 0.6, and 0.49 respectively. Similarly, cubosomes made with diluents other than ethanol tended to be larger before dialysis, as shown in Table 2, suggesting that diffusion of these solvents out of the cubosome particles is slower than for ethanol, particularly for ChCl:Gly and lactic acid, although the large standard deviation resulting from high polydispersity makes generalisation difficult. All of the diluents are water miscible, although they are all much more viscous than ethanol and will have a variety of different intermolecular interactions with the

phytantriol molecules meaning diffusion into the surrounding aqueous solution may be delayed, resulting in the large, and polydisperse particle size distributions seen at this stage.

Samples were put through a 0.45 μm syringe filter to reduce polydispersity and allow the signal from any smaller particles to be more visible compared to the larger aggregates. As shown in Table 2, filtering resulted in a general reduction in particle size and polydispersity, as expected, except in the case of particles made from lactic acid. Cubosomes made using lactic acid as the diluent were an order of magnitude larger than any of the others even after filtration, and were bigger than the filter size, demonstrating that these particles are soft, rapidly aggregating and very unstable compared to those made using the other diluents. In comparison, particles made from the ChCl:Gly diluent solution seem to have been almost entirely removed from solution by the filtration step, leaving only a few very small particles in solution. This suggests that these particles remain large and swollen with solvent before the dialysis step but are sufficiently rigid to be excluded by filtration. It is known that the solvent exchange rate has a significant effect on the internal particle structure, and particularly the location of initial precipitation of the insoluble phase during anti-solvent precipitation.[49] It may be that the different rates of exchange of diluents here result in differing initial particle structuration, before the final cubosome structure is formed by continuing lipid diffusion processes over time, or during the dialysis step.

Table 2. Z-average diameter measured using dynamic light scattering of cubosomes made from different diluents immediately after preparation, measured before and after filtration through a 0.45 μm syringe filter. Particles were also measured after dialysis against water or poloxomer 407 solution (filtered through a 0.45 μm syringe filter). Error is the standard deviation of triplicate measurements.

Before Dialysis				
	Unfiltered (nm)	**PDI**	**Filtered (nm)**	**PDI**
Ethanol	360±5	0.14	371±5	0.16
Choline Chloride:Glycerol	>10 000	0.71	58±1	0.15
Lactic Acid	>10 000	1.0	~10 000	0.94
Glycerol	2650±390	0.63	159±4	0.46
Honey	3560±170	0.49	940±30	0.55
After Dialysis (all filtered)				
	Against water (nm)	**PDI**	**Against poloxomer 407 solution (nm)**	**PDI**
Ethanol	211±2	0.32	218±3	0.21
Choline Chloride:Glycerol	244±6	0.15	237±22	0.31
Lactic Acid	2470±130	0.71	2770±9	0.98
Glycerol	263±5	0.28	211±1	0.11
Honey	355±6	0.39	271±2	0.22

After dialysis, DLS indicates a general reduction in particle size for particles created from all diluants except for lactic acid. Particles produced from lactic acid solution remain large, with

high polydispersity even after the dialysis step. It is possible that the acidic nature of lactic acid causes a change in the phytantriol which leads to softer particles, with lower stability, possibly due to formation of amorphous material as well as the desired cubic phase, observed in the SAXS data above. Dialysis against water or poloxamer 407 solution give very similar results for all of the other diluants, with particle diameters between 200-300 nm and lower polydispersities, generally around 0.2-0.3. Other than for particles prepared from lactic acid, these particle sizes are similar to the domain sizes measured in SAXS, with cubosomes prepared from honey again having the largest sizes.

These DLS results demonstrate that the cubosomes made using diluents other than ethanol require a longer period of time and possibly also dialysis to completely remove the solvent and reach the final particle sizes. During dialysis some of the phytantriol could also be lost from the particles, since it does have some solubility in water. This could have implications for applications and this synthesis method would benefit from further optimisation when targeting specific particle properties, such as changing the volume of anti-solvent (water) compared to that of diluant, concentration of poloxamer 47, using different needle gauges, injection speeds or temperatures and extent of dialysis.

Zeta potential measurements showed only small variations for cubosomes made in each solvent (Figure S3), ranging from a maximum of -17.2±0.6 mV for cubosomes made using glycerol as the diluant compared to -2.2±0.1 mV for cubosomes made from solutions in honey. While all the particles have a negative surface charge, the values are small, and are not of sufficient magnitude to allow electrostatic stabilisation to be the sole stabilisation mechanism.[50] Particle stability is therefore likely to be determined by the non-ionic poloxamer stabiliser. Photographs of the particle suspensions taken 2 months after initial preparation (Figure S4), demonstrate that they remain stable without creaming or sedimentation. This suggests that steric hindrance by the polymeric species is enough to prevent aggregation of the cubosomes after the dialysis stage.

Cryo-TEM

Cryo-TEM was used to further confirm cubosome formation after dialysis against water and to gather structural and size information. As shown in Figure 5, all three diluents tested resulted in cubosome formation (i.e. using ethanol, or glycerol, or ChCl-Gly) (see Supplementary Information, Figures S5-7 for higher resolution images where the crystalline structure is clearer). The samples using lactic acid or honey as the diluent have not yet been assessed by cryo-TEM.

The sample prepared using glycerol had larger cubosomes than either of the other two, consistent with the DLS results for particles dialysed against water. This may be because glycerol has a higher viscosity and therefore dispersal into the water phase takes slightly longer, and the droplet size during addition is bigger, which could lead to greater clumping of phytantriol.

The image of the sample prepared using ethanol has a significant number of liposomes in addition to cubosomes. This is consistent with results reported by Akhlaghi et al. who used the same method as reported here.[34] Liposomes were not observed in the samples made from either glycerol or ChCl-Gly, and as demonstrated by the above SAXS results, this is not due to

an effect of the dialysis procedure or a random result of the Cryo-TEM procedure. It is possible that the dilution trajectory from glycerol or ChCl-Gly to water does not pass through a transitional liposomal/bilayer phase, unlike the trajectory from ethanol.

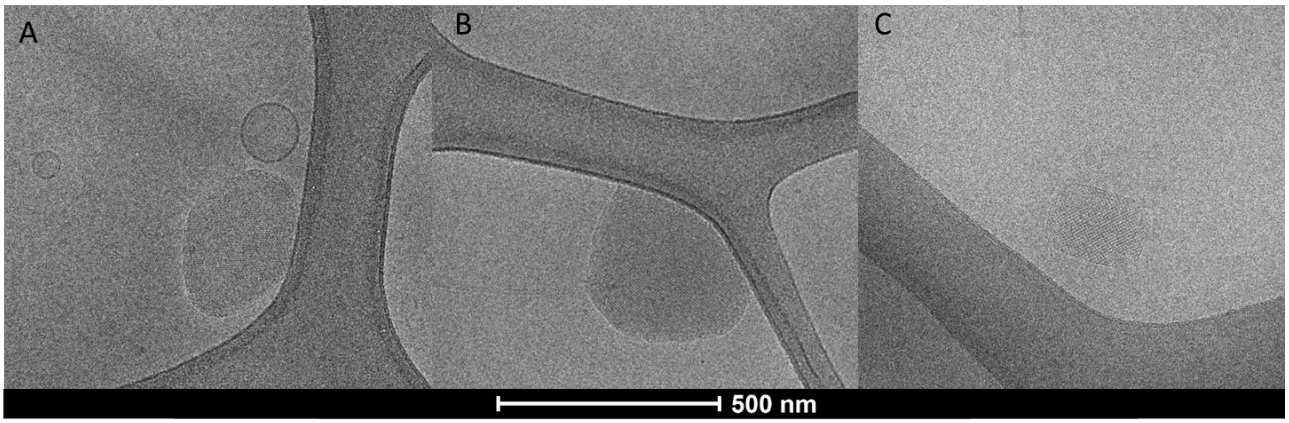

Figure 5. Cryo-TEM images of phytantriol cubosomes prepared from ethanol (no dialysis) (A), glucose (B), and ChCl-Gly (C) respectively as the dispersing phase. See SI for full size images.

Conclusion

This work demonstrates the formation of cubosomes using a bottom-up approach without the need for organic solvents. As far as the authors are aware, this is the first time that either honey, glycerol, lactic acid, or a deep eutectic solvent has been used to create cubosomes using the bottom-up approach. This work proves that low-energy methods of cubosome synthesis are not limited to organic solvents/hydrotropes and provides a means for pharmaceutical development of cubosomes using relatively non-toxic materials such as honey, glycerol and lactic acid. This permits the elimination of potentially harmful organic solvents from the synthesis process. Furthermore, removal of organic solvents may allow encapsulation of drugs that would otherwise be destroyed or be insoluble (e.g. in ethanol) during cubosome formation. In addition, using these other diluents allows formation of cubosomes without concurrent formation of liposomes, unlike those formed from ethanol.[34]

As mentioned in the introduction, phytantriol cubosomes are reportedly more toxic than those made from other lipids such as GMO.[10] The diluents presented in this paper are likely to be just as capable of solubilising other surfactants—like GMO—as they are phytantriol and could therefore be used in the preparation of more biocompatible nanoparticles.

Work is ongoing to find other non-toxic and renewable materials that can be used as dispersing agents for cubosome synthesis. Investigations are also underway to explore the effect of droplet size during addition of the phytantriol mixture (to the aqueous solution) on the final cubosome size and subsequent release of model drug compounds.

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