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Cubosome© Formation via Dilution – Kinetic Effects and Consumer Product Implications

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Optical microscopy is used to study the mechanism of cubosome formation via dilution of ethanol solutions of the monoglyceride monoolein. When water is used for dilution, large cubosomes form that require further dispersion. When aqueous Poloxamer 407 solution is used for dilution, spontaneous emulsification occurs, forming numerous sub-micron particles as well as larger particles that slowly crystallize into cubosomes. Unique intermediate myelin structures are observed as emulsion droplets hydrate and form cubosomes. The formation of cubosomes by dilution of isotropic liquid phase is controlled by the kinetics of the relevant liquid crystalline phase transitions.

Introduction

Cubosomes are dispersed particles of bicontinuous cubic liquid crystalline phase in equilibrium with excess water (*1*). Bulk cubic phase is formed by hydration of monoolein at levels between 20-40% w/w (*1-3*). Cubic phase is unique and desirable as a result of its mesoscale structure: a contorted lipid

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bilayer separating two continuous but non-intersecting water regions (*4, 5*). The tortuous structure of bulk cubic phase provides controlled release of solubilized active ingredients (*6*), while cubosomes exhibit burst release because of their sub-micron length scales (*7*). Cubosomes have been patented for use as active delivery vehicles (*8*), emulsion stabilizers (*9*), and pollutant scavengers (*10, 11*) in various pharmaceutical and personal care products (*12-15*).

Two main approaches are used to produce cubosome particles. The top-down approach applies high energy to fragment bulk cubic phase (*16-18*). The bottom-up approach forms cubosomes from molecular solution by, for example, dilution of an ethanol-monoolein solution (*19*). Top-down or high-energy techniques require formation of cubosomes prior to their use in a product. Bottom-up techniques avoid high-energy drawbacks and allow formation of cubosomes in use by a consumer or during product formulation. Both techniques require a colloidal stabilizer, like the tri-block copolymer Poloxamer 407 (*20*), to prevent cubosome aggregation. Cubosome formation by any method, even dispersion of bulk cubic phase, requires some time for the viscous cubic phase to crystallize from less-ordered precursors (*21*). Figure 1 shows a cryo-TEM image taken several days after ultrasonic treatment of bulk cubic phase (40% w/w water and 60% monoolein) in aqueous Poloxamer 407 solution (*19*). Well-formed cubosomes with regular cubic lattices are visible in Figure 1, as are less ordered cubosomes and simple vesicles, indicating the kinetic dependency of cubosome formation. The mechanism of cubosome formation by high energy dispersion is clearly the fragmentation of bulk cubic phase into smaller pieces. The dilution process produces sub-micron cubosomes in the absence of fluid shear by dilution of an isotropic liquid precursor, but the exact cubosome formation mechanism is not known (*19*).

Previous work has been limited in its ability to quantify cubosome formation mechanisms and rates because time-resolved observations with cryo-TEM are difficult. In this chapter we study the formation of cubosomes by dilution of monoolein-ethanol solutions with water and with aqueous Poloxamer 407 solution. When Poloxamer 407 solutions are used, the dilution process results in immediate interfacial turbulence of the type associated with spontaneous emulsification (*22*), producing numerous sub-micron particles. Some large isotropic liquid (L_1) phase droplets remain after the initial spontaneous emulsification, and their transformation into cubosomes is followed using optical microscopy. Unique intermediate myelinic particle morphologies form as water and Poloxamer 407 diffuse into the droplets and ethanol diffuses out. By characterizing the intermediate behavior of cubosomes formed by the dilution technique, we are better able to predict and understand the mechanisms and rate of cubosome formation. For cubosomes in consumer products and pharmaceuticals, the most commonly envisioned uses are diffusive uptake/release of materials and/or deposition onto skin or tissue. In some cases, the bottom-up dilution process (*19*) is favored so that cubosomes form only

during consumer use, such as by dilution via ingestion or sweating. Cubosome performance in such applications is then a function of the mechanism and rate of cubosome formation by dilution.

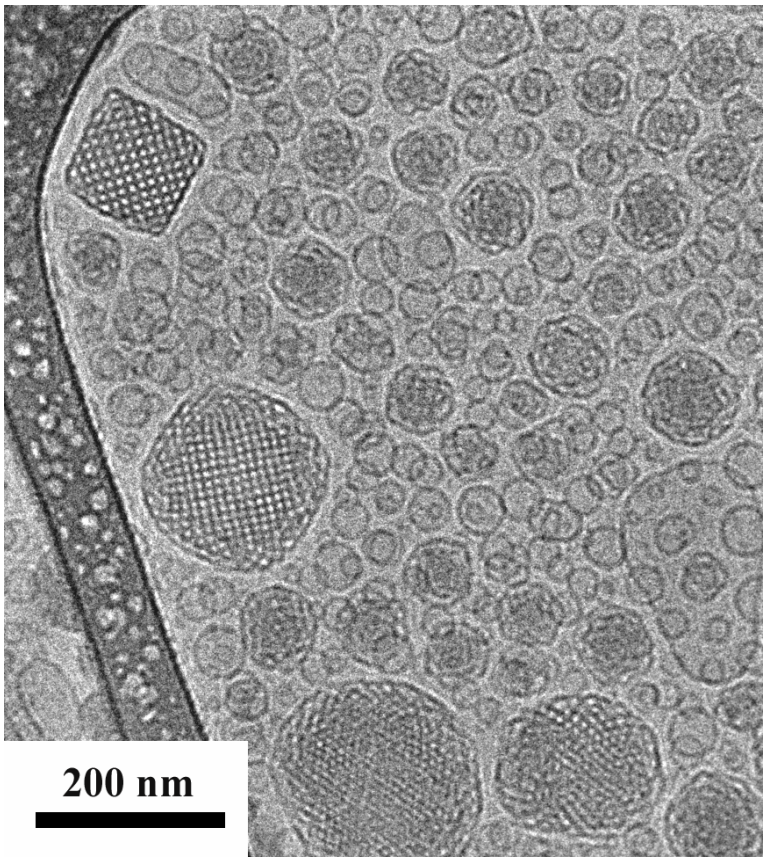

Figure 1. Cryo-TEM image of cubosomes formed by ultrasonic treatment of bulk cubic phase. (Reproduced with permission from reference 19. Copyright 2001 Am. Chem. Soc.)

Experimental

Materials

The system under investigation is a mixture of monoolein (Nu-Chek Prep), ethanol (Sigma Aldrich), Poloxamer 407 (PEO_{98}-PPO_{67}-PEO_{98}, with an average formula weight of 12,500, Spectrum), and deionized water (Millipore). The monoolein and ethanol are >99% pure.

Methods

Microscopy

All optical microscopy is carried out using a Zeiss Axioscop, equipped with differential interference contrast (DIC) optics, following alignment for Koehler illumination. Images are digitized using a Coolsnap camera (Photometrics) and a Flashbus MV frame grabber card (Integral Technologies) controlled by the software package Metamorph (v. 5.0, Universal Imaging Corp.).

Quantitative surfactant phase identification is difficult using only optical microscopy, although it is reasonable to assign some phases based on the previously established behavior of a given system and the optical defect textures exhibited by liquid crystalline phases (*23, 24*). For example, very viscous isotropic phases are assumed to be bicontinuous cubic phase. High viscosity is assessed by observations of low to no Brownian motion of tracer particles. The absence of birefringence between crossed polarizers indicates optical isotropy.

Cubosome Formation Experiments

Observations of cubosome formation are made directly on a microscope slide immediately following addition of either aqueous Poloxamer 407 solution to ethanol-monoolein solutions, or the opposite. Order of addition is not found to affect the general conclusions drawn for all cases examined. Cover slips are added to the sample immediately following cessation of the large amplitude interfacial turbulence and are not found to affect the conclusions drawn.

Results and Discussion

Cubosomes are formed by sufficient dilution of liquid precursors prepared in the binary monoolein-ethanol system. Such processes can be visualized using the ternary phase diagram determined by Spicer et al. (*19*), shown in Figure 2. In Figure 2, four single phase regions exist, including two cubic phases, one lamellar phase, and one isotropic liquid phase at high ethanol levels. Cubosomes form in the lower left portion of the phase diagram where cubic phase is in equilibrium with isotropic liquid phase. Dilution is represented by a straight line drawn from the starting composition toward the water apex of the triangle (Figure 2).

Cubosomes from the Ethanol - Monoolein -Water System

The formation of stable, discrete cubosomes is only possible when a colloidal stabilizer like Poloxamer 407 is used. The addition of 5 μL of 33% w/w monoolein solution in ethanol to 50 μL of deionized water on a microscope slide produces large pieces of cubic phase (Figure 3). Cubic phase forms when the monoolein-ethanol droplet contacts the water, ethanol diffuses out, and water diffuses in to hydrate the monoolein. In a matter of seconds, the system is essentially at equilibrium in the cubic phase-water region of the ternary phase diagram, the lower left portion of Figure 2. In this ternary system, dispersion of the cubosomes by fluid shear or another energy source is required to produce sub-micron particles. Less dispersion energy is needed than for the monoolein-water system because ethanol significantly reduces the cubic phase viscosity (*19, 25*). In most practical cubosome dispersion processes, a colloidal stabilizer like Poloxamer 407 is needed to provide steric stabilization against close approach and agglomeration. Beyond simple colloidal stabilization, however, the Poloxamer 407-monoolein-water system phase behavior significantly differs from that of the monoolein-water system (*20*). It is reasonable then to expect the amphiphilic polymer to affect the cubosome formation mechanism during its use in the dilution process.

Cubosomes from the Poloxamer 407- Ethanol-Monoolein -Water System

The inclusion of Poloxamer 407 in the ethanol-monoolein-water system has a significant effect on the mechanism of cubosome formation versus the ternary system without polymer. When 5 μL of 33% w/w monoolein solution in ethanol is added to 50 μL of 1.5% w/w aqueous Poloxamer 407 solution on a

slide, violent interfacial turbulence immediately occurs that is visible unaided. The

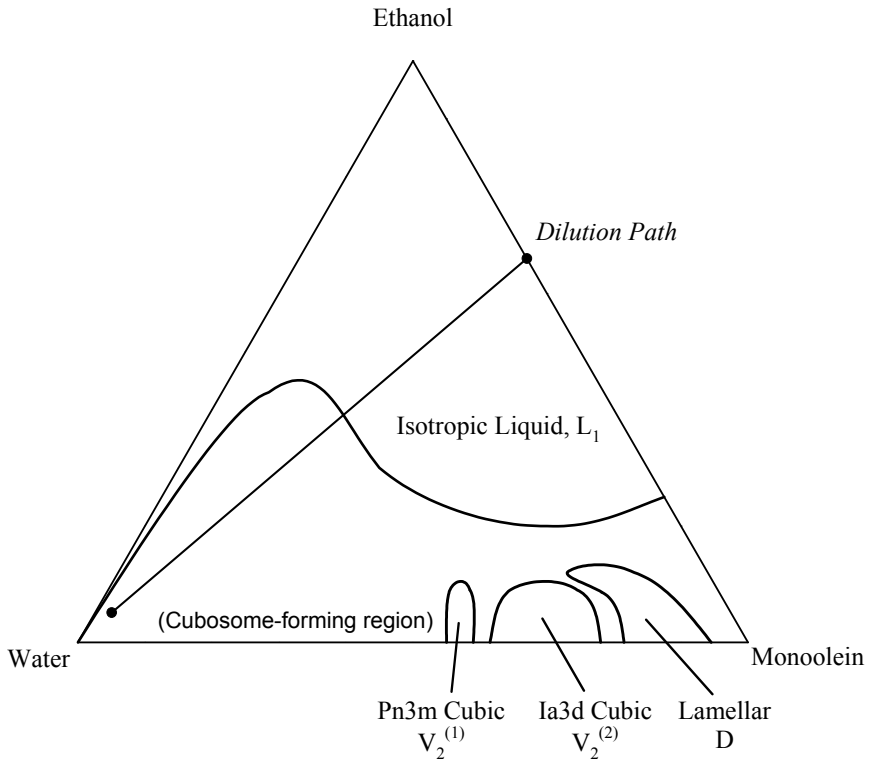

Figure 2. Equilibrium ternary phase diagram for the ethanol-monoolein-water system. The dilution path illustrates the process used to form cubosomes in this work. (Reproduced with permission from reference 19. Copyright 2001 Am. Chem. Soc.)

disturbance subsides in several seconds, leaving a dispersion of mostly sub-micron particles that can not be resolved well optically. Observations of the larger micron-scale particles provide insight into the driving force for the interfacial turbulence observed and thus the operative cubosome formation mechanism.

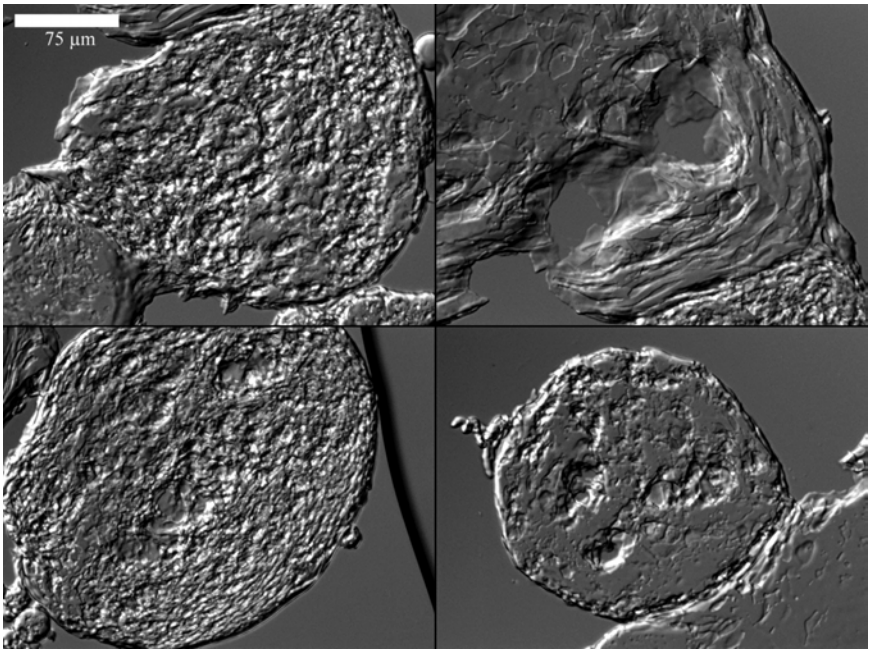

Figure 3. Optical micrographs of cubosomes produced without Poloxamer 407 polymer present.

The optical micrograph in Figure 4 shows a typical interface between the bulk solution and a droplet of concentrated ethanol-monoolein solution. Optical microscopy allows careful study of only the larger particles produced, but the background texture and Brownian motion observed indicate that the majority of the particles formed are sub-micron. The dilution process occurs with essentially zero fluid shear, so the production of such small particles must be the result of interfacial forces.

The interface in Figure 4 has surface protrusions resembling the myelin structures formed at surfactant-water interfaces far from equilibrium (*26-28*). Myelins are kinetically stable cylindrical structures, often appearing in surfactant-water systems with large miscibility gaps between the lamellar liquid crystalline phase and water (*29, 30*). Myelins are also observed when a surfactant-oil-water system crosses liquid crystalline phase boundaries during dilution, resulting in a type of spontaneous emulsification (*31, 32*). Very small emulsion droplets can be produced via myelinic spontaneous emulsification in the complete absence of shear (*33*). Droplets "bud" off of the tips of myelins,

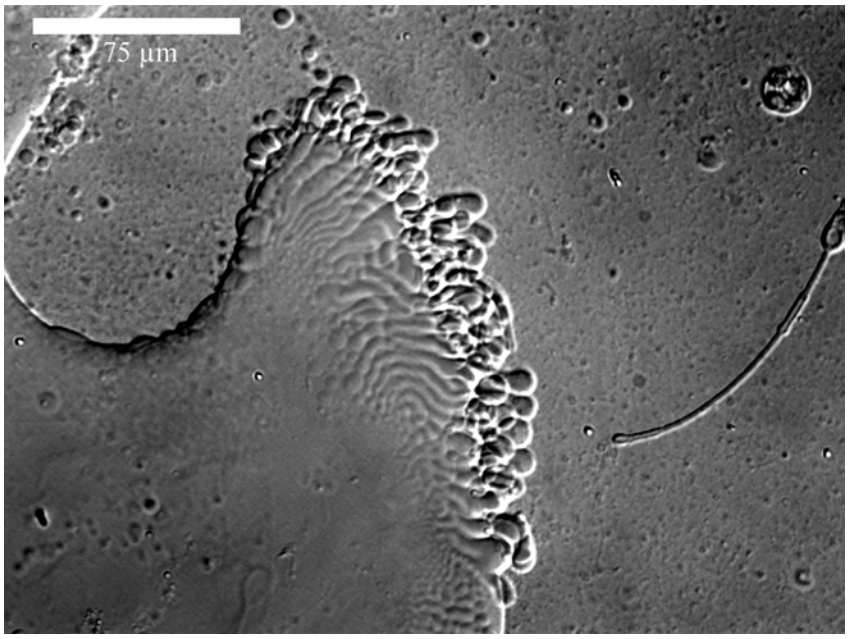

Figure 4. Interface between a droplet of monoolein-ethanol solution and aqueous Poloxamer 407 solution. Myelins are visible at the interface.

producing fine dispersions of droplets or liquid crystalline particles (*31, 34*). Myelin growth is only observed in the presence of Poloxamer 407 for the systems studied here. The associated spontaneous emulsification, caused by dilution through liquid crystalline phase regions, is apparently the mechanism of sub-micron cubosome formation.

Figure 5 shows four additional particles from the above quaternary system in various stages of myelin formation. The initial emulsification occurs almost instantaneously, forming numerous sub-micron particles. The larger droplets remaining continue to evolve, although more slowly as a result of their greater diffusion length scales and the formation of the more viscous cubic phase. The driving force for spontaneous emulsification and myelin formation is concentration gradients. As diffusion continues following the initial emulsification, the system heterogeneities decrease in magnitude and the driving force decreases for continued dispersion of particles. Nevertheless, all four particles in Figure 5 have myelinic tubules protruding from their surfaces, all in different stages of growth from the parent droplet. In some cases, such as the

upper right and lower left images in Figure 5, smaller particles are visible that have just budded off of the parent myelin but retain their tubule morphology.

The myelins observed in Figures 4 and 5 are unique in that they form in a system with bicontinuous cubic phase compositionally adjacent to the lamellar liquid crystalline phase (Figure 2). Most studies examine myelins in the context of lamellar phase swelling toward equilibrium with water, while here a phase transition from lamellar to cubic occurs as the system continues to hydrate. As a result, the bilayer flexibility of the myelin structures decreases as the viscous cubic phase forms from the less viscous lamellar phase. This may explain why no coiled myelins are observed. Haran et al. (*35*) found that hydrotropic additives like toluene sulfonic acid enhance myelin coiling by increasing bilayer flexibility. Although ethanol possesses hydrotropic properties as well, any flexibility increase is likely offset by the formation of the viscous cubic phase, preventing coiling.

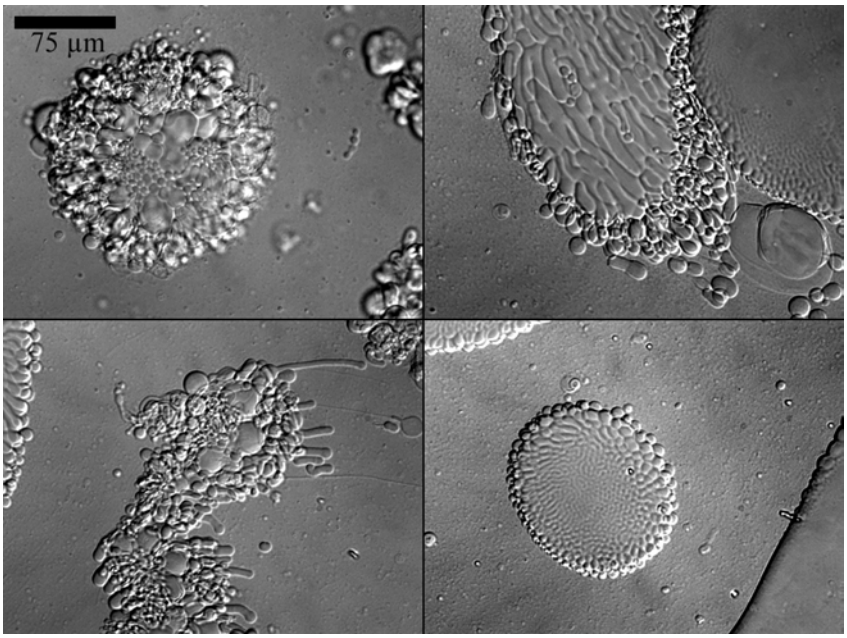

Figure 5. Examples of particles formed upon addition of monoolein-ethanol solution to aqueous Poloxamer 407 solution. All particles display myelin formation at various stages.

The order of addition is often a variable during non-equilibrium processes. For the example applications of the dilution process cited above, a preparation like ethanol-monoolein solution could be added to the mouth or skin where saliva or sweat induce dilution and cubosome formation. In such cases the order of addition is fixed: organic phase added to aqueous phase. In other applications, however, the order may be reversed. Application of organic phase to plant surfaces followed by rainfall is an example of the reverse case of aqueous phase added to organic phase. Figure 6 shows examples of particles formed by adding 50 μL of 1.5% w/w aqueous Poloxamer 407 solution to 5 μL of 33% w/w monoolein solution in ethanol. Spontaneous emulsification is again observed on the slide upon combination of the two phases. Comparison of the particles in Figure 6 with those in Figure 5 indicates no obvious differences. Most of the particles in Figure 6 exhibit some degree of myelin formation as a result of swelling by diffusion into the droplets.

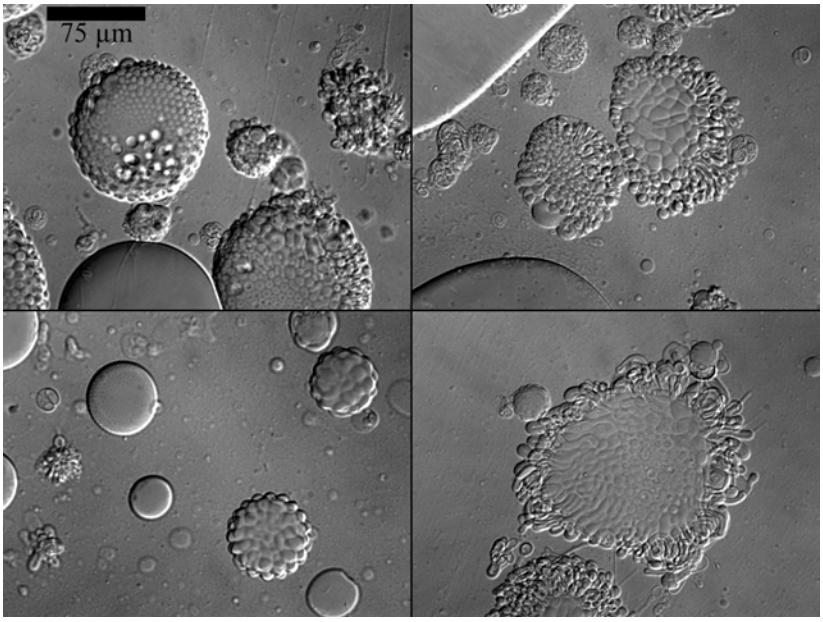

Figure 6. Particles formed by addition of aqueous Poloxamer 407 solution to monoolein-ethanol solution. Order of addition does not appear to affect particle morphology.

Some of the particles in the left hand side of Figure 6 exhibit a "raspberry" texture similar to particles observed by Buchanan et al. (*29*) when sheared lamellar phase particles, or onions, merged to form sponge, or L_3, phase. There is no evidence of such a transition here given the qualitative nature of these observations. However, a transition from isotropic, to lamellar, to sponge, to cubic phase is plausible on a structural basis. Sponge phase is simply disordered bicontinuous phase and is known to form in the Poloxamer 407-monoolein-water system (*20*) and may also form in the ethanol-monoolein-water system (*19, 36*). What is more likely is that the raspberry textures result from the early emergence of cylindrical myelins from the surface of a spherical droplet as hydration occurs. Buchanan et al. (*29, 30*) find that myelin formation proceeds via diffusive flux of water into myelin roots. They also calculate a packing density assuming the myelin cylinders order hexagonally. Figure 6 agrees with their finding as the upper left image shows the emerging myelins are ordered hexagonally. Also visible in Figure 6 are droplets that have not begun the transition through myelinic intermediates to cubic phase. It is useful to follow such a transition in order to better characterize the cubosome formation mechanisms and their rates.

Figure 7 shows a series of images taken at one minute intervals as the center droplet "crystallizes" from the isotropic liquid phase to the (presumed) cubic liquid crystalline phase. The first six images in Figure 7 illustrate development of the raspberry texture as myelins emerge from the droplet. Hydration occurs as water diffuses in between the myelins. After six minutes the droplet suddenly elongates, indicating a significant density or structural change, likely as a result of a phase transition. Image analysis indicates a linear change in droplet area with time and an area increase of 40% from the first to last image in Figure 7. Separate experiments with tracer particles support the formation of a very viscous, likely cubic, phase at the end of such a transformation. A similar change occurs in the particle partially visible in the lower right hand corner of each image, in this case the appearance changes less dramatically than the center particle but is also consistent with formation of a cubic phase. Clearly there is a distribution of times in which these transformations occur, consistent with the kinetic nature of transitions to the cubic phase (*37*). It is possible that the sub-micron particles also require some induction time between their spontaneous formation and their equilibration as cubosomes. The shorter length scales and thus diffusion times for smaller particles may speed things considerably, but cryo-TEM work indicates kinetic limitations to cubosome formation at the nanometer scale as well (*21*).

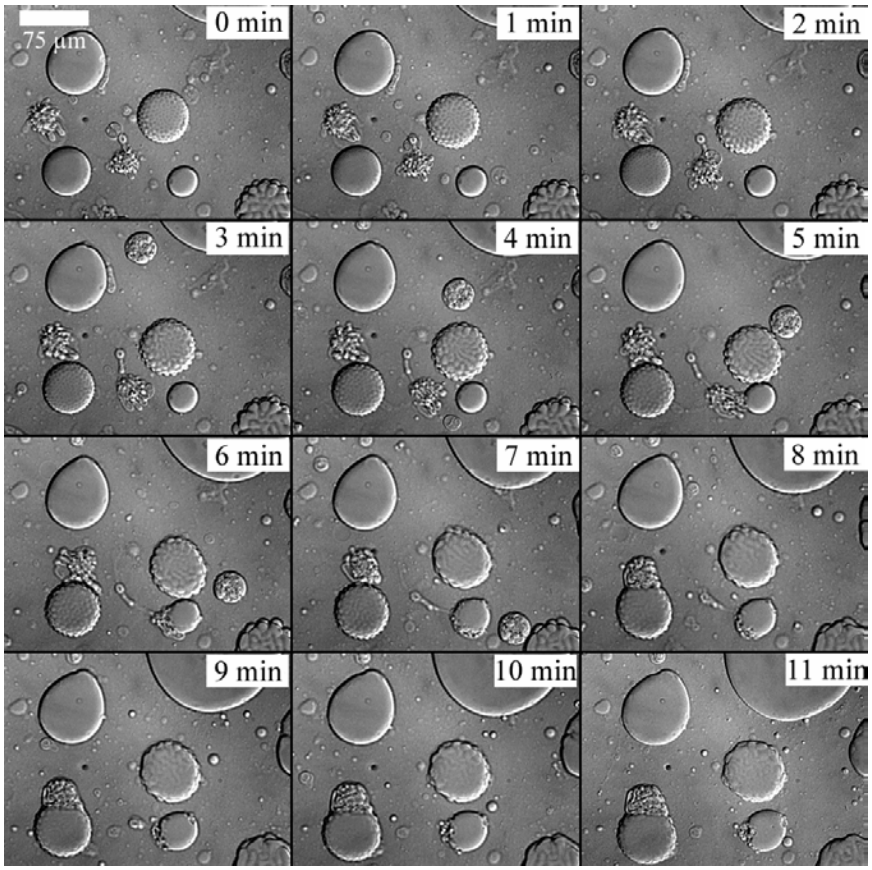

Figure 7. Montage of images of a droplet of isotropic liquid crystallizing into cubic phase.

Conclusions

Cubosome formation via dilution of the monoolein-ethanol system is described using the concepts of spontaneous emulsification and the associated myelinic interfacial instabilities. Upon dilution of the monoolein-ethanol system with aqueous Poloxamer 407 solution, spontaneous emulsification produces numerous sub-micron particles that form cubosomes and vesicles with time. Larger droplets with slower diffusion length scales form unique

particulate structures as a result of myelinic interfacial instabilities that slowly transform into the viscous bicontinuous cubic liquid crystalline phase.

The formation of cubosomes via dilution is limited by the kinetics of the relevant phase transitions. Some of the most desirable properties of the bicontinuous cubic liquid crystalline phase, including complex microstructure and bioadhesion, will thus also be present only after an induction time characteristic of the cubic phase. The interfacial properties of the quaternary Poloxamer 407-ethanol-monoolein-water system need to be studied in more detail to better understand and control the kinetics of the relevant phase transitions.

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