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Morphological Study of Emulsion-Assisted Cholesterol Precipitation Processes

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Crystallization of cholesterol in chemical conditions that involve the presence of emulsions of three different types of surface active agents—cationic, anionic, and nonionic—was investigated by means of scanning and transmission electron microscopic analyses. In contrast with the previous attempts to modify the typical plate- and needle-shaped character of biaxially grown cholesterol crystals, the present study indicates that spherical templates of fine emulsion droplets may impose spherical morphologies to cholesterol particles nucleated within.

1. INTRODUCTION

Cholesterol is one of the essential biochemical compounds in the animal world, involved in numerous body functions, ranging from the synthesis of bile acids and steriod hormones *in vivo* to maintaining a proper transport across cellular membranes [1]. On the other hand, because of disbalanced relationships between cholesterol intake and deposition rate with the rate of its solubilization through the action of micellar, vesicular, and bilayer cleansing agents in bile and specific lipoprotein complexes in blood, cholesterol may form excessive deposits on the flow-paths of some body fluids and cause problematic health issues that range from gallstone formation to intestinal lumen deposits to atherosclerotic plaque. Thereupon, because of potential development of reversible control, investigation of cholesterol crystallization into novel morphologies presents a task with large significance for both the areas of biomedicine and chemotherapeutics and the fundamental understanding of complex biochemistry of life.

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All of the previous attempts to obtain any morphology of cholesterol crystalline deposits other than the ones composed of typical plate-shaped and needle-shaped crystals by means of mere chemical manipulation did not succeed. Despite the fact that the effects of solvent type [2–4], nonsolvent phases [5], temperature [2], pH [6], electrolytes [7], dynamics of solvent systems [8], magnetic field [9], mineral substrates [10], and co-existing phases [11,12] (such as hydroxyapatite deposits, often found interspersed within cholesterol layers in atherosclerotic plaques [13,14], model bile composition [15–17], various medicinal plants [18], and synthetic biochemical compounds (including phospholipids [19], cholic acid [20], and other sterols [21]) on the process of crystallization and dissolution of cholesterol were previously evidenced, the inherent structural tendency of cholesterol molecules to adopt biaxially grown formations in solid state defied all the imposed environmental constraints. The observed variations in surface polarity of cholesterol crystals with the nature of the solvent [22] and the multifunctional biological role of cholesterol, which includes cell membrane flexibility mediation and transmembrane signal messaging [1], may be indicators of this intrinsic structural flexibility. The reason for the ostensibly inevitable formation of biaxially grown crystals can be found in the disparity between interlayer and intralayer interactions between cholesterol molecules in solid state. Namely, comparatively stronger hydrogen bonds predominantly figure as intermolecular links within the individual layers of cholesterol molecules, whereas weaker van der Waals' bonds are predominantly involved in stacking the layers along the c axis. Faster crystal growth in the bilayer plane compared to the growth in the direction of the c axis is, therefore, energetically more favorable within these conditions. However, as shown throughout the following text, because of the existence of micellar effects that—via limiting and templating nuclei and particle growth—decrease, globulize, and uniformize the resulting crystal forms, emulsion-assisted crystallization can be regarded as an effective means to decrease the dimensions of cholesterol plate-shaped crystals to almost nanosized level and obtain well-dispersed cholesterol crystals with spherical morphologies.

2. EXPERIMENTAL

2.1. Materials

The chemicals used in the synthesis were cholesterol (99+%, *Alfa Aesar*), Triton X-100 (*Rohm and Haas*), cetyltrimethylammonium

bromide (CTAB, 99%, *Alfa Aesar*), sodium n-dodecyl sulfate (SDS, 99+%, *Alfa Aesar*), and 1-hexanol (*J. T. Baker*).

2.2. Methods of Synthesis

In the first method, 1 ml of Triton X-100, 10 ml of 1-hexanol, and 2.2 ml of distilled water were poured successively into a beaker, resulting in the formation of a white turbid mixture. Twenty milligrams of commercial cholesterol were introduced in the prepared mixture, followed by the addition of 32 ml of 1-hexanol. The mixture was then stirred for 30 min by means of a magnetic bar, until the liquid became completely transparent. Twelve milliliters of distilled water were then rapidly poured into the emulsion, resulting in the formation of an opaque white dispersion. After 10 min of agitated aging, samples of the suspension were taken through the pipette, poured on top of the sample carriers for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analyses, and let dry in vacuum.

In the second method, 36 mg of sodium n-dodecyl sulfate (SDS) were dissolved in 10 ml of 1-hexanol and 2 ml of distilled water. Twenty milligrams of commercial cholesterol were introduced to the emulsion, with the later addition of 42 ml of 1-hexanol and 2 ml of water. In the ceaselessly agitated emulsion, which might be otherwise settling into thoroughly transparent biphase mixture, 12 ml of distilled water was abruptly introduced. The emulsion was further stirred for 5 min, let stand still for 10 min (with a dispersion sample poured on top of an SEM sample carrier afterward), dried in air, and viewed under the SEM.

In the third method, 33 mg of cetyltrimethylammonium bromide (CTAB) were dissolved in 10 ml of 1-hexanol and 2 ml of distilled water. Twenty milligrams of commercial cholesterol were introduced to the emulsion. Into this transparent but visibly emulsified liquid, additional 5 ml of 1-hexanol were introduced. To the prepared, magnetically agitated emulsion, 5 ml of distilled water were then abruptly added, resulting in the formation of a white dispersion of cholesterol particles. After 10 min of agitated aging, a dispersion sample was poured on top of an SEM sample carrier, dried, and observed under the SEM.

In the fourth method, 1 g of CTAB was dissolved in 18.5 ml of 1-hexanol and 0.5 ml of distilled water. To such prepared emulsion, 330 mg of cholesterol were introduced, followed by the addition of 3 ml of water, resulting in the formation of a white, emulsified dispersion. Magnetic stirring was subsequently discontinued, and the emulsion remained unstirred for the following 24 h, when the sample for SEM analysis was taken.

2.3. Characterization

Morphologies of the prepared cholesterol particles were examined with using SEM and field-emission scanning electron microscopy (FE-SEM), whereas the process of melting of submicron-sized cholesterol particles was observed by means of TEM.

3. RESULTS AND DISCUSSION

In the previous study [23], the isoelectric point of cholesterol crystals was determined as existing in a relatively low acidic pH range. This observation is consistent with the previous results reported in the literature [24], according to which the isoelectric point of cholesterol particles might have been deduced as present at pH = 2.7. Negative surface charge of cholesterol particles at neutral pH values is a consequence of the constitutive hydroxyl groups that typically come to protrude crystal surfaces when the crystallization is performed in polar solvents. Microemulsions and emulsions based on octylphenol ethoxylate (Triton X-100), SDS, and CTAB, as nonionic, anionic, and cationic surfactants, respectively, were chosen as emulsified media for the study of emulsion-assisted precipitation of cholesterol particles. Triton X-100, a biodegradable nonionic surfactant, has previously been known as an excellent dispersant for oil-in-water emulsions. On the other hand, the modification of physical effects that govern the phase-transition interaction of negatively charged cholesterol particles at neutral pH conditions was investigated by the introduction of electrically charged molecular moieties of CTAB and SDS within cholesterol precipitation processes.

After 3 min of aging of the precipitate, preparation of which is described in Section 2.2, involving the use of Triton X-100-based emulsion, cholesterol crystallizes in form of square-shaped particles

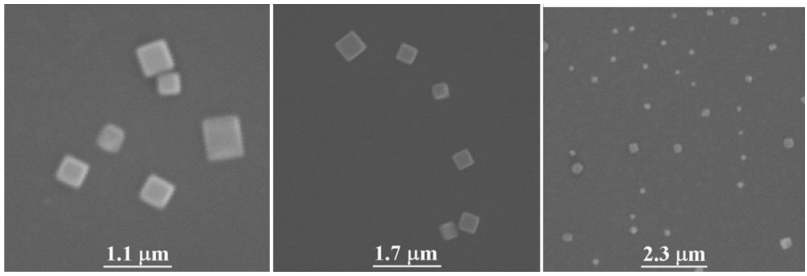

FIGURE 1 Submicron-sized, square-shaped cholesterol particles obtained by precipitation in Triton X-100/1-hexanol/water microemulsion.

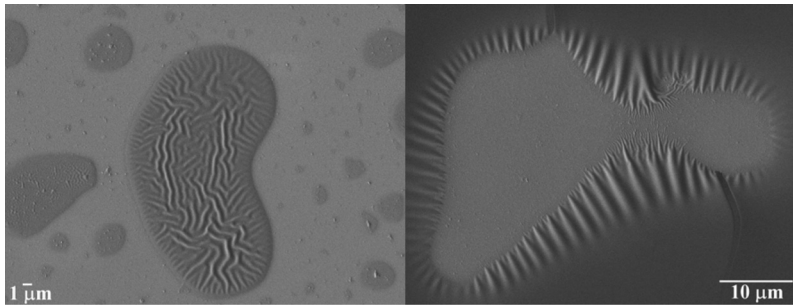

FIGURE 2 FE-SEM images of the self-assembled patterns in the emulsion system comprising Triton X-100, cholesterol, 1-hexanol, and water.

0.1–0.5 μm in size, as can be evidenced from SEM images in Fig. 1. In addition to square-shaped particles, bilayered-substructured micelles and other complex self-assembly patterns that incorporate the former solids were observed by means of FE-SEM analysis (Fig. 2). As a regular constituent of cellular membranes and an amphiphilic compound, cholesterol has a tendency for positioning at the oil–water interface (which was evidenced by using centrifugation treatment and consequently inducing segregation of aqueous and alcoholic phases with a thin white layer of dispersed cholesterol particles in between, although easily redispersed in a re-emulsified mixture, obtained again by only mild shaking), which might present the background of origins of the observed complex self-assembly patterns. The cholesterol particles, however, melt and undergo shape and phase modification under the influence of a high-voltage accelerated TEM electron beam, as can be seen from TEM images in Figs. 3–5. Nanosized melted cholesterol spheres are 50–100 nm in diameter, whereas an increased density of

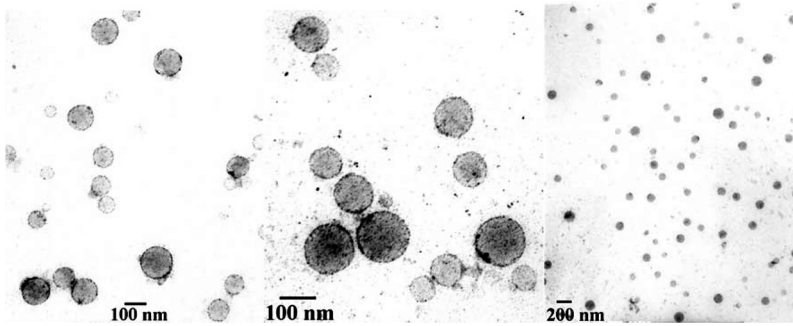

FIGURE 3 TEM images of melted and narrowly dispersed cholesterol droplets.

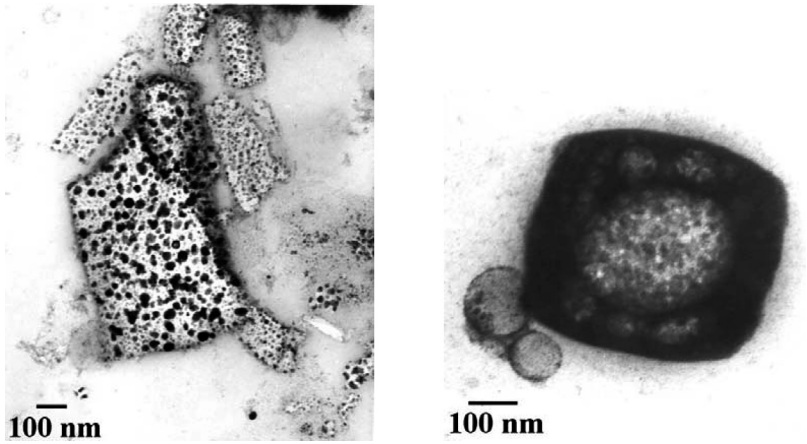

FIGURE 4 Cholesterol crystals preserving their sharp edges, albeit with the decomposition of their inner layers into spherical particles (left) and a square-shaped cholesterol particle melting to form spherical droplets during TEM analysis (right).

the constitutive matter toward edges of the particles compared to the particle core is consistent with the previous observation of similarly structured melted cholesterol droplets [25]. As the heat content of cholesterol deposits increases, sharp-edged crystallites become

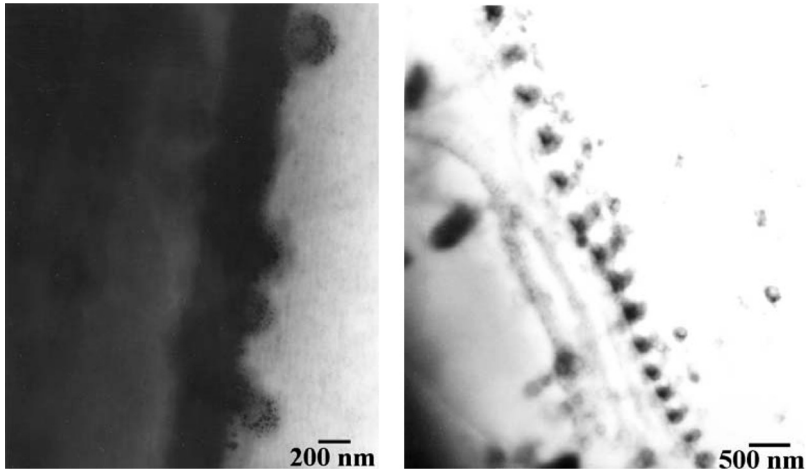

FIGURE 5 Needle-shaped cholesterol crystal transforming into an array of melted spherical droplets in the course of TEM analysis.

rounded and, in vicinity of the melting point, form spherical aggregates [25].

Significant charging effects evidenced during the SEM analyses of cholesterol samples at regular acceleration voltages (~ 15 kV) and frequently observed bending, yielding, and ripping of cholesterol platelet-shaped crystalline structures presented an immediate indication of significant susceptibility of cholesterol crystalline deposits upon the influence of electron beams. Therefore, thermal absorption of electrons during TEM analyses presents the reason for the formation of melted cholesterol droplets, typically composed of distinct spherical membranes that correspond to boundary outlines of cholesterol droplets. So far, Debuigne et al. have been the only research team that has reported a microemulsion-assisted cholesterol preparation route [26], as well as the corresponding formation of nanosized spherical cholesterol particles 3–7 nm in size. However, taking into account that a) TEM was in this case also used to characterize the morphology of recrystallized cholesterol particles and calculate the average particle size thereof and b) individual cholesterol molecule are ~ 1.7 nm in length leads to the conclusion that the observed were probably only the remnants of melted or even decomposed initial cholesterol crystals under the influence of sufficiently high local temperatures produced by the TEM electron beam under standard measurement conditions. More of the interesting features of the process of melting typical, plate- or needle-shaped cholesterol crystals into individual cholesterol spheres may be evidenced by employing TEM analysis and are presented in the following images. Cholesterol crystals preserving their sharp edges, albeit with the decomposition of their inner layers into spherical particles during the TEM imaging, are presented in Fig. 4a, whereas Fig. 4b shows a single, less than 10-nm-sized cholesterol particle transforming into melted spheres under the influence of TEM observation. The process of transformation of a needle-shaped cholesterol crystal into spine-like array of spherical particles is presented in Fig. 5.

Crystallization of cholesterol within SDS and CTAB comprising 1-hexanol/aqueous emulsions under specific conditions also results in the formation of well-dispersed and uniformly sized particles. Particles of unidentified composition, 1–2 μm in size, prepared in an unstable, biphase-settling SDS/1-hexanol/water emulsion according to the procedure presented in Section 2.2, are shown in Fig. 6. However, after prolonged aging of the still biphase mixture (up to ~ 4 h), the initial particles, typical of their poriferous, spongy substructure and irregular but curved shapes, transform into larger, flower-like structures (~ 10 μm in radii), followed by the transition from whitish

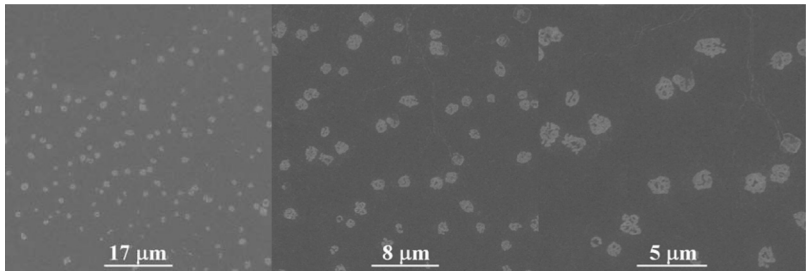

FIGURE 6 Particles obtained by precipitation of cholesterol in SDS/1-hexanol/water emulsion.

cholesterol curtain positioned at the alcohol–water interface on the bottom, aqueous phase side to white-colored upper, alcoholic phase (switching the medium transparency from alcoholic to aqueous phase, and its opacity in the opposite direction). SEM images of flower-shaped cholesterol crystals obtained by such aging of SDS-emulsified, hexanol/water biphase dispersion, consisting of platelet-shaped substructural units, are shown in Fig. 7. Previously, many cases of cholesterol preparations, particularly within model bile environments and lecithin-containing

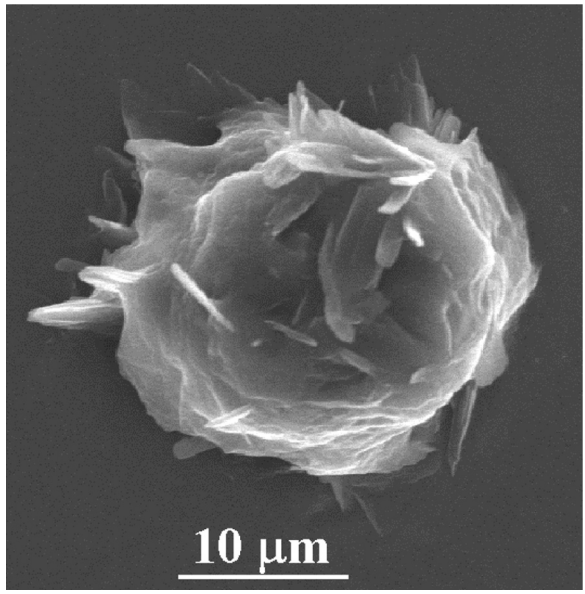

FIGURE 7 Individual flower-shaped cholesterol crystal obtained by 4 h of aging of nonagitated SDS-emulsified, hexanol/water biphase mixture.

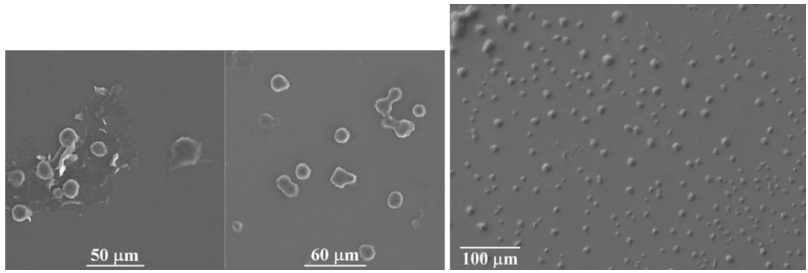

FIGURE 8 Round-shaped cholesterol crystals obtained by their precipitation in CTAB/1-hexanol/water emulsion.

emulsions, wherein transient micellar, vesicular, and lamellar structures as metastable cholesterol carriers transform to plate-shaped cholesterol morphologies with time, were reported [27,28]. The spongy globules obtained herein could similarly present either surfactant-comprising particles or an intermediate phase in the process of formation of larger aggregates composed of elongated cholesterol platelets.

SEM images of spherical cholesterol crystals, almost uniformly measuring $\sim 10\,\mu m$ in diameter, prepared with using CTAB/1-hexanol/water emulsion as a parent precipitation medium, and evidently composed of layered, cake-like substructure, are shown in Figs. 8–9. Compared to the previous presented cases, nuclei growth rate is herein supposedly favored more than the rate of seed formation, resulting in the initial crystallization of millisized, plate-shaped cholesterol deposits that may only subsequently break into numerous spherical cholesterol islands. The mechanism of cleavage of cholesterol particles through

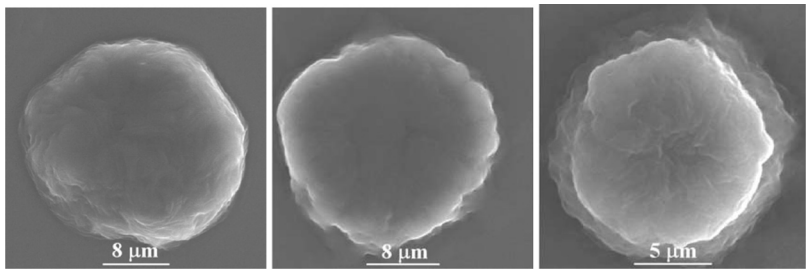

FIGURE 9 Individual round-shaped cholesterol crystals obtained by their precipitation in CTAB/1-hexanol/water emulsion, evidently composed of layered, cake-like substructure.

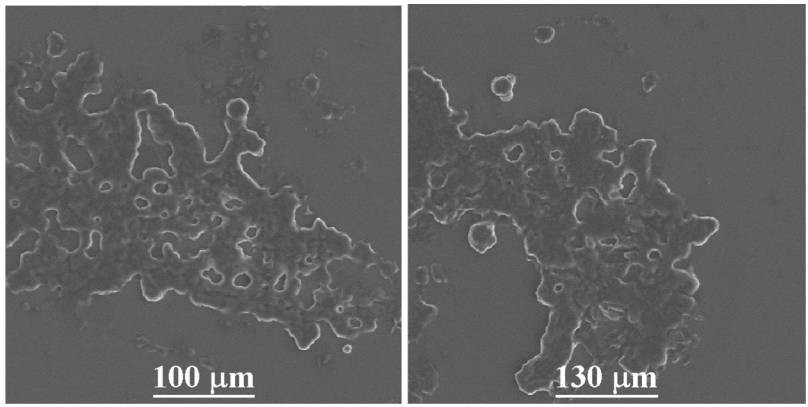

FIGURE 10 Cholesterol islands being chopped off from millisized initial cholesterol layers by the action of emulsion droplet dynamics.

the collisions of spherical emulsion droplets with cholesterol layers may be invoked in this case. Typical flat cholesterol deposits, being broken into numerous spherical islands by the influence of emulsion droplets, are presented in Fig. 10.

The fourth method of precipitation presented in Section 2.2, employing CTAB/1-hexanol/water water-in-oil emulsion yields similar aggregates of spherical cholesterol islands $\sim 50\,\mu m$ in size, as can be evidenced from Fig. 11.

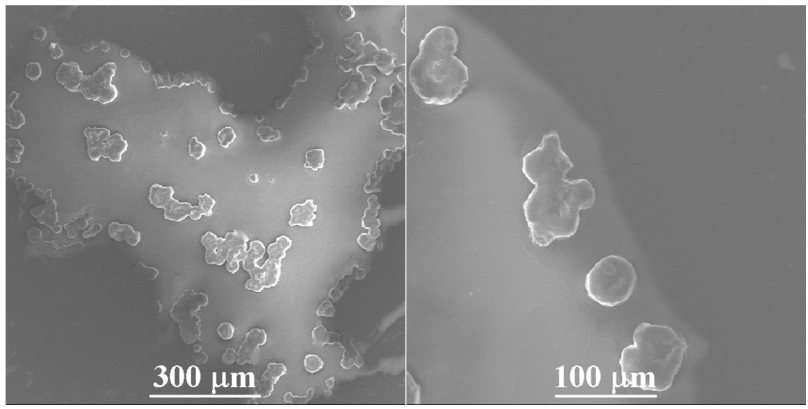

FIGURE 11 Microsized cholestrol spheroids prepared in accordance with the fourth method in Section 2.2.

4. CONCLUSION

In accordance with the presented results, spatial restriction effects of micellar multimolecular configurations during cholesterol crystallization can be considered significant enough to either limit and direct growth of cholesterol crystals into circular micellar interior shapes or break initial or transient layered cholesterol precipitates into oval-shaped particles. All the investigated emulsion compositions under particular conditions offer possibilities for obtaining smaller and even submicron-sized cholesterol particles instead of large, millisized cholesterol crystalline deposits. Such an observation is consistent with the biological role of *in vivo* micelles and vesicles involved in the natural solubilization of gallbladder cholesteric deposits. Whereas previous investigations aimed at production of spherical cholesterol morphologies based on the application of various solvents, surface active agents, and synthesis approaches in each given case resulted in plate-shaped cholesterol crystals and aggregates, it is shown hereby that cholesterol precipitation from dilute solutions may, within specific chemical environments and compositions thereof, indeed yield spherical cholesterol particles.

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