Co-operative self-assembly of cholesterol and γ-oryzanol composite crystals

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Cholesterol and γ-oryzanol co-crystallization occurs when equal ratios of the two compounds or excess γ-oryzanol is present in the solution. Excess cholesterol produces macroscopic fibrillar crystals from the melt prior to the co-crystallization and formation of radial fine fibers under slow cooling conditions. The cholesterol fibrillar crystals then facilitate cholesterol-γ-oryzanol co-crystallization producing fine fibers that nucleate from the surface of the cholesterol crystals. Equal ratios of cholesterol and γ-oryzanol or excess γ-oryzanol allow the radial fine fibers to nucleate from the melt without the need of an internal crystal template.

Introduction

Fabrications of molecular biomaterials frequently self-assemble via the ‘bottom-up’ approach, in which materials are assembled, molecule-by-molecule, resulting in the formation of novel supramolecular architectures.1 These supramolecular structures are stabilized by weak, noncovalent interactions including: hydrogen bonds, ionic bonds, hydrophobic interactions, and/or van der Waals interactions.1 Functionalized biomaterials found in nature, formed via self-assembly, have an array of novel, beneficial functionalities (i.e., silk,2 collagen,3 and tubulin4) as well as detrimental architectures that lead to disease (i.e., prion proteins and atherosclerotic plaques5). An understanding of these self-assembled supramolecular architectures allows for the future exploitation of nature to develop novel synthetic materials with unique functionalities as well as possible treatments for diseases arising from these adverse architectures.

To utilize molecular fabrication chemical complementarily and structural compatibility are required which need to confer weak, noncovalent interactions that bind the building blocks together resulting in the formation of supramolecular aggregates.1 In nature, the complexity of self-assembly if often taken for granted, and the ability to recreate these supramolecular structures in the laboratory often eludes scientists. However, nature has inspired our attempts to not only understand these processes but also to harness this technology creating new and exciting nano-materials.

Considerable attention has recently focused on the ability of γ-oryzanol and different phytosterols, including cholesterol, to co-crystallize forming hollow tubules that 7 nm diameter and just under 1 nm wall thickness.6 Under certain conditions, these compounds may co-assemble establishing a continuous three-dimensional network capable of immobilizing apolar solvents over macroscopic length scales.6a,6d Fiber formation occurs when a synergistic interaction occurs between γ-oryzanol + phytosterol. Individually, neither γ-oryzanol nor phytosterol are capable of immobilizing the oil phase.6d Interest in γ-oryzanol and phytosterol co-crystals is due to their ability to act as hardstock lipid replacements in foods. This system circumvents the need for trans or saturated fats, normally required to structure edible oils as a colloidal dispersion that provides the desirable elastic properties of soft materials. Although this is an exciting potential application of these systems there may be more important physiological consequences of these structures; since it is believed that cholesterol absorption may be reduced via the formation of co-crystals.7 The ability of γ-oryzanol to complex with cholesterol may be an effective mode of reducing cholesterol from being absorbed and circulated in the body. It is imperative to reduce the elevated cholesterol levels as it is now responsible for approximately 50% of deaths in the developed world.8

The aim of this manuscript is to develop an understanding of why certain ratios of γ-oryzanol and cholesterol (Fig. 1) form

Fig. 1 Chemical structure of (A) cholesterol and (B) γ-oryzanol.
thin fibers comprised of co-aggregates while other ratios form macroscopic crystals.

Methods

Sample preparation

Cholesterol was acquired from Sigma-Aldrich (Oakville, ON, Canada) and \( \gamma \)-oryzanol was obtained from Tsuno Rice Fine Chemicals (Wakayama, Japan) and each were dissolved in heavy mineral oil obtained from Sigma-Aldrich (Oakville, ON, CAN) by heating the samples to 120 °C to erase the crystal history. The \( \gamma \)-oryzanol is a mixture of 45.9% 24-methylene cycloartenyl ferulate, 26.8% cycloartenyl ferulate, 13.1% campesterol ferulate, 7.1% sitosterol ferulate, 1.4% \( \beta \)-avenasteryl ferulate, 1.3% stigmasteryl ferulate, 1.0% campesterol ferulate (Tsuno Rice Fine Chemicals Co, Wakayama, Japan). Samples of cholesterol and \( \gamma \)-oryzanol were prepared with varying concentrations in mineral oil and heated to 120 °C for 30 min and a drop of molten sample was placed between two CaF₂ optical windows (25 mm diameter, 2 mm thick) separated with a 15 \( \mu \)m Teflon spacer. The samples were transferred onto a Linkam LTS120 controlled temperature stage (Linkam, Surrey, United Kingdom) and where isothermally cooled to 10, 20, 30 and 40 °C.

Equipment

Polarized light micrographs were acquired using a Nikon Eclipse E400 light microscope equipped with a Nikon DS-FiL color camera and a long working distance 10X lens and condenser with a resolution of 2560 by 1920. Samples were cooled at 1 °C min⁻¹ to 30 °C using a temperature controlled stage (LTS 120 and PE94 temperature controller (Linkam, Surrey, United Kingdom). Fourier transform infrared spectroscopy (FT-IR) spectra were collected using the end station of the mid-IR beamline (beamline 01B1-01, Canadian Light Source, Saskatoon, SK). The end station is comprised of a Bruker Optics IFS66v/S interferometer coupled to a Hyperion 2000 IR microscope (Bruker Optics, Billerica, MA, USA). Light is focused on the sample using a 15X magnification Schwarzschild condenser, collected by a 15X magnification Schwarzschild objective with the aperture set to a spot size of 40 \( \mu \)m by 40 \( \mu \)m and detected by a liquid nitrogen cooled narrowband MCT detector utilizing a 100 \( \mu \)m sensing element.

A KBr-supported Ge multilayer beamsplitter was used to measure spectra in the mid-infrared spectral region. Measurements were performed using OPUS 6.5 software (Bruker Optics, Billerica, MA). The measured interferograms were an average of 32 scans and were recorded by scanning the moving mirror at 40 kHz (in relation to the reference HeNe laser wavelength of 632.8 nm). Scans were taken every 10 s. The wavelength range collected was 690 to 7899 cm⁻¹ with a spectral resolution of 4 cm⁻¹. Single channel traces were obtained using the fast Fourier transform algorithm, without any zero-filling, after applying a Blackman-Harris 3-Term apodization function. For single spectra, measurements reference single channel traces were carried out in the molten state.

All Focal-Plane Array (FPA) imaging measurements were done with a Bruker Optics Hyperion 3000 microscope connected to a Tensor 27 spectrometer, using the internal globar source. The 64 × 64 pixel FPA Mercury-Cadmium Telluride (MCT) detector was used to map an area of 171 × 171 microns at one time (40 × 40 microns per pixel/15X objective = 2.67 × 2.67 microns x64 = 171 × 171 microns in the sample plane). Depending on the sample, each spectrum used between 64–256 co-added scans, divided by 64 co-added scans taken for the background. The Norton-Beer Medium apodization function was used, with a Mertz phase correction. The resolution used was 4 cm⁻¹. Samples were mapped at 30 °C.

Discussion

The crystal morphology for 8 wt% cholesterol in mineral oil is long fiber-like crystals radiating from central nuclei and 8 wt% \( \gamma \)-oryzanol develops small, dense plate-like crystals (Fig. 2). Pure
Fig. 4  FT-IR spectrum of 8 wt% γ-oryzanol in mineral oil (A) and 2D (B) and 3D (C) spectromicrographs for 8 wt% γ-oryzanol in mineral oil. FT-IR spectrum of 8 wt% cholesterol in mineral oil (F) and 2D (D) and 3D (E) spectromicrographs for 8 wt% cholesterol in mineral oil. Spectromicrographs represent the integrated area under the peak associated with hydrogen bonding (3000 to 3550 cm⁻¹).

Fig. 5  FT-IR spectrum of 4% γ-oryzanol – 8% cholesterol in mineral oil (E, F) and 2D (A, C) and 3D (B, D) spectromicrographs representing the area integrated between 3000 and 3550 cm⁻¹ (A, B) and between 3400 and 3475 cm⁻¹ (C, D). Arrows represent approximate area in which the spectra were extracted from.
cholesterol or γ-oryzanol form highly birefringent macroscopic crystals, capable of diffracting visible light resulting in highly turbid samples. The pure systems have a uniform crystal structure, which contain a single supramolecular morphology. Upon the addition of both cholesterol and γ-oryzanol a new crystal morphology results consisting of fine tubules radiating from central nuclei (Fig. 3). However, under slow cooling conditions, depending on the ratio of cholesterol and γ-oryzanol, there may be one or multiple crystal morphologies (Fig. 2). Excess cholesterol results in dense crystals as well as very fine tubules radiating from the central nuclei (Fig. 3). However, when the ratio of cholesterol and γ-oryzanol is equal or excess γ-oryzanol is present then very fine fibers are observed as the predominate crystal morphology (Fig. 2). This trend is more likely prevalent at slow cooling rates compared to rapid rates of cooling; because, at fast cooling rates less time is allowed for ordering of molecules. However, at this point it is unknown which molecules each crystal morphology contains even though previous research infers that the new fine fibers are co-aggregates of γ-oryzanol and cholesterol.

The composition of the crystals, present in Fig. 2, was probed using FTIR spectromicroscopy carried out using a Focal Plane Array (FPA) microscope (Fig. 4–7). 8 wt% γ-oryzanol (Fig. 4A–C) and 8 wt% cholesterol (Fig. 4D–F) in mineral oil were mapped and the individual spectrum, for both, indicate hydrogen bonding between 3000 and 3550 cm$^{-1}$ (Fig. 4). Hydrogen bonding corresponding to the area between 3000 and 3550 cm$^{-1}$ is associated with hydrogen bonding that does not have a specific orientation and hence a difference chemical environment experienced by the hydroxyl group. The individual spectra were integrated between 3000 and 3550 cm$^{-1}$ and the area was used to generate spectromicrographs. 2-D spectromicrographs of the hydrogen bonding area were overlayed with the corresponding light micrographs (Fig. 4B, D). It is obvious that the hydrogen bonding corresponds to the crystals present in the light micrographs as the two structural features overlay. Fig. 4C, E are 3D illustrations of the hydrogen bonding area overlaid on the light micrographs, which allow the crystal morphology to be easily discerned as well as the corresponding hydrogen bonding. Fig. 4D illustrates that the γ-oryzanol platelets (Fig. 4B, C) and cholesterol fibers (Fig. 4D, E) have a relatively even distribution of hydrogen bonding suggesting that very few solvent inclusions are present within the crystals. It is important to note that the hydrogen bonding, which occurs in cholesterol and γ-oryzanol does not have a highly specific orientation, illustrated by broad FTIR peak which arises from the varying chemical environments of the hydroxyl group (Fig. 4A, F).

A drastic change in the crystal morphology occurs when cholesterol and γ-oryzanol are mixed (Fig. 2). In the mixed system, cholesterol and γ-oryzanol may co-crystallize causing changes in the crystal morphology. The macroscopic platelets or fibers, in the pure systems, are modified to a combination of macroscopic crystals and very fine fibers that

![Fig. 6](image-url)
radiate from central nuclei in the mixed system (Fig. 2). The new morphology (Fig. 3) appears similar to maltese crosses (i.e., indicative of spherulites); however, upon close examination they are not the characteristic shape of the maltese cross. Instead they appear as radial fiber growing from a central nucleus. As well, previous X-ray scattering indicates that the new aggregates are hollow tubules that are 7 nm in diameter and the wall thickness is approximately 1 nm.

Excess cholesterol produces both highly birefringent, macroscopic crystals and small birefringent crystals (Fig. 2). The small crystals are not observed in either the pure cholesterol nor γ-oryzanol systems. When 4 wt% γ-oryzanol and 8 wt% cholesterol in mineral oil were crystallized the light micrograph indicated that two “spherulitic-like” crystals on the ends of a fibrillar crystal (Fig. 5). Two different FTIR spectrums were observed in this system unlike the individual spectrum for

Fig. 7 FT-IR spectrum of 8% γ-oryzanol and 8% cholesterol in mineral oil (E) and 2D (A, C) and 3D (B, D) spectromicrographs representing the area integrated between 3000 and 3550 cm$^{-1}$ (A, B) and between 3400 and 3475 cm$^{-1}$ (C, D).
cholesterol or γ-oryzanol (Fig. 5E, F). For the mixed system, a broad peak between 3000 and 3550 cm\(^{-1}\) and a narrow peak at 3450 cm\(^{-1}\) were observed (Fig. 5E, F). Previous research indicated that the narrow peak at 3450 cm\(^{-1}\) corresponds to the new co-aggregate of cholesterol and γ-oryzanol. During crystallization of cholesterol and γ-oryzanol the interactions are highly specific giving rise to a similar chemical environment of the hydroxyl groups that corresponds to a narrow FT-IR peak.

Conversely, crystallization of cholesterol or γ-oryzanol has less specific ordering in the crystal and corresponds to a varying chemical environment of the hydroxyl group and a broad FT-IR peak. The non-specific hydrogen-bonding peak (i.e., 3000 to 3550 cm\(^{-1}\)) and specific hydrogen-bonding peak (i.e., 3400 to 3500 cm\(^{-1}\)) were integrated and 2D (Fig. 5A, C) and 3D (Fig. 5B, D) spectromicrographs were overlaid with the corresponding light micrograph. Upon integrating the non-specific hydrogen bond peak integral of the corresponding 2D (Fig. 5A, C) and 3D (Fig. 5B, D) spectromicrographs.

Fig. 8 FT-IR spectrum of 12% γ-oryzanol and 8% cholesterol in mineral oil (E) and 2D (A, C) and 3D (B, D) spectromicrographs representing the area integrated between 3000 and 3550 cm\(^{-1}\) (A, B) and between 3400 and 3475 cm\(^{-1}\) (C, D).
formed. FT-IR indicated that a single spectral pattern was observed within the co-crystal (Fig. 7E). These co-crystals are comprised of both cholesterol and γ-oryzanol (Fig. 7A–D). Further, when excess γ-oryzanol is present the fine fibers are predominantly observed (Fig. 8). Originally, it was believed that the fine fibers had to have alternating cholesterol and γ-oryzanol molecules; however, this work suggests that excess γ-oryzanol may be incorporated into the co-aggregates. At this point, it is unclear why excess cholesterol causes fine fibers to crystallize from the surface of the cholesterol fiber.

The molten sols, at 120 °C, for the various cholesterol-γ-oryzanol mixtures were used as the background for subsequent spectra collected during crystallization. The samples were cooled isothermally from the sol phases to different crystallization temperatures below the sol–gel transition temperatures and spectra were recorded every 11 s in the hydroxyl regions (Fig. 9). It is evident that when the cholesterol and γ-oryzanol are mixed in equal ratios (Fig. 9A, B) the specific hydrogen-bonding peak evolves rapidly from the sol. However, when cholesterol is in excess it takes several minutes before the peak at 3450 cm$^{-1}$ evolves (Fig. 9E, F). This suggests that excess cholesterol or γ-oryzanol crystallizes out prior to co-crystallization. The area associated with the hydroxyl hydrogen-bonding peaks (Fig. 9) was integrated between 3000 and 3550 cm$^{-1}$ and between 3440 and 3550 cm$^{-1}$ and plotted as a function of time at each of the crystallization temperatures (Fig. 10). The sigmoidal curves, typical of crystallization events, were fitted to the Avrami equation (eqn (1))$^{10}$ as shown in Fig. 10 to determine the Avrami rate constant plotted as a function of time (Fig. 11). In eqn (1), $Y$ is the phase volume ($i.e.$, the area under the peaks), $k$ is the rate constant, $x$ is time, and $n$ is the Avrami exponent. The Avrami model has been utilized previously to analyze the kinetics and mode of gelation in other systems,$^{10}$ and has been used recently to model the crystallization process of numerous crystalline materials.$^{11}$

$$Y = 1 - e^{-kx^n}$$  \hspace{1cm} (1)

The Avrami exponent is a measure of the type of nucleation and dimensionality of crystal growth and typically has an integer value between 1 and 4. The values of $n$ did not change over the range of crystallization temperatures explored with each of the cholesterol γ-oryzanol samples.

The rate constants from the Avrami fits ($k$) can be plotted in an Arrhenius fashion (eqn (2)) to calculate activation energies of crystallization (Fig. 11).

$$\ln k = \ln A + \frac{E_a}{RT}$$  \hspace{1cm} (2)

where $\ln A$ is the $y$-intercept, $E_a$ is the activation energy, $R$ is the ideal gas constant and $T$ is an incubation temperature corresponding to a value of $k$. The activation energy may only be calculated in this way if the dimensionality of growth, $n$, is the same at each crystallization temperature (as it is here). A linear regression between the $\ln k$ versus the inverse of temperature yielded $R^2$ values greater than 0.88 for all fits (Fig. 11). From the rate constants, the activation energy was calculated for the specific hydrogen bonding (Fig. 11A–D) and for the non-specific hydrogen bonding (Fig. 11E–H). It is apparent for the system with excess γ-oryzanol or equal amounts of cholesterol and γ-oryzanol the activation energy of nucleation are similar for

![Fig. 9](image_url) Changing FT-IR signal for 8 : 12 wt%:wt% cholesterol:γ-oryzanol (A, B) at 10 °C (A) and 40 °C (B); 8 : 8 wt%:wt% cholesterol:γ-oryzanol (C, D) at 10 °C (C) and 40 °C (D); 12 : 8 wt%:wt% cholesterol:γ-oryzanol (E, F) at 10 °C (E) and 40 °C (F); 8 : 4 wt%:wt% cholesterol:γ-oryzanol (G, H) at 10 °C (G) and 40 °C (H). The time between displayed spectra is one minute. Graph adapted from [6e].

bonding peak (Fig. 9A, B), it is evident that significant hydrogen bonding, in both the “spherulite-like” structures and along the fiber, is present. However, upon integrating the highly specific peak at 3450 cm$^{-1}$, that corresponds to the cholesterol-γ-oryzanol co-aggregate, it is apparent that this spectral feature corresponds solely to the spherulitic aggregates that are radiating from the fiber (Fig. 9C, D). This confirms that the highly birefringent crystals are comprised solely of either cholesterol or γ-oryzanol while the fine fibers are co-crystals of cholesterol and γ-oryzanol. In all spectromicrographs, the fine fibers appear at an edge or off a surface of the single compound fiber. Another system with excess cholesterol (i.e., 8% γ-oryzanol and 12% cholesterol in mineral oil) was imaged (Fig. 6). Once again, the radial co-aggregate of cholesterol and γ-oryzanol contained both the non-specific and specific hydrogen bonding (Fig. 6C, D) while the fibers contained only the non-specific hydrogen bonding (Fig. 6A, B).

Cholesterol and γ-oryzanol mixed in a 8 wt%: 8 wt% ratio result in a single crystal morphology consisting of fine fibers radiating from a central nuclei (Fig. 2). When the 1 : 1 cholesterol and γ-oryzanol system is compared to systems that contain excess cholesterol or γ-oryzanol much larger aggregates are formed. FT-IR indicated that a single spectral pattern was
both the non-specific hydrogen bonding peak as well as the specific hydrogen bonding peak. This would suggest, thermodynamically, that either crystal or co-crystal could form from the melt. However, when excess cholesterol is present the activation energy, of the non-specific hydrogen bonding, is much larger than the activation energy of the new phase that consists of the specific hydrogen bonding (Fig. 11C, G, D, H). This suggests that the co-crystal would thermodynamically be favorable to form from the melt, which is not the case. Images suggest that when excess cholesterol is present, macroscopic crystals form (Fig. 2). However, the low activation energy of the new co-crystal may be explained by the spectromicrographs (Fig. 5, 6). Upon cooling, the crystallization event begins by cholesterol crystallizing out of solution forming macroscopic crystals. This is evident by the early formation of the FT-IR peak between 3000 and 3550 cm\(^{-1}\). Here homogenous nucleation (i.e., nucleation from the melt) occurs. Upon subsequent cooling the generation of specific peak at 3450 cm\(^{-1}\) suggests co-crystallization occurs after cholesterol has crystallized out of solution. Further, the presence of the co-crystals on the ends or surfaces of the cholesterol fibers suggests that the existing macroscopic fibers act as a surface for nucleation of the co-crystals allowing the subsequent crystallization to occur with a lower activation energy (Fig. 11C, G and D, H).

**Conclusions**

Co-crystallization of cholesterol and γ-oryzanol occurs with equal ratios of the two compounds or excess γ-oryzanol. Excess cholesterol results in the formation of macroscopic fibrillar crystals prior to the co-crystallization and formation of fine fibers radiating from a central nucleus. The cholesterol fibers act as an internal template to allow the cholesterol-γ-oryzanol co-crystals to nucleate from the surface to facilitate crystallization with a lower activation energy. Further, equal amounts of cholesterol and γ-oryzanol or excess γ-oryzanol allow for the radial fine fibers to crystallize from the melt without the need of an internal crystal template.
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Works cited