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The microstructure of β-sitosterol: γ-oryzanol edible organogels

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Rheology and atomic force microscopy (AFM) were employed to examine the microstructure of β-sitosterol: γ-oryzanol organogels in sunflower oil. Using time resolved rheology we followed gel formation, paying specific attention to the fibril aggregation process, which had not been studied in detail previously for this system. Using AFM we observed gel structures directly, and obtained detailed information of the gel structure, far exceeding previous studies. Our analysis suggests that though gels are formed by the self-assembly and aggregation of one-dimensional fibrils, the manner in which these fibrils aggregate into ribbons results in complex structures of higher dimensionality. We emphasize that it is a surprise to find ribbons and not twisted strands. Comparing AFM images of 10 % w/w and 20 % w/w gelator systems, we observed differences in the degree of branching which are consistent with the rheology. We also observed the individual self-assembled fibrils which make up these gels with much greater clarity than in previous
microscopy studies, and the fibril diameters of ~ 9.8 nm we measured agree excellently with those obtained from existing small angle neutron scattering data. These results provide new insight into the structure and formation kinetics of this important organogel system.
**Introduction**

An organogel is a system in which a solid network exists within a continuous oil phase resulting in elastic behaviour. There is much interest in organogels especially within the field of food science due to the possibility of replacing saturated fat crystal with structured oil phases. Several different approaches have been employed to structure oil phases \(^1,2,3,4,5\), including phytosterol based gels \(^6\).

![Image of chemical structures of β-sitosterol and γ-oryzanol](image)

**Figure 1** - Chemical structures of β-sitosterol (top) and γ-oryzanol (bottom).

Phytosterols are a class of materials which reduce blood cholesterol levels and may be incorporated into foodstuffs \(^7\). When phytosterols such as β-sitosterol are blended at a 1:1 molar ratio with the sterol-ester γ-oryzanol (structures given in Fig. 1), the molecules self-assemble into ~10 nm diameter fibrils which produce strong organogels in a range of edible oils \(^8,9,10\). The macroscopic properties of this system have been studied in some detail \(^1,8,11,12\), as have the nanoscale structure of the fibrils and the chemistry and physics behind their formation \(^9,10,13,14\). However, there is still much to be understood about the intermediate scale, i.e. how these nanoscale fibrils form a microstructure, which in turn results in a change in macroscopic behaviour. Additionally, there are technical difficulties encountered if food manufacturers formulate β-sitosterol and γ-oryzanol into
organogel products, due at least in part to the limited number of sterol esters that can form gels with the sterol and the sensitivity of the system to water. In this article, we used a combination of bulk oscillatory rheology and atomic force microscopy with the aim to understand more fully the structure and growth mechanisms of this system, paying special attention to the formation and structure of the microstructure; this insight could suggest new molecules that might function as organogelators.

**Experimental Section**

AFM measurements were performed using a Bruker Multimode/Nanoscope IIIa (Bruker, Santa Barbara, CA) atomic force microscope operating in tapping mode. The instrument was equipped with a J-Scanner (lateral scan range of ~140 µm), Bruker cantilevers (model MPP-11220-10) with nominal spring constant of 40 N/m and resonant frequency of 300 kHz, and tips with nominal tip radius of 8 nm. Images were post-processed and analysed using the Gwyddion software package.

For rheology measurements, a TA instruments AR2000 rheometer was used, with parallel cross-hatched plate geometry and a gap set at 1 mm. An oscillatory stress of 175 Pa at 10 Hz was applied to the samples and strain recorded for the duration of measurements.

The β-sitosterol and γ-oryzanol were provided by Unilever Research and Development, Vlaardingen, The Netherlands. Sunflower seed oil from Helianthus annuus was provided by Sigma Aldrich. Samples were prepared by mixing sitosterol and oryzanol at a mass ratio of 4:6 (molar ratio ~1:1) in sunflower oil, which was heated at 90 °C and stirred until the sterols had fully dissolved to produce sterol in oil solutions of the required concentration. For the rheology
measurements, these solutions were poured directly onto the bottom plate of the rheometer. For AFM measurements, freshly cleaved mica sheets were dipped into the hot organogel solutions then left at room temperature for 24 hours for gelation to occur. Once this time had elapsed, the samples were dipped in ethanol for ~1 minute to wash off the top layer of oil from the surface of the gel and expose the sterol network. This technique was adapted from that used previously by others to prepare sitosterol: oryzanol gels for cryo-SEM imaging\textsuperscript{10}.

Results & Discussion
Figure 2- Rheology data for a) a 10 % w/w sample and b) a 20 % w/w sample undergoing the sol-gel transition. c) Plots used to extract n values, along with the linear fitting to the data to yield Avrami parameters of $n = 0.9$ and $n = 1.6$ respectively.

Figure 2 a) and b) show the evolution of the complex modulus during the gelation process, after molten gel is poured onto the rheometer plate. The sol-gel transition does not happen immediately, consistent with previous observations \(^8\), until a time $t_g$ when there is a sharp initial growth in $G'$. The value of $t_g$ is considerably larger for the 10% w/w gel than the 20 % w/w gel ($\sim 2500$ s and $\sim 100$ s respectively), and once this time has elapsed the growth rate is much slower. By analysing the change in $G'$ with time it is possible to link the bulk properties of the gel to the microstructure and the manner in which the gel transition occurs.

\[
X_{crit}(t) = \frac{G'(t)}{G'(_\infty)} = 1 - \exp[-k(t - t_g)^n]
\]

Equation 1 is the Avrami relation for aggregate nucleation and growth; it has provided a means of analysing and comparing the processes which drive gelation in a range of organogel systems previously \(^{13, 16, 17, 18, 19, 20, 21, 22}\). It relates the degree of gelation in the system ($X_{crit}(t)$) at a time (t) after $t_g$, to n, the Avrami growth parameter and k, the rate constant of growth. Conveniently, $X_{crit}(t)$ can be defined relative to a range of rheological or optical properties of the system \(^{19}\). In our case, we define $X$ relative to the storage modulus at time t, $G'(t)$ and its plateau value once gelation is complete, $G'(_\infty)$. The evolution of $G'(t)$ has been used previously as a means of tracking the self-
assembly process in sterol-ester organogels, and the re-formation of gel networks in thixotropic materials. The physical significance of \( n \) is the matter of some debate. In some quarters, \( n \) has been taken to be equivalent to the fractal dimension \( D_f \). However, it is generally agreed that although closely related to fractal dimension, \( n \) is also sensitive to the manner in which the crystal is nucleated. For example, a value of \( n = 1 \) signifies 1-dimensional crystal growth with instantaneous nucleation while a value of \( n = 2 \) signifies either 2-dimensional growth with instantaneous nucleation or 1-dimensional growth with sporadic nucleation. Equation 1 may be rearranged to give equation 2 which linearizes the relation, and thus allows the parameter \( n \) to be extracted easily:

\[
\ln \left( -\ln \left( 1 - \frac{G'(t)}{G'(\infty)} \right) \right) = \ln(k) + n \ln(t - t_g)
\]

Data plotted in this manner is presented in Figure 2c for both 10 % w/w and 20 % w/w gels. Fitting equation 2 to the data obtained for the 10 % w/w gel during the initial growth period yields a value of \( n = 0.9 \). As previously stated, a value of \( n = 1 \) corresponds to 1-dimensional growth with purely instantaneous nucleation. The obtained value is sufficiently close to 1 to suggest that the 10 % w/w sample may undergo gelation of this type, but the fact than \( n < 1 \) does require some consideration. Values of \( n < 1 \) have been observed before in other systems and it has been suggested that it may be a consequence of super-cooling of the system, or the growth of highly anisotropic crystals. This system has previously shown to support significant super-cooling, and the fibrils formed in the self assembly process are highly anisotropic, so both may have a contribution to the \( n = 0.9 \) value observed. Regardless, this may be compared to the value of \( n = 1.6 \) obtained for the 20 % w/w gel, which is significantly larger. A similar increase in \( n \) with gelator concentration
has been observed for other organogel systems, and was assigned to a shift from purely instantaneous nucleation to some sporadic nucleation occurring as concentration was increased\textsuperscript{25}. Rogers et al. obtained Avrami parameters for a very similar system, but where cholesterol was used rather than sitosterol and heavy mineral oil was used as a solvent rather than sunflower oil\textsuperscript{13}. For a 20 % w/w gelator system, they observed a value of $n = 2$ via the evolution of FTIR signal strength. They observed this figure changed somewhat if the oryzanol/cholesterol ratio was modified, but did not explore how the Avrami exponent changed with total gelator concentration at a fixed oryzanol/cholesterol ratio. They concluded that as it has previously been shown that sterol:γ-oryzanol systems co-crystallize into 1-dimensional fibres, the system must undergo one-dimensional growth with sporadic nucleation. However, when comparing this result to our own system, it is worth noting two things. Firstly, the high $G'(t)$ values given in Figure 2a) and b), and the fact that $G'(t)$ exceeds $G''(t)$ almost immediately in the gelation process, suggests a system with a yield stress and thus a degree of network percolation from very early times. It has also previously been shown that mechanical agitation such as shearing decreases $t_g$ and substantially increases $G'(\infty)$ in sitosterol-oryzanol gels\textsuperscript{8}, this shearing presumably increases the likelihood of discrete fibrils coming into contact while the system is percolating. Therefore, it is reasonable to assume the majority of the growth in $G'(t)$ may not be due to the formation of fibrils, but instead due to the aggregation of fibrils into larger structures. Secondly, as Rogers et al. extracted their Avrami exponent from the FTIR signal associated with sitosterol-oryzanol hydrogen bonding, it relates only to the formation of individual fibrils, and not how those fibrils interact to form the larger microstructure. Although the growth of sitosterol-oryzanol fibrils is an inherently 1-D process, the structures these fibrils aggregate into need not necessarily be. This is not to suggest that the results obtained by Rogers et al. are invalid or incorrect, but that they may relate to a
different process to the one we observe by measuring the rise in G’(t). Despite the volume of research carried out on sitosterol-oryzanol gels, the manner in which fibrils aggregate has largely been overlooked, with most studies focusing on the processes behind fibril formation. Therefore, these results relate to a hitherto unexplored aspect of the gel formation.

We do not believe this aggregation and percolation step should preclude the use of equation 2 as an analytical tool. The aggregation of pre-formed flocs, fibres or gel fragments into a gel system is similar to the reformation process which occurs in thixotropic gels; as noted above, equation 2 has successfully been used to characterise the reformation kinetics of thixotropic gels 20, and specifically the re-aggregation of spherulites of self-assembled sterol-ester fibrils to form an organogel 19. Taking all of these results into account, we believe the shift from n = 0.9 to n = 1.6 as concentration is increased may correspond to a change in the growth of the larger structures formed during the subsequent fibril aggregation process.

Having used rheology to observe how changes in concentration alter the growth process, we directly observed gels using AFM to see if these changes could be correlated with final structure. Figure 3 shows height and phase mode AFM images for 10 % w/w (a and b) and 20 % w/w (c and d) gelator samples respectively. These images show the structure of this gel system in never before seen detail, which allows for several qualitative and quantitative conclusions to be drawn.
Figure 3- AFM height (left column) and phase (right column) images of 10% w/w (top) and 20% w/w (bottom) gels. Both samples show that fibrils aggregate into thick bundles.
Looking at the AFM images, we immediately see fibrous structures. The thickness of the fibres we observe is far from uniform, spanning a range of 30 - 400 nm in diameter. As individual fibrils are thought to be ~ 10 nm in diameter and the fibrous structures we observe are much thicker than this, it is immediately clear that the fibrils do not exist in isolation, but as part of larger bundles, as was previously observed in SEM images. Additionally, there are many three-point junctions in the system, i.e a point where a bundle splits into two branches forming a “Y” shape, whereas a system in which fibrils remained discrete would be dominated by 4 point junctions where fibrils cross over each-other to form an “X” shape. This suggests that there must be a driving force for fibrils to aggregate into bundles, and is consistent with a system in which gel formation is driven not only by the self-assembly of individual fibrils, but also the aggregation of these fibrils. The chemistry of fibril bundle aggregation is not entirely clear, but it is thought that the sitosterol and oryzanol self-assemble in such a way that the ferulic acid group of the oryzanol hangs off the exterior of the fibril. This moiety is aromatic and also features both hydroxyl and methoxyl groups, it may be possible that the propensity for aromatic groups to aggregate in stacks, or the formation of inter-fibril hydrogen bonds, will cause fibrils to stick together. We observe these fibre bundles running continuously from one side of the field of view to the other, although the thickness of the bundles varies significantly over these distances, this implies a length for the bundles > 10 µm, although it is unclear how long the individual fibrils which make up the bundles are. Due to the high density of the system and the complex structure, it is not possible to extract mechanical information such as bending modulus from these images, in the manner previously used to analyse polymer networks. Attempts to obtain AFM images at lower concentrations proved unsuccessful, presumably due to the fact the less dense network allowed oil to flow in response to the motion of the AFM tip. We do, however, observe that the bundles exhibit a degree
of flexibility, with some of the smallest bundles (i.e those with a radius ~ 30 nm) achieving radii of curvature of ~ 300 nm. The largest curving bundle observed is 400 nm in diameter and able to maintain a radius of curvature of ~800 nm.

Comparing the 10% and 20 % gels, we see that the increase in gelator density appears to result in a change in the overall structure. The 10 % gel appears to be largely made up of bundles ~ 100 nm in diameter. The 20 % gel seems to be dominated by a few core sections of very thick bundles with peak diameter of ~500 nm, from which other smaller bundles similar to those which dominate the structure of the 10 % gel (i.e. those with a ~ 100 nm diameter) radiate. The degree of branching may also be used to characterise the two systems, if we define a junction point as any point where a fibre peels away from its parent bundle we may characterize the 10 % gel as having 6.8 ± 0.2 junction points per µm², whereas the 20 % gel has 15.6 ± 0.6 junction points per µm². If increasing the gelator concentration resulted in the same total number of bundles, but with the bundles doubling in diameter, the junction point density would remain constant. That it doesn’t remain constant demonstrates quantitatively that the structure has changed somewhat. Qualitatively, the structures observed in Figure 3 a) and b) are largely fibrous but with some branching, whereas those observed in Figure 3 c) and d) are more highly branched, with some objects of higher dimensionality than 1-D fibres. As discussed previously, fractal dimension is intimately linked to the Avrami exponent 16, 19. The fractal dimension expected for gels based on linear fibrous structures such as those shown in Figure 3 a) and b) is 1 < D_f < 1.5. Gels based on growth which is highly branched yet not truly fractal, such as that apparent in Figure 3 c) and d), are referred to as fern-like, with a fractal dimension of 1.5 < D_f < 2 29,30,31,32. These values are similar to the n = 0.9 and n = 1.6 values obtained for the 10 % and 20 % gels respectively, which suggests that
changes in the structures into which the fibrils aggregate may contribute to the change in Avrami exponent.
We performed further AFM measurements on the 10 % gel with higher spatial resolution to look at the internal structure of the fibril bundles. A topographic image is shown in Figure 4 a), in this image we can see bifurcation and fusion of bundles. Within the bundles, it is just possible to see the individual self-assembled fibrils. This is consistent with previously published cryo-SEM images of this system 10 and far exceeds the resolution of previously published AFM data 33. The structure of the bundles is even clearer when the phase image of the same field of view is examined, as in Figure 4b. Here the individual fibrils show up a lighter colour than the thin layer of oil trapped between adjacent fibrils, giving excellent contrast. Previous SEM studies of this system were barely able to distinguish individual fibrils within bundles, so these phase images offer new insight into the structure of the gels. Interestingly, despite models suggesting fibrils have an inherent helicity to their own structure 9, they appear to run parallel to each other rather than twisting like a rope to form the bundle as has been observed in some organogel systems 34. The resulting bundle thus resembles a ribbon. Despite being a well-studied system, this interesting property of the gel micro-structure has not been reported previously, and it is not clear to us why the fibrils should aggregate into ribbons rather than twisted strands. It suggests that any chirality at the single fibril level is not asserting itself as larger structures are formed. Figure 4c gives the profile taken across one of these bundles both in height mode and in phase mode. Taking the average peak to peak distance in these profiles gives a diameter of 9.8 nm which is in very good agreement with the value obtained from small angle neutron scattering 9. Scattering data and subsequent modelling suggests a circular fibril cross-section, but this is not observed in the height profile where the peak
to trough depth is only on the order of ~ 1 nm, suggesting a highly anisotropic fibril cross-section. This is however, to be expected when imaging close packed fibrils where the diameter of the tip is similar to that of the fibrils as the tip may only penetrate a small way between fibrils before coming into contact with the fibril sides \(^{35}\). In this instance, it can be shown from elementary geometry that if an AFM tip with radius = 8 nm, is placed between two circular cross-section fibrils of radius = 4.9 nm as we believe we have here, the tip will only penetrate ~1 nm.

**Conclusions**

These results show the importance of fibril aggregation on the formation and structure of this important gel system. We observe the complicated structure is extremely hierarchical, with 0-D molecules self assembling into 1-D fibrils, these aggregate into bundles which may form complex structures with higher dimensionality. Our images of the gel micro-structure are far superior to any obtained for this system previously, and further confirm the previously calculated values for fibril diameter. Intriguingly, no fibril twisting in multi-fibril bundles is observed, and thus the bundles more closely resemble ribbons than twisted strands, the reason for this is unclear. We show that the Avrami parameter obtained from rheology may be correlated to AFM images very effectively, allowing a bridge from the macro to micro scale for this important system, and giving insight on the fibril aggregation process, which has not previously been studied for this system. Our rheology and AFM results both suggest an increasing gelator concentration results in the formation of more highly branched structures, and thus a change in the dimensionality of the larger microstructure formed by the fibrils. These results suggest that fibril-fibril interactions may be the key to understanding the structure and growth of this system.
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