Effect of water on self-assembled tubules in β-sitosterol + γ-oryzanol-based organogels

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Abstract. Mixtures of β-sitosterol and γ-oryzanol form a network in triglyceride oil that may serve as an alternative to the network of small crystallites of triglycerides occurring in regular oil structuring. The present x-ray diffraction study investigates the relation between the crystal forms of the individual compounds and the mixture in oil, water and emulsion. β-Sitosterol and γ-oryzanol form normal crystals in oil, in water, or in emulsions. The crystals are sensitive to the presence of water. The mixture of β-sitosterol + γ-oryzanol forms crystals in water and emulsions that can be traced back to the crystals of the pure compounds. Only in oil, a completely different structure emerges in the mixture of β-sitosterol + γ-oryzanol, which bears no relation to the structures that are formed by both individual compounds, and which can be identified as a self-assembled tubule (diameter 7.2±0.1 nm, wall thickness 0.8±0.2 nm).

1. Introduction
Over the years, it has been realized that complex molecules may form solid phases that are ordered in a fundamentally different way on a supra-molecular scale than according to simple crystal lattices (for which the simple, face-centered or body-centered cubic structures may serve as the archetypical example). Among these solid phases are those that do not form a space-filling structure. Some systems are known to form helical or twisted ribbons, resulting in the formation of fibrillar structures instead of forming a structure that expands in three dimensions [1]. This type of molecular aggregation is sometimes referred to as self-assembly. The exact ‘design rules’ for obtaining a particular type of supra-molecular ordering are not yet established, but the chirality of the molecule does seem to play a role, as most examples of self-assembly involve chiral molecules [1].

Figure 1: Chemical structure of the main components in β-sitosterol and γ-oryzanol. For comparison, the structure of cholesterol is also drawn.
Cholesterol (see Figure 1) is an example of a molecule that forms a fibrillar structure under specific conditions: the molecules aggregate to form a helical ribbon of which the wall is slowly filled up to form a solid tubule [2]. Many cholesterol derivatised compounds seem to show fibrillar aggregation as well: cholesterol substituted with anthracene [3-5], azobenzene [6,7], carbamate [8], stilbene [9-10], squaraine [9] and amino acid [11] groups have the ability to gel selected organic phases by themselves. Most of these successful organogelling agents contain an aromatic ring in the appended group. Next to this, a range of dicholesteryl compounds have been identified with various linking groups that show the capability to gel organic phases [12].

Mixtures of sterol molecules have been investigated less extensively. A system that received some attention is the mixture of γ-oryzanol with β-sitosterol in triglyceride oils (see Figure 1). The individual compounds cannot gel the oil [13]. The mixture can form tubules in oil with a diameter of only 7.2±0.1 nm and a wall thickness of 0.8±0.2 nm [14-16]. The triglyceride oil can be found both inside and outside the tubules. The exact molecular structure of the wall is not known, but circumstantial evidence allows some speculation: It has been shown that these molecules can stack, but that they do not order exactly parallel due to the presence of a hydrogen bridge [17]. As a consequence, a curved supermolecular structure can form, which may be characterized best as a tubule on longer length scales (see Figure 2). The wall of the tubule is assumed to consist of stacked sterol ring systems, and the hydrogen bond is located at the exterior wall of the tubule. As a consequence, the ferulic acid moieties in the oryzanol molecules would stick out of the tubule [15]. The tubules may stick together, most likely at locations that are relatively depleted in oryzanol as the ferulic acid moieties at the exterior wall seem to reduce aggregation of the tubules [15,17]. If sufficient aggregation of tubules occurs, the system would indeed form the firm, slightly hazy gel that has been observed experimentally [13]. The mostly transparent, slightly hazy appearance of these gels is explained by the small diameter of the tubules that form the gel.

Some alternatives to β-sitosterol have been identified, such as the chemically very similar compounds ergosterol, cholestanol, stigmasterol and cholesterol [15]. The identification of such alternatives, however, did not allow pin-pointing obvious molecular characteristics required to form the tubules. Until now, only two have been found. First, the presence of the hydroxyl moiety in the sterol (i.e. replacing β-sitosterol by 5α-cholestanol does not result in the formation of a tubule anymore.
Secondly, the number of double bonds in the sterol ring system seems to affect the diameter of the tubules: more double bonds correlate with more narrow tubules (see Figure 3). Interestingly, the chemical details of the alkyl chains do not seem to affect the formation of the tubules very much. A likely explanation is that these flexible alkyl chains probably blend in with the surrounding liquid triglyceride phase, and do not contribute much to the formation of the tubules in the first place.

No food-grade alternatives to γ-oryzanol have been identified yet. Replacing the ferulic acid moiety in γ-oryzanol by the fatty acids from sunflower oil prevents tubule formation [15]. A limited number of alternative esters are known to gel organic phases outside the realm of food-related ingredients (and food grade oils) [3-11].

The end vision for these organogelling systems would their application in the structuring of food emulsions [17-19]. Significant hurdles will have to be overcome to achieve this. Amongst these is the ability to combine the structuring functionality of these systems with the presence of water. Some initial scoping in this field has already been published [20]. The present paper will lay the groundwork for establishing a deeper understanding of the effect of water on the tubules, the building blocks of these organogelling systems.

2. Experimental

2.1. Materials

In the present experiments γ-oryzanol (Tsuno Rice Fine Chemicals) and tall oil sterol (Unilever, 78.1% β-sitosterol, 10.3% campesterol, rest minor but structurally very similar sterols [15]) were used in combination with refined sunflower oil (Cargill).
Samples were prepared by mixing the ingredients until all powder had dissolved in the sunflower oil (at ~100°C). Emulsions were prepared by mixing in the appropriate amount of water (no additional emulsifiers used). The samples were cooled and stored for 1 week at 5°C.

2.2. Measurement techniques
Small-angle and wide-angle x-ray scattering (SAXS, WAXS) experiments were performed at the high-brilliance ID2 beamline of the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. Details of the experimental set-up are given elsewhere [14,21]. SAXS data were collected in the range 0.069<q/nm\(^{-1}\)<4.3 and WAXS data in the range 3.6<q/nm\(^{-1}\)<33.1, where q = 4π·sinθ / λ is the wave vector (and θ the scattering angle and λ the wavelength of the incoming x-ray radiation). Scattering data from separately obtained water and sunflower oil reference samples were subtracted from the emulsion and dispersion data to obtain curves representing the sitosterol and/or oryzanol.

A number of complementary x-ray diffraction (XRD) measurements were performed at Unilever R&D Vlaardingen using a Bruker D8-Discover in a θ/θ configuration. Data was collected in the range 0.64<q/nm\(^{-1}\)<7.7 [15]. Data analysis was described elsewhere [15,22].

Fourier Transform Infrared (FTIR) experiments were performed on a Bio-Rad FTS-6000 spectrometer (V 4.0) which was equipped with a deuterated triglycine sulphate (DTGS) detector. The accessory used was a Micro-ATR equipped with a Si-crystal. For both samples and background, 64 interferograms with a resolution of 4 cm\(^{-1}\) were co-added and Fourier transformed. The spectral information was obtained over a frequency window from 4000 to 400 cm\(^{-1}\).

Differential Scanning Calorimetric (DSC) experiments were performed on a Perkin-Elmer Pyris 1. The samples (15-30 mg) were weighed into stainless steel sample pans and subjected to a heating-cooling-heating cycle between 20 and 120°C at a scanning rate of 10°C/min.

3. Results and discussion

3.1. Kinetics of sitosterol+oryzanol tubule formation
A previous study demonstrated that the tubule structure vanishes at the melting temperature of the gel, which showed that no mesophase structure persists above the melting temperature [15]. A similar experiment investigating the kinetics of tubule formation showed that most of the tubules (>85%) were formed within the time resolution of that particular experiment (~10 min at 5°C). This is in line with macroscopic observations that the gels develop very quickly, once gelation is initiated [13].

![Figure 4: (Left panel) XRD curves for organogels composed of 3.2% sitosterol + 4.8% oryzanol in sunflower oil (after correction for contribution from sunflower oil) as a function of storage time at 5°C after cooling from 100°C at a rate of 10°C/min. Vertical shifts were applied to the curves for clarity; (Right panel) Time dependence of the first maximum in the XRD curves near q=1 nm\(^{-1}\).](image-url)
3.2. Molecular structure of powder, lipid and aqueous slurries, and emulsions

3.2.1. Sitosterol. A number of SAXS curves were obtained for β-sitosterol in powder form, in lipid and aqueous slurries, and in an emulsion with 30% water (see Figure 5a and Table 1). It is not expected that the present preparation procedure produces neat single crystals, nor is it our aim to derive information on units cells from the data. The slurries and the emulsions were stored for 1 week before measurement. The data were compared to literature data [23-25] which distinguished three types of sitosterol crystals: anhydrous crystals (most pronounced and characteristic peaks at d = 2π/qi = 1.76, 0.880 and 0.523 nm), hemi-hydrates (0.5 mol water/mol sitosterol, most pronounced and characteristic peaks at d = 1.88 and 0.481 nm) and monohydrates (1 mol water/mol sitosterol, most pronounced and characteristic peaks d = 1.76 and 0.503 nm). Next to that, all crystal forms typically exhibit Bragg reflections for 1.2 and 0.59 nm. Note that the data in the present experiment accesses a wider q-range than the literature data (0.4<d<2 nm).

The close resemblance of the sitosterol chemical structure to that of cholesterol (see Figure 1) allows an interpretation of the main features of the diffractograms, despite the fact that a precise crystallographic analysis of sitosterol has not been done. The sitosterol crystals are expected to form a bilayer structure of about 3.8 nm thickness (cf. Reference [26]). The position of the characteristic sterol ring system in these structures is defined quite accurately, whereas the alkyl chains tend to have more conformational freedom. The relative orientation of the molecules in the various crystal forms seems to be determined by the need to maximise the hydrogen bonding between the molecules, explaining some of the differences between anhydrous and hydrated forms [26].

The presence of crystallographic reflections for the powder at 0.89 and 0.48 nm in Figure 5a, suggests the presence of anhydrous and hemi-hydrate sitosterol in the powder, respectively. On the other hand, the absence of a peak at 0.50 nm suggests that mono-hydrated sitosterol is not present in the powder [23].

Infrared spectroscopy (IR) can be used to investigate hydrogen bonding in these systems (see Figure 6) [27]. Generally, intermolecular hydrogen bonds should appear as medium-intensity bands in the 3550-3450 cm\(^{-1}\) wave number range, and intramolecular hydrogen bonds as medium intensity bands in the 3570-3540 cm\(^{-1}\) wave number range [28]. Focussing on the 3800-3000 cm\(^{-1}\) wave number range, the β-sitosterol powder only shows a broad band in around ~3350 cm\(^{-1}\), and a very weak band at the wave numbers for intermolecular hydrogen bonding, ~3420 cm\(^{-1}\) (Figure 7a). The spectra for sitosterol and oryzanol are in agreement with those of Qian and Xu [29] and Bucci et al [30], respectively.

In oil slurries, the pattern of crystallographic reflections suggests a mixture of anhydrous and hemi-hydrated sitosterol too (Figure 5a). However, some features become much more pronounced at both relatively large distances (d=5.19 nm) and small distances (d=0.46, 0.43, 0.22 and 0.21 nm), the intermediate range being relatively unaffected. It should be noted that most of the peaks did occur already in the powder spectrum, but at insufficient intensities to report them in Table 1. The infrared spectrum of a sitosterol slurry in oil shows a very small peak at 3441 cm\(^{-1}\), indicating some very weak intermolecular hydrogen bonding (Figure 7a).

In aqueous slurries, the absence of peaks at d = 1.88 and 0.48 nm suggests that the sample does not contain a hemi-hydrate, and the absence of peaks at d = 0.88 and 0.52 nm suggests (not surprisingly) that the sample is not anhydrous (Figure 5a). Although Christiansen et al [23] do not list a peak at 0.75 nm as characteristic for their mono-hydrate form, Argay et al [24] do list the 0.75 nm peak in their mono-hydrate crystal. The combination of data suggests therefore the presence of sitosterol monohydrate, possibly as a mixture of the forms observed by Christiansen et al [23] and Argay et al [24]. The infrared spectrum is similar to that of the powder (Figure 7a).

The most pronounced peaks for sitosterol dispersed in an o/w emulsion with 30% water coincide with those for the aqueous slurries, except that some of the characteristic mono-hydrate peaks tend to be weaker than in the aqueous slurry (Figure 5a). This suggests that the alternative hydrate forms more
easily in emulsions, and one can speculate that the triglycerides play a role in this. Generally, however, peaks in the emulsion are more pronounced than in the oil slurry, which can be explained by the reduced solubility of sitosterol in oil in the presence of water from the emulsion [31].

Figure 5: SAXS data for (a) sitosterol and (b) oryzanol, dissolved/dispersed in a number of different phases. From top to bottom: (—) powder, (—) 16% in oil, (—) 16% in water, and (—) 16% in an o/w emulsion containing 30% water after 1 week storage (20°C for oil slurry, 10°C for water slurry and emulsion).

Figure 6: IR spectra of the raw materials. From top to bottom: (—) sitosterol powder, (—) oryzanol powder, (—) sunflower oil and (—) water.
3.2.2. Oryzanol. Cholesteryl esters form complex crystal structures, sometimes dominated by the intermolecular interactions between the sterol ring systems (as in so-called ‘type-II monolayers’) or by the balance between the interaction of the esterified acid moiety with the sterol ring system on the one hand and the interaction of the sterol ring systems on the other hand (as in ‘type-I monolayers’) [26]. Systems that are dominated by interactions between the esterified acid moieties tend to organise in bilayers, but this seems to happens mainly in systems where the esterified moiety consist of long fatty acid chains [26].

The SAXS data that was obtained for γ-oryzanol powder, in lipid and aqueous slurries, and in an emulsion with 30% water is shown in Figure 5b (see also Table 1). Bragg peaks for γ-oryzanol powder confirm that the main Bragg reflection is moved to much smaller d values around 2.5 nm, suggesting that no bilayer structure is formed. The absence of reference data makes it difficult to interpret the data in terms of hydration state, although it seems likely that the two peaks at d=2.50 and 2.34 nm should be assigned to different hydration states of the oryzanol crystals. It should be noted however, that the oryzanol powder cannot be wetted very well in water, more or less in contrast to sitosterol powder. Infrared spectroscopy on γ-oryzanol powder shows a weak band indicating intramolecular hydrogen bonding at 3532 cm⁻¹ (Figure 7b).

Oil slurries do not show very pronounced Bragg reflections, signalling the high solubility of oryzanol in the triglyceride oil. The reflections in the spectrum of the aqueous oryzanol slurries match the powder spectrum best, because the oryzanol does not dissolve very well in water. Like in the sitosterol case, we can see the main reflection moving to somewhat bigger d values – it is tempting therefore to identify these peaks at d = 2.60 en 2.74 nm as features of an oryzanol hydrate (Figure 5b). The infrared spectra suggest the presence of two different types of crystals in the dispersion. Transparent crystals contain traces of water, whereas the whitish crystal appear to be anhydrous. Both show very weak intramolecular hydrogen bonding peak as the powder (Figure 7b).

The diffractogram for emulsions is simpler than for the aqueous solutions, because a large fraction of the oryzanol dissolves in the oil (Figure 5b). The infrared spectrum for the aqueous dispersion is dominated by water, and does not show any pronounced intermolecular of intramolecular features (Figure 7b).
3.2.3. Sitosterol+oryzanol mixtures. The experiments reported until now were intended to have the data available to determine whether oryzanol or sitosterol show any special compound formation in triglyceride oil, or in water, or in their mixture. Furthermore, the effect of water on such mixtures was considered to be of interest. The data is shown in Figure 8 (see also Table 1).

The data on oryzanol + sitosterol in triglyceride oil shows the broad interference pattern of self-assembled tubules that has been presented before [14,15]. It is of interest to note that the scattering pattern does not show any of the sharp reflections that are characteristic for the diffractograms of the pure compounds. Instead, the data shows very smooth, broad features, except for a few very weak wiggles in the WAXS region of the spectrum (d = 0.68 and 0.62 nm). Thus, it can be concluded that the molecular order in the tubules is very different from that in the pure components. The absence of additional peaks in WAXS is a strong argument to support the claim that no bilayer is formed in the wall of the tubules. In addition, the infrared spectrum of sitosterol+oryzanol in oil shows a small peak at 3441 cm$^{-1}$, indicating intermolecular hydrogen bonding (Figure 9). It can be shown that this is not a feature of any of the single components in the mixture by subtracting the contributions of sitosterol, oryzanol and sunflower oil from the spectrum, because the resulting corrected spectrum is essential identical in this frequency window to the uncorrected spectrum. This peak was already observed in the spectrum for the sitosterol oil slurry, but at much lower intensity. It is interesting to note that the intermolecular peak can be found at a different position than the intramolecular peak for oryzanol powder and in the aqueous sitosterol + oryzanol slurry. The data on the aqueous slurry contains a mixture of sitosterol and oryzanol crystals, as can be concluded from inspection of Table 1.

The scattering data on the emulsion reveals reflections that can be traced back to sitosterol crystals in most cases, and to oryzanol crystals in a smaller number of other cases. This can be explained by the high solubility of oryzanol in the oil phase of the emulsion, leading to greater contribution of sitosterol crystals to the diffractogram. More importantly, however, we see no evidence for the
presence of tubule formation under the present conditions. This indicates that the tubule structure is extremely sensitive to the presence of water.

![Figure 9: From top to bottom, IR spectra of the (---) sitosterol, (----) oryzanol, (-----) sitosterol+oryzanol in oil, (-----) sitosterol+oryzanol in oil (individual components subtracted), and (-----) sitosterol+oryzanol in water (water subtracted). The mixture in oil shows a small peak at 3441 cm⁻¹, which is ascribed to intermolecular hydrogen bonding.](image)

| d (nm)            | sitosterol | oryzanol | sitosterol+oryzanol |
|-------------------|------------|----------|---------------------|
| powder            | 3.76, 3.57, 3.04, 1.88, 1.78, 1.19, 0.89, 0.73, 0.59, 0.52, 0.48, 0.40, 0.32, 0.24 | 4.95, 2.50, 2.34, 1.53, 0.99, 0.90, 0.77, 0.56, 0.52, 0.40 | not applicable |
| dispersed in oil  | 5.19, 3.76, 3.55, 1.88, 1.78, 1.17, 0.89, 0.71, 0.59, 0.52, 0.48, 0.46, 0.43, 0.40, 0.32, 0.22, 0.21 | 3.90, 0.76, 0.56, 0.52 | 6.68, 3.19, 2.28, 0.68, 0.62 |
| dispersed in water| 5.19, 3.61, 1.81, 1.18, 1.04, 0.75, 0.60, 0.51, 0.45, 0.41, 0.38, 0.35, 0.31, 0.24 | 5.07, 4.33, 2.74, 2.60, 2.36, 1.50, 1.34, 1.04, 0.97, 0.90, 0.77, 0.66, 0.56, 0.51, 0.46, 0.40 | 5.15, 4.30, 3.59, 2.72, 2.60, 1.80, 1.05, 0.98, 0.90, 0.77, 0.66, 0.60, 0.56, 0.51, 0.49, 0.46, 0.43, 0.41 |
| dispersed in emulsion | 5.19, 3.59, 1.79, 0.75, 0.60, 0.51, 0.49, 0.46, 0.41, 0.35, 0.28, 0.24 | 2.61, 1.28, 1.03, 0.82, 0.67, 0.54, 0.50 | 5.19, 3.59, 2.72, 2.59, 1.80, 0.60 |

Table 1: Most pronounced Bragg spacings d = 2π/q in SAXS and WAXS diffractograms for sitosterol, oryzanol and a 40:60 mixture of sitosterol+oryzanol (total sterol(esters)s at 16% by weight). A more or less equal number of weaker reflections are present in the diffractograms. Numbers in bold refer to the strongest reflections.
3.2.4. Behaviour at higher temperatures. The fact that the mixture of sitosterol and oryzanol in the presence of water does not behave differently from the single components at low temperatures (below ~70°C) is also clear from the behaviour at a differential calorimetric scan (DSC) in the emulsion. The data is shown in Figure 10.

For sitosterol, there are clear features at ~45°C, ~80°C and ~100°C. The monohydrate is the most stable from below 45°C, the hemi-hydrate between 45 and 80°C, and the anhydrous form between 80 and 110°C [23] (but note that the small differences between the x-ray diffractograms after chilled storage for one week reported in the previous sections and the ones reported by Christiansen et al [23]). Sitosterol is completely dissolved above ~110°C. There is qualitative agreement with the phase behaviour of hydrated cholesterol as a function of temperature [32]. Oryzanol shows three features: peaks at ~70°C, ~100°C and ~115°C. Although reference data is lacking, it seems likely that the transitions are also related to (de)hydration phenomena as oryzanol shares many of its structural features with sitosterol (see Figure 1). The emulsion with the sitosterol + oryzanol mixtures shows the same behaviour as the single components, i.e. a mono-hydrate to hemi-hydrate transition for the sitosterol at ~45°C and a transition at ~70°C in oryzanol.

Surprisingly, however, the mixture does behave differently at higher temperatures (above ~70°C). No further transitions are observed for the sitosterol+oryzanol mixture dispersed in emulsion, whereas the individual components do show a number of transitions in what is assumed to be their anhydrous state.

![Figure 10: DSC data for ( ) sitosterol, ( ) oryzanol and ( ) mixed sitosterol+oryzanol in an emulsion containing 30% water after 1 week storage at 10°C. The scanning rate was 10°C/min.](image)

4. Conclusions
The mixture of γ-oryzanol with β-sitosterol forms a network of tubules in oil that may serve as an alternative to the network of small crystallites of triglycerides occurring in regular oil structuring [17-19,33-37]. The tubule diameter is found to be 7.2±0.1 nm, the wall thickness 0.8±0.2 nm [15]. The results are consistent with a previously proposed helical ribbon structure. The present experiments demonstrate that the tubules form rapidly upon sufficiently deep cooling.

The x-ray diffraction experiments have shown that for the present experimental conditions 16% sitosterol and oryzanol form regular crystals in oil, in water, and in emulsions. The crystals are sensitive to water. The mixture of sitosterol + oryzanol (16% in total, ratio 40:60) forms crystals in water and in emulsions that can be traced back to the crystals of the pure compounds. Only in oil, a completely different structure is formed, which bears no relation to the structures that are formed by
both individual compounds, and which can be identified as a self-assembled tubular structure involving both molecules. Obviously, the fact that different molecular structures are formed in the presence of water does not exclude the possibility that such systems do develop firmness as well (cf Reference [20]).

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