§1. Details of crystal structure determination of monoclinic cholesterol monohydrate.

As mentioned in the main text, the three-dimensional structure of the monoclinic polymorph was determined based on only three cholesterol bilayers. Structure elucidation of a thin molecular layered system at the air-water interface is generally not a straightforward process for complex systems, requiring a multi-pronged approach. Such thin films can be characterized to near-atomic resolution by synchrotron grazing incidence x-ray diffraction (GIXD), a method that has been applied to study molecular self-assembly of crystalline films ranging from one to several layers thick, such as amphiphiles, and thin film crystallites of supramolecular architecture.\(^{(1, 2)}\) Cholesterol monoclinic films composed of 1-3 bilayers, azimuthally misoriented on the water surface,\(^{(3)}\) were amenable to detailed structural characterization, via GIXD, because the diffraction peaks were by and large sharp and intense, as a result of pronounced molecular ordering. Thus, each of these crystalline films yielded well-defined \(\{h,k\}\) Bragg ‘rods’, which were indexed in terms of a two-dimensional (2D) rectangular \(10 \times 7.5\ \text{Å}^2\) unit cell, parallel to the plane of the water surface. The single bilayer is of symmetry \(p2_1\), namely the two leaflets are related by twofold screw symmetry. A film of three bilayers yielded intensity maxima along the Bragg rods, which were sufficiently intense, sharp, and well-separated to be regarded as regular \(\{hkl\}\) reflections of a 3D crystal (with unit cell dimensions of \(a = 10.15(2)\), \(b = 7.57(2)\), \(c = 68.2(3)\ \text{Å}, \beta = 94.8(5)\degree\).\(^{(3)}\)

The symmetry of this crystal structure of monoclinic monohydrate was assigned based on 48 reflections, the \(hkl\) indices of which obeyed the condition \(k + l = 2n\). The cholesterol molecular packing was then generated by utilizing the cholesteryl myristate crystal structure\(^{(4)}\) as an initial guess. This is because the cholesteryl myristate crystal structure exhibits a bilayer motif with \(a,b\) axial dimensions and a \(\beta\) angle, very similar to those of monoclinic cholesterol.H\(_2\)O and because
its 32.9 Å bilayer thickness almost matches half the length of the $c$-axis of the monoclinic cholesterol (assuming the latter is a monohydrate phase with a 1.5 Å thick water layer). With the structure thus determined, the calculated density of the monoclinic form (1.029 g/mL) almost matches the density of the triclinic form of cholesterol (1.048 g/mL).

Even with this structural information several unknowns remained. Firstly, the cholesterol bilayer was generated via a twofold (2) axis as in the cholesteryl myristate crystal structure, with space group $A2$. However, because this monoclinic space group incorporates rows of twofold (2) and twofold screw (2$_1$) axes parallel to $b$ and alternating along the $c$ axis, the cholesterol bilayer could have been constructed across the 2$_1$ axes instead. Indeed, the latter arrangement occurs in the organization of a single cholesterol bilayer on the air-water interface, and in the crystal structures of the tridecanoate and stearate derivatives of cholesterol. Most importantly, likely the H-bonding bilayer was poorly determined.

§2. Eight H-bonding motifs of the triclinic cholesterol.H$_2$O polymorph.

Fig. S2. Eight H-bonding motifs of the triclinic cholesterol.H$_2$O polymorph. The number before each dot refers to the configuration of the cholesterol molecules connected by hydrogen bonds between the two hydroxyl groups. In 1.1 to 1.4, one of the oxygens acts as a donor (D) and the other one as an acceptor (A). In motifs 2.1 to 2.4, cholesterol oxygen molecules switch their roles. The number after the dot refers to the different orientations of the acceptor hydrogen atom, as shown with colored arrows. The donor hydrogen bonding orientation remains unchanged within each donor-acceptor configuration. 1.3 corresponds to the motif originally determined by Craven, based on computational refinement of this structure by Frincu et al.
Table S2.1. Optimized unit cell parameters (in Å, degrees and Å³) for the proposed structural motifs at PBE-TS level of theory.

| motif | a    | b    | c    | α    | β    | γ    | V    |
|-------|------|------|------|------|------|------|------|
| 1.1   | 11.85| 11.95| 34.16| 92.1 | 98.8 | 102.0| 4663.3|
| 1.2   | 11.86| 11.94| 34.17| 91.9 | 98.8 | 102.0| 4669.0|
| 1.3   | 11.84| 11.96| 34.15| 92.3 | 98.8 | 101.9| 4662.9|
| 1.4   | 11.85| 11.96| 34.17| 92.1 | 98.8 | 101.9| 4668.9|
| 2.1   | 11.85| 11.94| 34.18| 92.0 | 98.9 | 102.0| 4663.4|
| 2.2   | 11.84| 11.95| 34.17| 92.2 | 98.8 | 101.9| 4662.9|
| 2.3   | 11.88| 11.93| 34.19| 91.8 | 98.9 | 102.0| 4671.1|
| 2.4   | 11.87| 11.94| 34.18| 91.9 | 98.8 | 102.0| 4670.3|

Table S2.2. Optimized average O···O bond lengths in Å for the proposed structural motifs of the triclinic polymorph at PBE-TS level of theory. The different H-bonded rings in Fig. 2C are labeled in grey by r_i and R_i, which refer to tetragons and octagons, respectively; subscript i from 1 to 4 designates the unique polygons of each type.

| motif | R_1      | R_2      | R_3      | R_4      | r_1      | r_2      | r_3      | r_4      |
|-------|----------|----------|----------|----------|----------|----------|----------|----------|
| 1^st  | 2.75 ± 0.07 | 2.73 ± 0.09 | 2.77 ± 0.08 | 2.76 ± 0.10 | 2.82 ± 0.13 | 2.77 ± 0.11 | 2.80 ± 0.11 | 2.75 ± 0.09 |
| 2^nd  | 2.74 ± 0.09 | 2.72 ± 0.10 | 2.79 ± 0.10 | 2.76 ± 0.10 | 2.83 ± 0.15 | 2.76 ± 0.12 | 2.81 ± 0.11 | 2.75 ± 0.11 |
| 3^rd  | 2.74 ± 0.08 | 2.72 ± 0.08 | 2.78 ± 0.06 | 2.77 ± 0.11 | 2.81 ± 0.09 | 2.76 ± 0.11 | 2.81 ± 0.14 | 2.74 ± 0.11 |
| 4^th  | 2.73 ± 0.08 | 2.72 ± 0.09 | 2.78 ± 0.07 | 2.78 ± 0.10 | 2.80 ± 0.09 | 2.76 ± 0.09 | 2.81 ± 0.14 | 2.77 ± 0.13 |
| 5^th  | 2.73 ± 0.06 | 2.74 ± 0.10 | 2.78 ± 0.08 | 2.77 ± 0.09 | 2.82 ± 0.13 | 2.76 ± 0.09 | 2.80 ± 0.11 | 2.77 ± 0.13 |
| 6^th  | 2.74 ± 0.06 | 2.73 ± 0.10 | 2.79 ± 0.10 | 2.77 ± 0.09 | 2.83 ± 0.15 | 2.76 ± 0.09 | 2.81 ± 0.11 | 2.75 ± 0.10 |
| 7^th  | 2.75 ± 0.06 | 2.73 ± 0.07 | 2.79 ± 0.10 | 2.77 ± 0.09 | 2.82 ± 0.12 | 2.77 ± 0.09 | 2.80 ± 0.11 | 2.76 ± 0.10 |
| 8^th  | 2.75 ± 0.09 | 2.72 ± 0.06 | 2.79 ± 0.10 | 2.76 ± 0.09 | 2.82 ± 0.12 | 2.77 ± 0.13 | 2.80 ± 0.11 | 2.76 ± 0.11 |

Table S2.3. Computed total energies for the proposed structural motifs, relative to the lowest energy motif, at PBE-TS level of theory.

| motif | 1.1   | 1.2   | 1.3   | 1.4   | 2.1   | 2.2   | 2.3   | 2.4   |
|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Δ, [kcal/mol]/molecule | 0.00  | 0.02  | 0.06  | 0.08  | 0.06  | 0.13  | 0.12  | 0.20  |
§3. Generation of H-bonding network in the monoclinic crystal structure of cholesterol.H_2O.

Each water molecule in the crystal structure of hexagonal ice participates in four H-bonds in a three-dimensional tetrahedral arrangement, incorporating two O-H proton donor bonds and two proton acceptor lone-pair electron lobes. The C-OH groups of, e.g. methanol molecules, each participates in two H-bonds forming a one-dimensional H-bonded network, incorporating one OH proton donor, which is linked to a proton acceptor O atom via its lone pair electron lobes. Hence, we may expect ideally that in the crystal structure of monoclinic cholesterol.H_2O, a two-dimensional H-bonded layer network of sterol C-OH groups and H_2O molecules would interlink such that each O atom forms three O-H…O bonds as in the crystal structure of the triclinic polymorph of cholesterol.H_2O and in the (002) bilayer arrangement of hexagonal ice. Each sterol O atom would donate one proton and accept two protons, whereas each water molecule would do the reverse, i.e., donate two protons and accept one proton. Thus, given the cholesterol:water molar ratio of 1:1, each water O atom would be bonded to two sterol O atoms and one water O atom, and each sterol O atom would be H-bonded to two water O atoms and one sterol O atom. Since the cholesterol molecules in each layer are too bulky to easily form H-bonds with each other, the H-bond between the two sterol C-OH groups must interlink the molecular layers across the twofold screw axes. This is indeed true for the starting model structure of the monoclinic form (Fig. S3). Given meagre information as to the C-O sterol bilayer arrangement in the monoclinic cholesterol, it is still possible to construct a feasible H-bond motif, which satisfies the above conditions, as depicted in Fig. S3. In this figure, we have drawn circles of a 3 Å radius about the atomic centers of the sterol O atoms, to generate the possible positions of the water O atoms.
Fig. S3. Various steps (1-3) in the generation of the H-bonded $ab$ bilayer in monoclinic cholesterol.$H_2O$.

1. Apply the twofold screw elements shown to generate the symmetry-related positions of the two asymmetric sterol O atoms (in red), which belong to the opposite leaflet of the bilayer. Note that the neighboring sterol O atoms, belonging to opposite leaflets, are separated by $\sim 3$ Å and so H-bonded to each other.

2. Draw circles of radius 3 Å about the centers of two symmetry-related sterol O atoms as shown. These two circles intersect at two positions. The position labeled with a red $\times$ cannot accommodate a water O atom, because it would form a close-packed isosceles triangle with two sterol O atoms. The other position of intersection is occupied by a water O(1) atom (blue), which clearly can form H-bonds with two neighboring sterol O atoms. Now generate the position of the symmetry related water O(1) atom, marked by an arrow.

3. Draw circles of radius 3 Å about the atomic centers of sterol O atom and the generated water O(1) atom from step 2 as shown. These two circles intersect at two positions. The position labeled with a red $\times$ cannot accommodate a water O atom, because it would form a close-packed isosceles triangle with two sterol O atoms. The other position of intersection is occupied by a water O(2) atom (light blue), which clearly can form H-bonds with two neighboring sterol O atoms.
§4. Four H-bonding motifs of monoclinic cholesterol.H$_2$O polymorph.

Fig. S4. Four H-bonding motifs of the monoclinic cholesterol.H$_2$O polymorph. In motifs 1 and 2, one of the sterol O atoms acts as a donor (D) and the other one as an acceptor (A), and in motifs 3 and 4 their roles are reversed. The H$_2$O molecules adapt to these configurations, yielding a maximum of 4 H-bonding motifs.

Table S4.1. Computed total energies for the generated structural motifs, relative to the first motif at PBE-TS level of theory.

| motif | 1$^{st}$ | 2$^{nd}$ | 3$^{rd}$ | 4$^{th}$ |
|-------|----------|----------|----------|----------|
| Δ, [kcal/mol]/molecule | 0 | 0.18 | 0.23 | 0.67 |

Table S4.2. Optimized unit cell parameters (in Å, degrees and Å$^3$) and corresponding averaged H-bonding OH–O distances in the two hexagonal rings, R$_1$ and R$_2$, for all generated H-bonding motifs of the monoclinic cholesterol.H$_2$O polymorph at PBE-TS level of theory.

| motif | $a$ | $b$ | $c$ | β | $V = abc \cdot \sin \beta$ | Ro-o (R$_1$) | Ro-o (R$_2$) |
|-------|-----|-----|-----|----|-----------------|-------------|-------------|
| 1$^{st}$ | 9.63 | 7.46 | 66.91 | 96.3 | 4778.7 | 2.74 ± 0.10 | 2.87 ± 0.11 |
| 2$^{nd}$ | 9.62 | 7.46 | 67.01 | 96.2 | 4781.7 | 2.76 ± 0.08 | 2.85 ± 0.16 |
| 3$^{rd}$ | 9.63 | 7.47 | 66.78 | 96.3 | 4773.7 | 2.78 ± 0.07 | 2.83 ± 0.13 |
| 4$^{th}$ | 9.62 | 7.49 | 66.66 | 96.0 | 4772.3 | 2.83 ± 0.03 | 2.77 ± 0.07 |
§5. Model structures used to study the contribution of intra-molecular forces on the molecular packing of cholesterol.H₂O crystals.

Fig. S5. Model structures used to study the contribution of intra-molecular forces on the molecular packing of cholesterol.H₂O crystals: Isolated single cholesterol molecule for the triclinic (A) and monoclinic (B) polymorphs.

§6. Structural parameters of the monoclinic cholesterol.H₂O optimized by DFT compared to experiment.

Table S6. Optimized structural parameters (in Å, degrees and Å³) for the lowest energy H-bonding motif of the monoclinic polymorph at the PBE-TS, MBD and MBD-NL levels of theory, compared to the experimental data of Solomonov et al.\(^{(3)}\)

| method | a  | b  | c  | α   | β   | γ   | V    |
|--------|----|----|----|-----|-----|-----|------|
| experiment | 10.15 | 7.57 | 68.20 | 90.0 | 94.8 | 90.0 | 5222.0 |
| TS     | 9.66 | 7.48 | 66.99 | 90.0 | 96.4 | 90.0 | 4809.4 |
| MBD    | 9.51 | 7.62 | 66.02 | 90.0 | 96.7 | 90.0 | 4749.2 |
| MBD-NL | 9.70 | 7.51 | 67.89 | 90.0 | 100.6 | 90.0 | 4861.5 |
| % TS   | 4.82 | 1.22 | 1.78 | 0.0  | 1.6  | 0.0  | 7.90  |
| % MBD  | 6.30 | 0.61 | 3.19 | 0.0  | 2.1  | 0.0  | 9.05  |
| % MBD-NL | 4.47 | 0.74 | 0.46 | 0.00 | 6.12 | 0.00 | 6.90  |
§7. Temperature dependence of the $d$-spacing of monoclinic cholesterol.H$_2$O.

Table S7. Temperature dependence of the $d$-spacing of monoclinic cholesterol.H$_2$O, measured by electron diffraction (ED) and grazing incidence X-ray diffraction (GIXD), and calculated by DFT at the PBE-TS level of theory.

| method     | T, [K] | $d_{200}$ [Å] | $d_{111}$ [Å] |
|------------|--------|---------------|---------------|
| TS         | 0      | 4.8           | 5.84          |
| Cryo ED$^{(10)}$ | 90    | 4.9           | 5.8           |
| GIXD$^{(3)}$ | 278   | 5.06          | 6.01          |

$\Delta$ in %, TS vs cryo ED  
2.04 -0.69

$\Delta$ in %, TS vs GIXD  
5.14 2.83

§8. Morphologies of the triclinic and monoclinic crystals of cholesterol.H$_2$O.

Theoretical morphology simulations

Theoretical crystal morphologies were obtained using the Materials Studio Morphology module 6.1.$^{(11)}$ The crystal shape was simulated by use of the “growth morphology” approach. Attachment energies and surface energies were calculated using the Dreiding force field.$^{(12)}$ We note, however, that the morphology simulation method does not take into account solvent effects and possible surface reconstructions, which could have a profound influence on experimentally observed morphologies.

Cholesterol crystals were grown on supported lipid bilayers (Fig. S8C and F1) following a procedure described in detail in our previous work.$^{(13)}$ The procedure for crystal growth from cell culture models (Fig. S8F2), following crystal characterization using cryo-transmission electron diffraction and cryo–soft X-ray tomography, is described in Varsano et al.$^{(14)}$

The theoretical growth morphologies of both the triclinic and monoclinic structures were determined using interatomic potential energy computations (see Methods section for details), which although qualitative, yielded results which by and large match the observed morphologies of the crystals grown in solution. The computational analysis predicts a crystal plate habit with a rhomb-like shape (Fig. S8), expressing a dominant (001) face, which corresponds to the plane parallel to the bilayer. All expressed faces correspond to low index flat planes. The crystal plate is delimited by the {100}, {010}, and {011} side faces, where the rhomb shape reflects the close similarity between the unit cell dimensions and the molecular interactions in the $a$ and $b$ directions. Two cut-off edges expose minor {$\overline{1}10$} and {$\overline{1}$1$\overline{1}$} side faces. This is in good agreement with the
The experimental morphology of triclinic cholesterol crystals grown from water solutions, which appear as thin quadrilateral plates (Fig. S8B, C), where a bi-axial growth along \( a \) and \( b \), forming an angle of 101°, is found. The crystals are so thin that accurate determination of the side faces is difficult to perform.

For the monoclinic structure, the growth analysis predicts a crystal habit with a rectangular shape (Fig. S8D), expressing the hydrogen-bonded (001) layer as a dominant plate face. The crystals elongate along the \( b \) direction and are delimited by \{102\} and \{100\} side faces along the \( b \)-axis and \{011\} side faces along the \( a \)-axis. Experimental information as to the facets exhibited by the 3D monoclinic polymorph is lacking. We only managed to examine 3D faceted monoclinic crystals when we nucleated cholesterol on supported mixed lipid bilayers with saturated phospholipids. Representative results for monoclinic crystals thus grown (Fig. S8E,F) show similarities to the theoretical prediction in the tendency to elongate along \( b \) (see model Fig. S8E).

The very thin crystals develop diagonal end faces \((111)\) and \((\overline{1}1\overline{1})\) with an angle between them of \(\sim106°\), rather than \(010\) end faces (Fig. S8E,F\(_1\)). The long aspect ratio results in the monoclinic crystals having a needle shape (Fig. S8E,F\(_1\)). Crystals grown from supported bilayers can also form tens of micrometer-long ribbons elongated in \( b \) (Fig. S8F\(_2\)). It is noteworthy that the habit of cholestanol crystals reported by D. Hodgkin (Fig. 5D and S13) matches the transmission electron microscope image of a monoclinic crystal grown on a supported lipid bilayer (Fig. S8F\(_1\)).

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**Fig. S8.** (A) and (D): Theoretical equilibrium growth morphology of the triclinic (panel A) and monoclinic (panel D) structures. The corresponding molecular packing arrangement for each crystal orientation is presented accordingly. (B) and (E): Model representation of the experimental morphology of triclinic (B) and monoclinic (E) crystals. (C): Optical images of triclinic crystals grown on supported lipid bilayer. (F\(_1\)): Transmission electron microscope image of a monoclinic crystal grown on a supported lipid bilayer; (F\(_2\)): Helical crystal of the monoclinic polymorph grown from a macrophage cell under conditions of cholesterol supersaturation. The image is a reconstructed segmented volume from a cryo-soft X-ray tomogram.\(^{(14)}\)
§9. Hypothetical $P2_1$ crystal structures of cholesterol.H$_2$O: structure and energy profile.

Fig. S9.1. A series of hypothetical $P2_1$ crystal structures of cholesterol.H$_2$O, viewed along the $b$-axis, generated by replacing the twofold axes of the $A2$ polymorph by twofold screw axes and subsequent (A) zero, (B) 0.1$a$ and (C) 0.2$a$ offsets of the adjacent cholesterol bilayers along the $a$-axis, at the hydrophobic interface. The hydrophilic interface was kept fixed as in the $A2$ crystal structure, thus maintaining the original H-bonded bilayer system across which the corresponding cholesterol layers are related by twofold screw symmetry.
Fig. S9.2. DFT-computed, static energy profile of a series of hypothetical $P2_1$ crystal structures of cholesterol.$H_2O$, generated by replacing the twofold axes of the $A2$ polymorph by twofold screw axes and subsequent offset of the adjacent cholesterol bilayers along the a-axis, at the hydrophobic interface. The hydrophilic interface remained the same as in $A2$ crystal structure, thus maintaining the original H-bonded bilayer system across which the corresponding cholesterol layers are related by twofold screw symmetry.

§10. Single cholesterol bilayers in which the two leaflets are related by a twofold screw axis $p2_1$.

Fig. S10. View along the b-axis of single cholesterol bilayers in which the two leaflets are related by a twofold screw axis $p2_1$ (A), as opposed to a twofold axis $p2$ (B), followed by a 10Å vacuum layer. $A_1$ and $B_1$ are $p2_1$ and $p2$ isolated cholesterol bilayers, respectively; $A_2$ and $B_2$ are $p2_1$ and $p2$ hydrated cholesterol bilayers, respectively.
§11. Transformation of the monoclinic form on increased interlayer growth.

**Fig. S11.** (A) Crystalline monolayer of cholesterol at the water surface. (Left to right) Bragg rod corresponding to broad single Bragg peak. The profile of this Bragg rod indicates that the long axis of the molecule is aligned perpendicular to the water surface. The full width at half maximum of the Bragg rod indicates that it is a monolayer. The side and top views of the monolayer structure, with proposed $p3$ trigonal symmetry. The molecule undergoes pronounced librational motion about its long axis. The molecules are arranged in an $a'b'$ super cell (——) constructed from the subcell (· · ·). (B) Crystalline bilayer of cholesterol at the water surface of hydrated at opposite sides. (Left to right) Several $\{h,k\}$ Bragg rod profiles corresponding to different Bragg peaks. The FWHM of the Bragg rods yielded the thickness of the bilayer; Side and top views of the packing arrangement of the crystalline bilayer with lattice symmetry $p2_1$. (C) Crystalline triple bilayer of cholesterol monohydrate with lattice symmetry $A2$. (Left to right) Several $\{h,k,l\}$ Bragg rod profiles corresponding to different Bragg peaks. The FWHM of the Bragg rods yielded the thickness of the film; Packing arrangement of the triple bilayer of cholesterol monohydrate; Cross-section through a cholesterol layer. (D) Transformation of crystalline cholesterol monohydrate from monoclinic $A2$ to triclinic $P1$ symmetry: Part of the packing arrangement viewed edge-on to the bilayer; Cross-section through a layer of cholesterol molecules.
§12. Model for the packing arrangement of the monoclinic cholestanol.2H₂O.

Fig. S12. Model for the packing arrangement of the monoclinic cholestanol.2H₂O unit cell, viewed along the $a$-axis (A) and $b$-axis (B). The atoms are color-coded in: white, hydrogen; brown, carbon; red, oxygen. OH···O bonds are represented as grey dashed lines. The unit cell is indicated by a black rectangle.
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