Supporting Information

Expanding the molecular recognition repertoire of antifreeze polypeptides: effects on nucleoside crystal growth†

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1. Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) at ACS grade or better, and were used without additional purification. Solvents and chemicals for the HPLC experiments were purchased at HPLC grade from Sigma-Aldrich. All of the aqueous solutions were prepared using Milli-Q water produced from a Synergy water system (Millipore) with a minimum resistivity of 18 MΩ·cm. All of the samples including the polypeptide samples were filtered through 0.2 μm filters before use unless otherwise indicated. 8 mL sample vials (National Scientific) were used for crystallization. All glassware and stir bars were first cleaned in a KOH/2-propanol bath. After rinsed with distilled water, the glassware and stir bars were soaked in 1 M HCl for 24 h and then rinsed with distilled water. Finally they were cleaned using RBS35 (Pierce), a surface-active detergent. After rinsed with distilled water and then with deionized water completely, the glassware and stir bars were air dried at room temperature before use.

2. AFP and control preparation

DAFP-1 was expressed and purified as described previously.[1] The purified DAFP-1 was characterized using SDS-PAGE gel electrophoresis, MALDI-TOF mass spectrometer, Circular Dichroism (CD) spectrometry, and differential scanning calorimetry (DSC), respectively, as previously described[2] and the identity of DAFP-1 was confirmed. The concentration of stock DAFP-1 solution was determined using a Cary 100 Bio UV-Vis spectroscopy (Varian) and the extinction coefficient of 5.47×10^3 M^{-1} cm^{-1} at 280 nm was used.[3]

BSA was purchased from Sigma-Aldrich (Item number A7030) and type III AFPs from fish were purchased from A/F Protein (Waltham, MA), which were used as received. The stock BSA and fish AFP solutions were prepared by weighing the solute and dissolving the solute in a known volume of water. The molecular weights, 66.5 kDa and 6.5 kDa, were used for BSA and type III fish AFPs, respectively. All the weight measurements were carried out with an Ohaus Voyager Pro analytical and precision balance (Parsippany, NJ).

In the denatured DAFP-1, all disulfide bonds were broken, resulting in the disruption of the structure of the conserved repeated threonine residues in the adjacent loops of DAFP-1 and thus loss of the hydrogen bonding interactions with the m^5U crystal surface. Thus, the denatured DAFP-1 with completely reduced disulfide bonds was used as a second unknown and prepared following the previously reported methods.[4] To fully reduce all the disulfide bonds in DAFP-1, purified DAFP-1 (~1 mM) was incubated in 0.10 M sodium citrate, pH 3.0, and 15.0 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) at 60 °C for 30 min. Then the denatured DAFP-1 was further purified using purified using ÄKTA Purifier 10 (GE Healthcare) with a Sephacryl S-100 gel filtration column (GE Healthcare).

3. Crystal growth procedure

m^5U was known to be crystallized from aqueous ethanol solutions.[5] We found that m^5U can crystallize from its aqueous solution directly. On day 1, each sample vial was first added 600 μL of 400 mM m^5U solution. Then 20 μL of water or polypeptide solutions at certain concentrations were added into each vial. The final m^5U concentration was 387 mM in each vial. The additive/m^5U molar ratios ($\times 10^{-5}$) were varied from 0, 0.04, 0.4, 1.0, 1.2, 3.0, 4.0, 7.8, 9.0, and 18.0. The vials were gently swirled after the addition and were left open in the air at the room temperature. At least three observations were recorded per day (every 8 hours) until the solutions in all the vials were dry. The experiments were repeated five times. Sample results were as listed in Table S1.

In previous reports, crystals of C were prepared by evaporating of its aqueous ethanol solutions.[6,7] Here we obtained C crystals by evaporating of its aqueous solution. It is known that fast evaporation from the aqueous solutions of I usually resulted in a mixture of its aggregates, α-, β-, and γ-form crystals.[8,9] By slowly evaporating its aqueous solution at 20 ˚C, the pure α-form I crystals (in this report referred to as I) were obtained. The experimental procedures for C and I were similar to those described above for m^5U, while the concentrations of C and I solutions were 252 mM and 92 mM, respectively, in each sample vial on day 1 and the additive/C molar ratios ($\times 10^{-5}$) were varied from 0, 0.04, 0.4, 1.0, 1.2, 3.0, 4.0, 7.9, and 10.0 and the additive/I molar ratios ($\times 10^{-5}$) were varied from 0, 0.05, 0.5, 1.1, 2.2, 4.4, 8.1, 10.8, and 21.7. Sample results were shown in Table S1. Photos of the vials were taken with a Canon EOS 30D camera during the crystallization process and when the process finished (data are not shown). Optical micrographs were taken under Nikon SMZ800 microscope with a Nikon Coolpix 5400 when the crystallization completed. The crystals were under Nikon SMZ800 microscope, and photos were taken by Nikon Coolpix 5400.

For the crystal habit study, crystallization conditions for the three nucleosides were used as described above. The formed nucleoside crystals in the absence of additives are homogeneous and reproducible with respect to their sizes and shapes. The same criterion was used for the study in the presence of each of the AFPs and controls. When the seed nucleoside crystals were first observed (less than 0.2 mg), DAFP-1 and type III AFP were added into the vials at final concentrations of 4.8 μM and 0.10 μM, respectively. Data were recorded as described above. The habit change can be stopped during the process. To stop the habit alteration, the mother liquor was removed from the vials and the seed crystals were washed quickly with cold water at 4 °C twice. The same volume of fresh saturated m^5U solution as the removed mother liquor was added into the vial.

To make saturated thymine solution, an excess amount of thymine was first added into water and the resulting sample was shaken for two days and filtered. Then 20 μL of water or polypeptide solutions at certain concentrations were added into each vial with 1000 μL saturated thymine solution. The final thymine concentration was 2.94 mM in each vial and the final additive

concentrations at the additive/thymine molar ratio of 1×10^{-4} or above. All the above mentioned experiments were repeated five times.

4. HPLC analysis

The resulting solids, both crystals and precipitates, were analyzed by HPLC. The HPLC analysis were performed on a Waters HPLC system consisting of a Waters 1525 binary HPLC pump, a BioSuite 125, 4 μm UHR SEC HPLC column (4.6 × 300 mm), and a Waters 2998 photodiode array detector. The SEC buffer contained 0.10 M Na_2SO_4, 0.10 M NaPi (pH 7.00), and 0.02% NaN_3. The solid m^5U samples, each at a weight of 0.5 ± 0.05 mg, were selected in at least four different locations in the same vial. The samples of precipitates were taken from the inside of solids. Each selected sample was dissolved in 100 μL HPLC buffer and diluted six-fold before HPLC analysis. For the solid thymine samples, the crystals were selected at three or more different locations in a vial. The selected crystals were dissolved in 1000 μL HPLC water and diluted to a final thymine concentration at about 0.600 mM. All the buffers and samples were filtered through a 0.1 μm filter and vacuum degassed before use. The flow rate was 0.30 mL/min at ambient temperature. The injection volume was 5.00 μL for all the HPLC experiments. Each experiment was repeated twice. Pure DAFP-1 and m^5U were eluted at 11.5 and 13.1 minutes, respectively. The HPLC analysis of all the thymine samples shows that only pure thymine was eluted at 14.5 minute (data are not shown).

5. X-ray diffraction

All the resulting solids were sent to X-ray crystallography laboratory at the Beckman Institute of California Institute of Technology for analysis. The qualities of the solids for single crystal X-ray diffraction were determined and listed in Table S1. The crystallographic data of m^5U reported below were from the m^5U crystals grown in the presence of BSA. The resolution of the data was improved compared with that of previously reported data of m^5U.[5] The crystallographic data of m^5U have been deposited in the Cambridge Database (CCDC) and the CCDC deposit number is 835399.

Powder X-ray diffraction (PXRD) data were collected at room temperature on a Rigaku Mini Flex II desktop diffractometer using Cu Kα radiation (30 kV, 15 mA) with a 2θ range of 5° – 50°, a step size of 0.010°, and a step time of 2s. Samples were lightly ground by hand using a mortar and pestle. The same amount of the resulting powders was then mounted and analyzed on a front loading sample holder.

The identity and quality of the final obtained nucleoside solids for single crystal X-ray diffraction and PXRD were determined and listed in Table S1. The PXRD patterns of the

crystalline thymine obtained in the presence of the AFPs are in agreement with the reported pattern of crystalline thymine.[10]

6. Supporting figures and table

Table S1. Sample results for crystal growth of m^5U in the presence of AFPs in the absence of m^5U seed crystals.

Sample[a]	Polypeptide concentration (μM)	Induction time (day)[b]	Crystal quality[c]
m^5U	0	6	Yes
m^5U + BSA/denatured DAFP-1	70.0	6	Yes
m^5U + DAFP-1	70.0	13	No
m^5U + DAFP-1	35.0	11	No
m^5U + DAFP-1	30.0	10	No
m^5U + DAFP-1	15.0	10	No
m^5U + Type III AFP	1.50	12	No
m^5U + Type III AFP	0.15	12	No
C	0	13	Yes
C + BSA/denatured DAFP-1	70.0	13	Yes
C + DAFP-1	20.0	21	No
C + DAFP-1	10.0	21	No
C + Type III AFP	20.0	20	No
C + Type III AFP	10.0	20	No
I	0	5	Yes
I + BSA/denatured DAFP-1	70.0	5	Yes
I + DAFP-1	2.0	15	No
I + DAFP-1	0.5	15	No
I + Type III AFP	20.0	14	No
I + Type III AFP	10.0	13	No

[a] Each sample contained 387 mM m^5U, 252 mM C or 92 mM I on day 1. Results of each nucleoside alone and in the presence of each of the two polypeptide controls, BSA and denatured DAFP-1, were listed for comparison. [b] The day that the first appearance of solid was observed. [c] The suitableness of the resulting solids for single crystal x-ray diffraction. The identity and quality of single crystals of m^5U, C, and I were examined using polarized microscope, PXRD, and single crystal x-ray diffraction. See Supporting Information for methods.

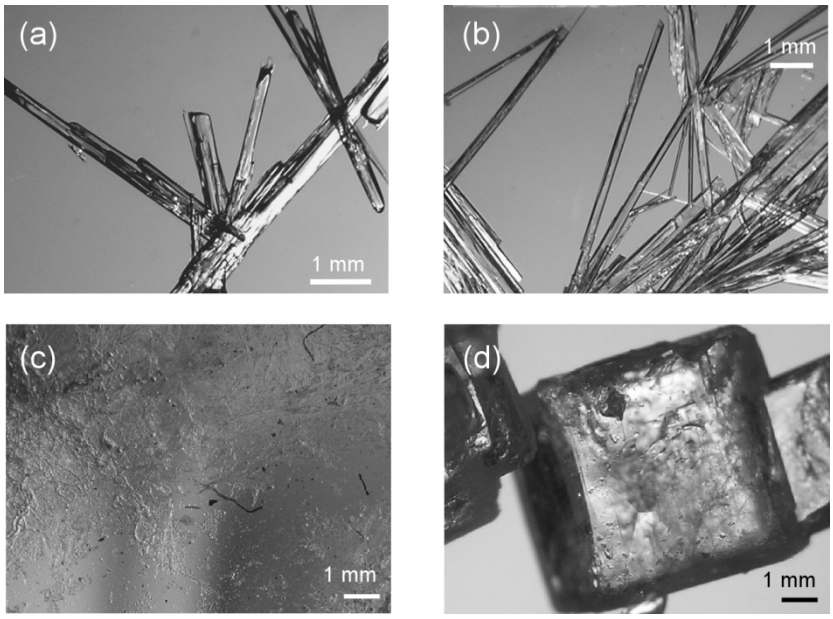

Fig. S1 Optical micrographs of the finally achieved crystals or amorphous solids of m^5U: (a) needle-shaped m^5U crystals obtained in the presence of BSA, (b) needle-shaped m^5U crystals obtained in the presence of the denatured DAFP-1 with completely reduced disulfide bonds, (c) gel-like amorphous m^5U solids obtained in the presence of type III AFP, (d) normal orthorhombic m^5U crystals obtained in the presence of type III AFP and seed m^5U crystals in a saturated m^5U solution.

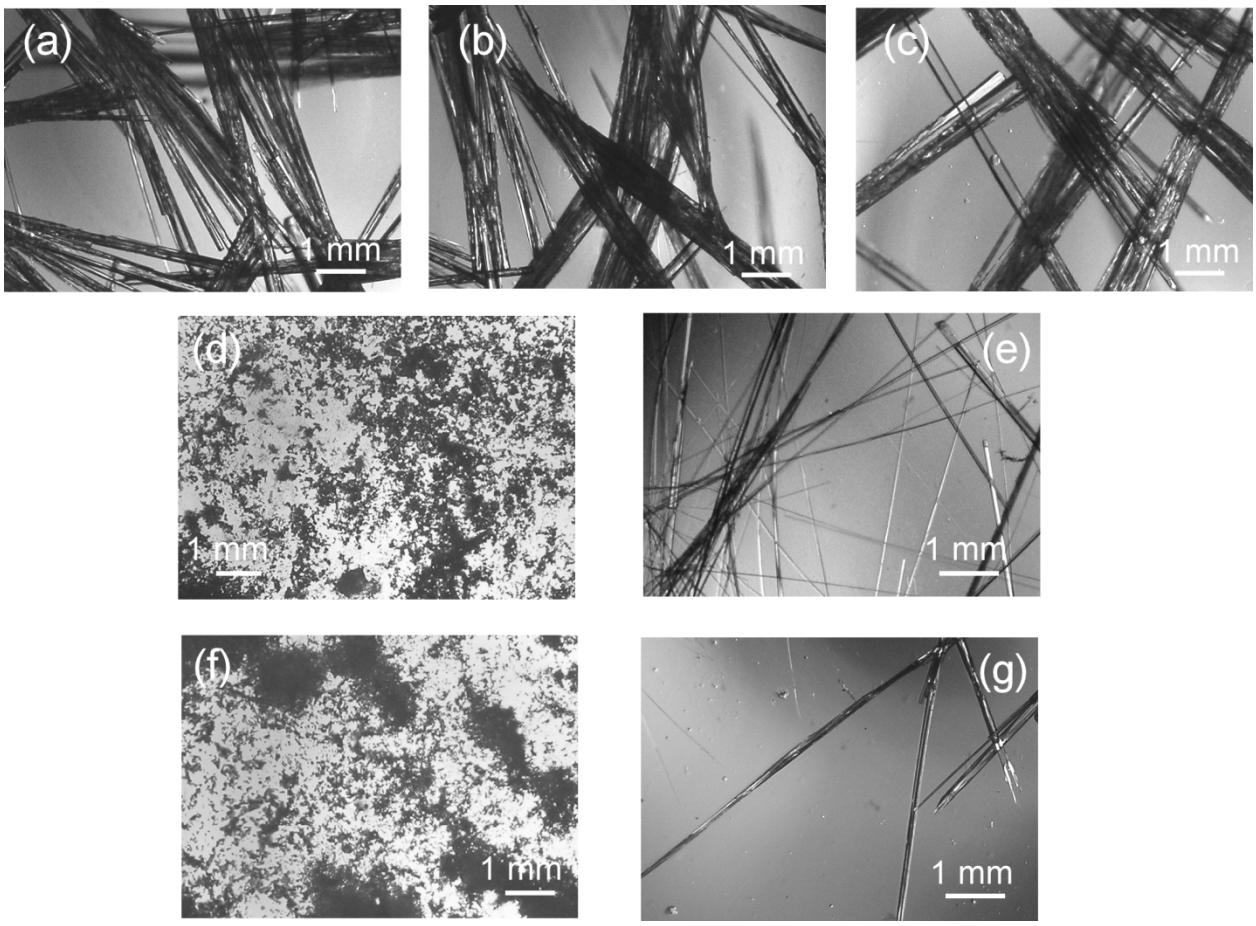

Fig. S2 Optical micrographs of the final achieved crystals or amorphous solids of C: (a) needle-like C crystals, (b) needle-like C crystals obtained in the presence of BSA, (c) needle-like C crystals obtained in the presence of the denatured DAFP-1 with completely reduced disulfide bonds, (d) powder-like amorphous C solids obtained in the presence of DAFP-1, (e) hair-like C crystals obtained in the presence of DAFP-1 and seed C crystals in a saturated C solution, (f) powder-like amorphous C solids obtained in the presence of type III AFP, (g) hair-like C crystals obtained in the presence of type III AFP and seed C crystals in a saturated C solution.

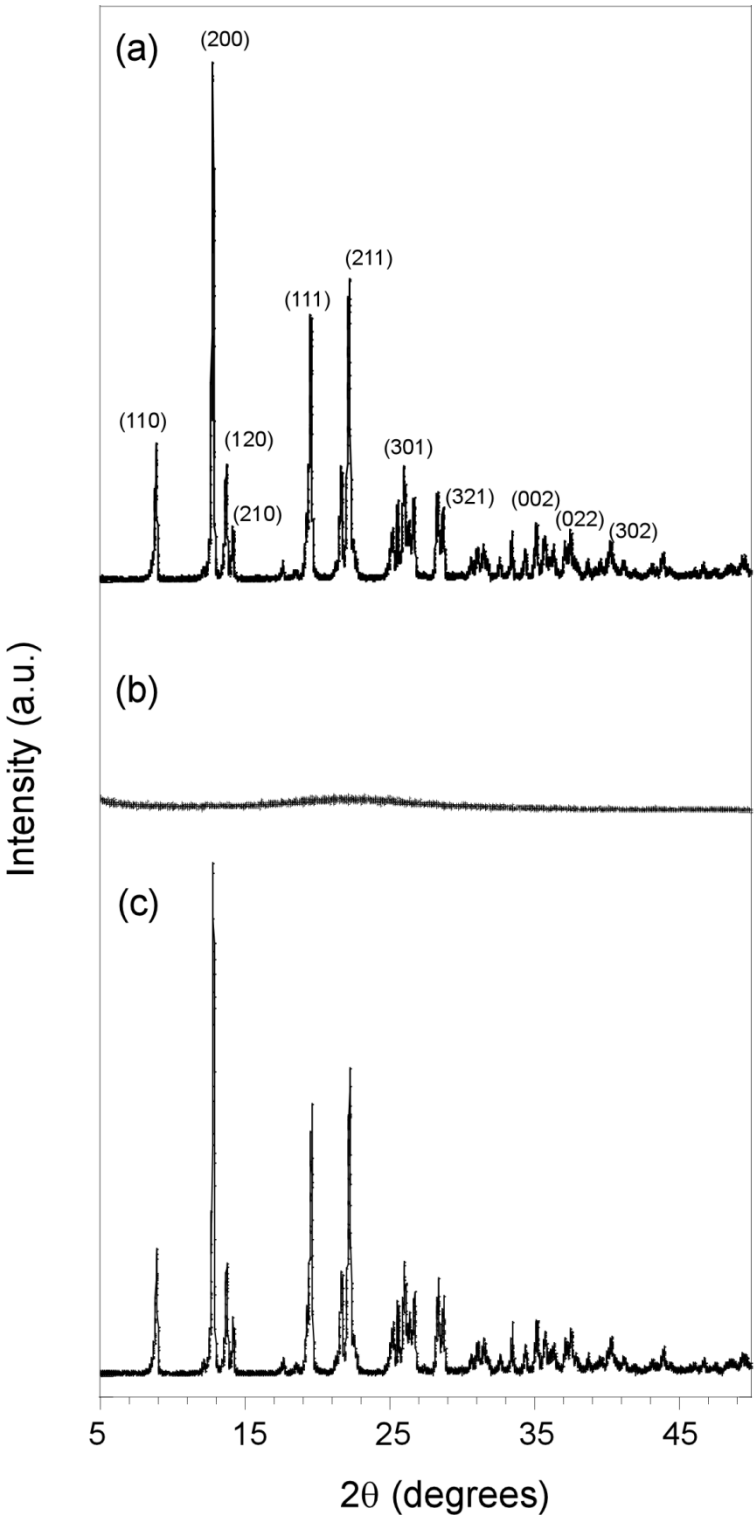

Fig. S3 Overlay of representative PXRD profiles of the samples from (a) needle-like C crystals, (b) amorphous C (obtained in the presence DAFP-1), and (c) hair-like C crystals (modified by DAFP-1). Major crystalline peaks in (a) are labeled with miller indexes (*hkl*).

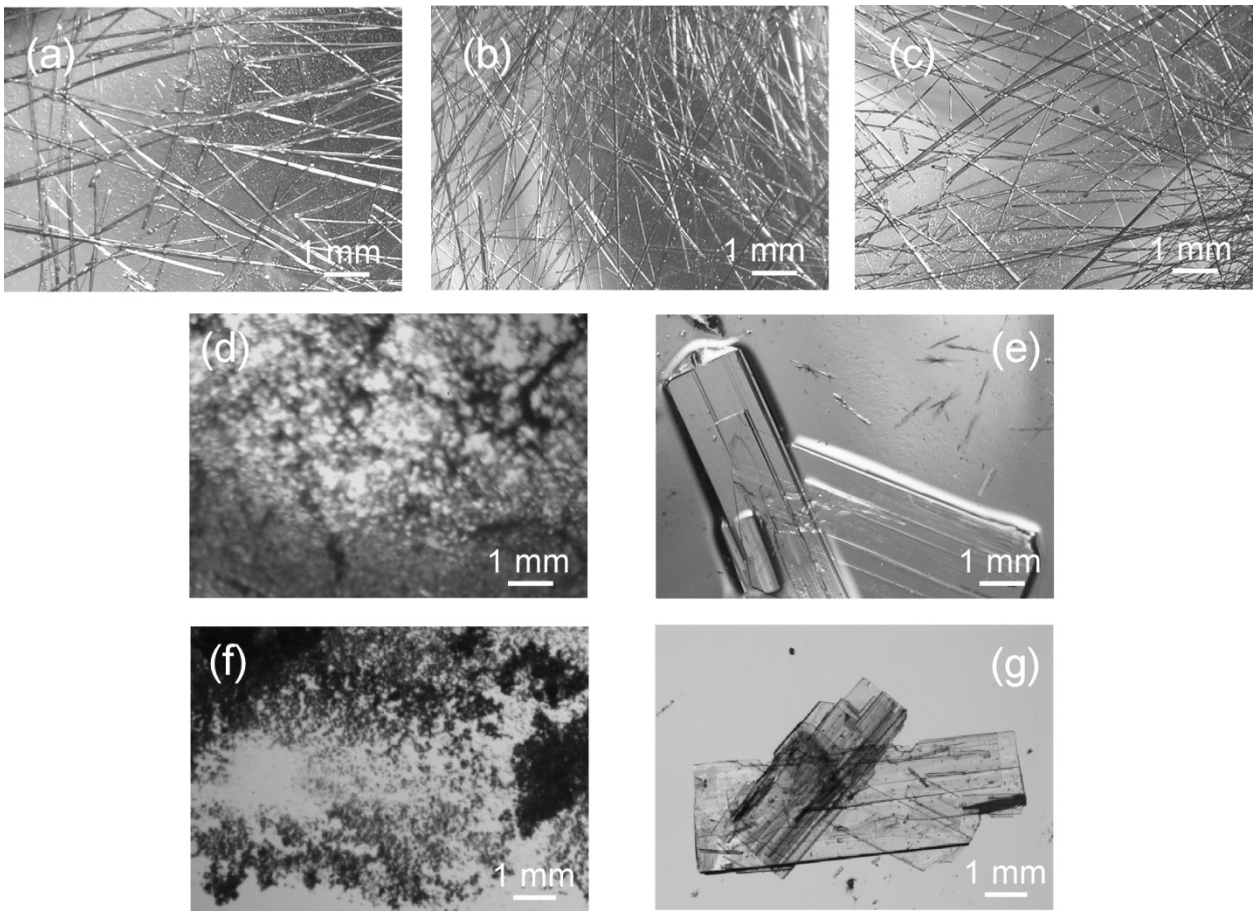

Fig. S4 Optical micrographs of the final achieved crystals or amorphous solids of I: (a) hair-like I crystals, (b) hair-like I crystals obtained in the presence of BSA, (c) hair-like I crystals obtained in the presence of the denatured DAFP-1 with completely reduced disulfide bonds, (d) powder-like amorphous I solids obtained in the presence of DAFP-1, (e) plate-like I crystals obtained in the presence of DAFP-1 and seed I crystals in a saturated I solution, (f) powder-like amorphous I solids obtained in the presence of type III AFP, (g) plate-like I crystals obtained in the presence of type III AFP and seed I crystals in a saturated I solution.

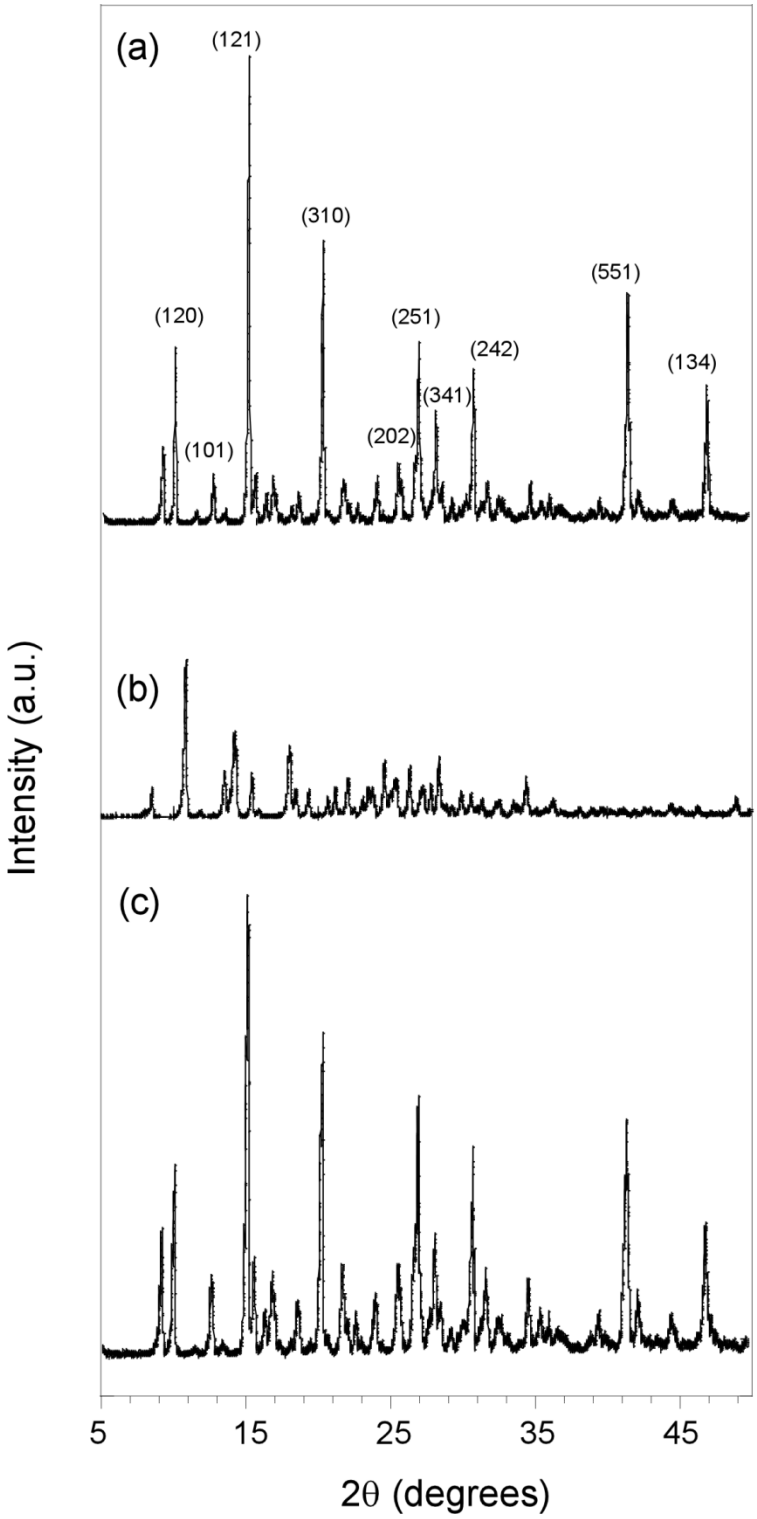

Fig. S5 Overlay of representative PXRD profiles of the samples from (a) hair-like I crystals, (b) amorphous I (obtained in the presence DAFP-1), and (c) plate-like I crystals (modified by DAFP-1). Major crystalline peaks in (a) are labeled with miller indexes (*hkl*).

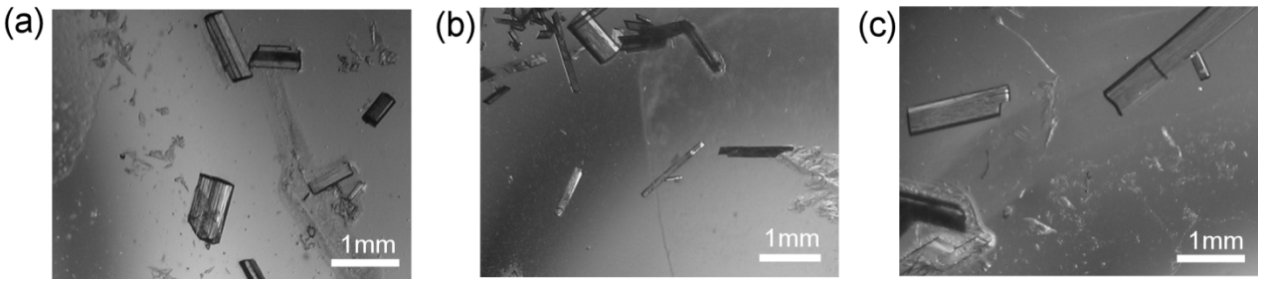

Fig. S6 Optical micrographs of the final achieved thymine crystals (a) in the absence of AFPs, (b) in the presence of DAFP-1, (c) in the presence of type III AFP.

7. References

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