Genetically fused charged peptides induce rapid crystallization of proteins

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Materials and Methods:

Construction of expression vectors of SAs with charged peptides.

pET22b+ carrying the genes of SA-D_4Y or SA-R_4Y was used as the template DNA for vector construction. The number of C-terminus Asp or Arg residues in the charged peptides was changed by either deleting or introducing the DNA sequence coding those amino acid residues by site-directed mutagenesis using inverse PCR and subsequent self-ligation of the PCR products. SAs possessing two to six Asp or Arg residues at the C-terminus were constructed; the SA derivatives are named SA-D_nY or SA-R_nY, where n is the number of introduced charged amino acid residues. Also, the expression vectors of SAs with charged peptides without a C-terminus Tyr residue were constructed in the same manner (denoted SA-D_n and SA-R_n). In addition, we constructed SAs with E_6Y or K_6Y sequences at the C-termini (denoted SA-E_6Y and SA-K_6Y, respectively).

Expression of SAs with charged peptides

The expression vectors of SAs were transformed into *E. coli* BL21 Star (DE3) by heat shock and seeded on lysogeny broth (LB) agar plates containing 100 μg/mL of ampicillin sodium. A single colony was picked up and used to inoculate LB medium (5 mL). The suspension was precultured at 37 °C and 220 rpm overnight. The cell suspensions were then poured into LB medium (250 mL) containing the same antibiotic in a baffled Erlenmeyer flask and cultured at 37 °C and 220 rpm until optical density at 600 nm reached around 0.9. Isopropyl β-D-1-thiogalactopyranoside was added to induce protein expression, and culturing was continued at 37 °C for 5 h with shaking at 220 rpm. Cells were harvested by centrifugation at 5,000×g for 20 min and kept at −30 °C until purification. The cells were suspended in 30 mL of the Wash Buffer 1 (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0). Lysozyme (20 mg), 60 units of Cryonase™ Cold-active Nuclease (Takara Bio Inc., Japan) and 1 M $MgCl_2$ (120 μL) were added to the cell suspension. After incubation at 4 °C for 1 h, the cells were completely lysed by sonication for 3 min three times with 3 min of cooling between each sonication. The insoluble materials containing the inclusion body of SAs were separated by centrifugation at 5,000×g for 10 min at 4 °C. The precipitates were resuspended in 30 mL of Wash Buffer 2 (30 mM Tris-HCl, 2 mM EDTA, 0.1% Triton-X100, pH 8.0) using a pipette. The inclusion body was centrifuged again at 5,000×g for 10 min at 4 °C to remove supernatant. This washing process was repeated at least three times to obtain the inclusion body of SAs. The same washing process was repeated using the Wash Buffer 1 to completely remove surfactant. Finally, the inclusion body was dissolved in 6 M guanidinium hydrochloride (1.2 mL, pH 1.5) and centrifuged at 20,000×g for 10 min at 4

°C. The supernatant containing denatured SAs was gradually added dropwise into vigorously stirred Tris-buffered saline (TBS) (50 mL, 25 mM Tris-HCl, 150 mM NaCl, pH 7.4) on ice to induce refolding. The TBS solutions were then stirred gently for 3 h at 4 °C. The precipitates were removed by centrifugation at 20,000×g for 10 min at 4 °C. Finely ground ammonium sulfate (24.6 g) was added to the solution with gently stirring to precipitate the refolded SAs. The precipitates were collected by centrifugation at 20,000×g for 10 min at 4 °C. SAs were dissolved in Milli-Q water (10 mL) and then subjected to ion-exchange chromatography. SAs possessing negatively charged peptides were isolated using a HiTrap DEAE FF column 5 mL (GE Healthcare Life Sciences, UK) pre-equilibrated with 10 mM Tris-HCl (pH 8.0). The column was washed with the same buffer (five column volumes) and then SAs were eluted by a salt gradient of the same buffer containing 1 M NaCl. SAs with positively charged peptides were purified using a HiTrap SP FF column, 5 mL (GE Healthcare Life Sciences) equilibrated with 10 mM Tris-HCl (pH 7.4). Unbound proteins were washed out with the same buffer (five column volumes). The SAs were eluted using a salt gradient of 1 M NaCl in 10 mM Tris-HCl (pH 7.4). The fractions containing SAs were collected and concentrated using an ultrafiltration membrane (30 kDa MWCO). SAs with negatively charged peptides were further purified and dialyzed into 10 mM Tris-HCl (pH 8.0) by size-exclusion chromatography (SEC) using a HiLoad 16/600 Superdex75 pg column (GE Healthcare Life Sciences). The fraction containing the SAs was collected and concentrated using a 30-kDa MWCO ultrafiltration membrane then stored at −30 °C. SAs with positively charged peptides strongly adsorbed to the SEC resin, so after cation-exchange chromatography, these SAs were simply buffer-exchanged into 10 mM Tris-HCl (pH 8.0), concentrated using an ultrafiltration membrane (30 kDa MWCO) and then stored at −30 °C.

Preparation of crystals of SAs with charged peptides

Equimolar amounts of SAs with positively or negatively charged peptides were mixed together in 10 mM Tris-HCl (pH 8.0) in a 1.5-mL plastic tube and incubated at 25 °C for 6 h. To determine the phase diagram of SAs, the concentrations of SAs and NaCl were varied. The reaction mixtures (5 μL each) were set up in the wells of a Terasaki plate and the surface was covered with paraffin oil to prevent evaporation. Each set of conditions was run in five individual wells. After setting up the reaction mixtures, the plates were left at 25 °C overnight, and then each well was observed with a microscope (IX 81, Olympus, Japan). The supernatant of each solution was collected and its absorbance at 280 nm was measured using a spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific Inc., USA) to obtain the solubility curves of the SAs. Time-lapse observation of crystallization was conducted in time-lapse observation mode of the IX 81 microscope. SA-D_4Y and SA-R_4Y were mixed in 10 mM Tris-HCl (pH 8.0) containing an appropriate amount of NaCl and the mixture was placed as a droplet at the center of a 35-mm culture dish. The edge of the 35-mm culture dish was covered with water to increase the humidity within the dish and slow evaporation of the crystallization solution. Time-lapse observation was conducted for 200 min acquiring an image every 5 min. In addition, SA crystals were prepared using charged oligopeptides. Peptides of YD_8Y and YR_8Y were mixed with SA-R_4Y and SA-D_4Y, respectively, in equimolar

ratios in 10 mM Tris-HCl (pH 8.0) and incubated at 25 °C overnight. Oligo-DNAs (5'-GAGATATCG-3' and its complementary oligo-DNA) were mixed with SA-R_6Y (final concentration of 10 μM) to give a final concentration of 10 μM in 10 mM Tris-HCl (pH 8.0) and incubated at 25 °C overnight. SA crystals were prepared by mixing dendrimers (PAMAM dendrimer, cystamine core, generation 2.0 [P-Dendrimer] or PAMAM dendrimer, ethylenediamine core, generation 1.5 [N-Dendrimer], both from Sigma Aldrich, USA) with oppositely charged SAs in 10 mM Tris-HCl (pH 8.0) and incubation at 25 °C overnight. Concentrations of SAs and dendrimers were 10 μM each.

Measurement of SA concentration in crystallization solutions during crystal growth

SA-D_4Y and SA-R_4Y were mixed in 10 mM Tris-HCl (pH 8.0) at a concentration of 10 μM each to give a total volume of 200 μL. Aliquots of supernatant (2 μL) were taken every 10 min and the concentration of SAs in each solution was estimated by measuring its absorbance at 280 nm using the NanoDrop 1000 spectrophotometer.

Structure determination

A single crystal suitable for structure determination was obtained by the sitting drop vapor diffusion method at 293 K. Equal volumes of 1 μM SA-D_6Y and SA-R_6Y were mixed. An aliquot of this mixed protein solution (200 nL) was further mixed with 1,000 nL of reservoir solution containing 100 mM MES-NaOH (pH 6.5), 100 mM ammonium sulfate and 30% w/v polyethylene glycol monomethyl ether 5,000 from Crystal Screen-II kit (Hampton Research, USA). A crystal was immersed in reservoir solution containing 30% v/v glycerol. The cryoprotected crystal was flash-cooled in liquid nitrogen. Diffraction data were collected at 100 K on beamline BL38B2 at SPring-8 (Harima, Japan). The wavelength used was 1.0 Å. Data were indexed, integrated and scaled using the XDS program suite.[1] The complex structure of SA-D_6Y and SA-R_6Y was solved by molecular replacement with a search model (PDB entry 3RY1) using the program Phaser in the CCP4 Suite.[2,3] The model was built using XtalView.[4] The structure was refined to R_{work}/R_{free} of 18.1%/21.9% using the program PHENIX.[5] Ramachandran plot values were within limits with 97.1% in the favoured region, 2.5% in the allowed region and only 0.4% in the outlier region, as analysed with Rampage.[6] Images in Fig. 2 were prepared with PyMOL.[7]

Preparation of biotinylated EGFP and DsRed monomer

EGFP and DsRed monomer(mDsRed) were expressed in *E. coli* BL21(DE3) star with pET22b+ vector and purified by Ni-NTA affinity chromatography, anion exchange chromatography and size exclusion chromatography. The concentrations of purified EGFP and mDsRed were determined by UV absorbance values at 488 nm ($E_{488\ nm}$=55,000 $M^{-1}cm^{-1}$) and 556 nm ($E_{556\ nm}$=35,000 $M^{-1}cm^{-1}$). Both proteins were buffer-exchanged into PBS (-) and reacted with 10 eq. of biotin-LC-LC-NHS (Tokyo Chemical Industry Co., Ltd., Japan) at 4˚C for overnight. The final concentrations of proteins and biotin-LC-LC-NHS in the reaction mixture (0.5 mL) were 100 μM and 1 mM, respectively. Then 100 μL of 1 M Tris HCl pH 8.0 was added to the reaction mixture to

quench the unreacted biotin-LC-LC-NHS. Finally, the buffer was exchanged to 10 mM Tris HCl (pH 8.0) to remove any excess biotin-LC-LC-NHS by using ultrafiltration membrane (10 kDa MWCO).

Modification of crystals of SAs

SA crystals were prepared from a mixture of 10 μM SA-D_6Y and 10 μM SA-R_6Y in 10 mM Tris-HCl (pH 8.0) containing 50 mM NaCl with a total volume of 100 μL. After crystallization, 25% glutaraldehyde solution (20 μL) was added to the mixture to fix the SA crystals. After incubation at 25 °C for 12 h, the supernatant was carefully removed using a pipette. Milli-Q water (100 μL) was carefully added to the SA crystals and then removed to flush out the remaining glutaraldehyde. This washing process was repeated twice. A hundred microliters of 10 μM biotin-4-fluorescein (B4F, Sigma Aldrich) in 10 mM Tris-HCl (pH 8.0) was then added to the SA crystals and incubated for 6 h at 25 °C. The B4F solution was removed and the concentration of remaining B4F in the solution was estimated by measuring UV absorbance at 494 nm with extinction coefficient of 68,000 M^{-1} cm^{-1}. The modified SA crystals were washed with Milli-Q water twice. The SA crystals modified with B4F were immersed in 10 mM Tris-HCl (pH 8.0) and observed with a confocal laser microscope (LSM710, Carl Zeiss Co. Ltd., Germany). The SA crystals were also modified with biotinylated EGFP and biotinylated mDsRed in the same manner as B4F before CLSM observation was carried out. EtBr solution (2.5 mg/mL, 2 μL) was added to the solution containing SA crystals and observed with CLSM (LSM510, Carl Zeiss Co. Ltd., Germany) without washing. For the CLSM observations of the modified SA crystals, a 488-nm laser was used for SA crystals modified with B4F and EGFP, while 543-nm laser was used for crystals modified with mDsRed and EtBr with appropriate emission filter sets.

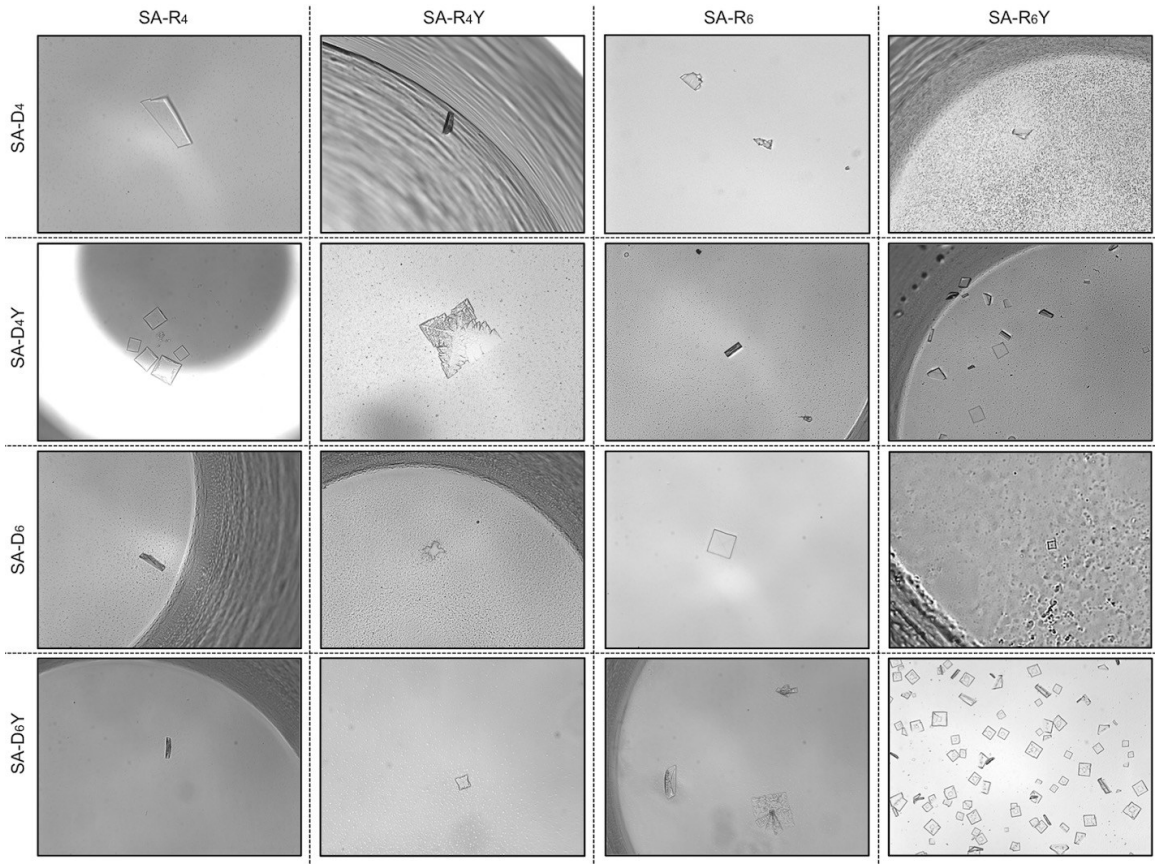

Fig. S1 Observation of SA crystals. Microscopy images of crystals obtained by mixing SAs possessing more than four charged amino acids. Scales are different in each picture.

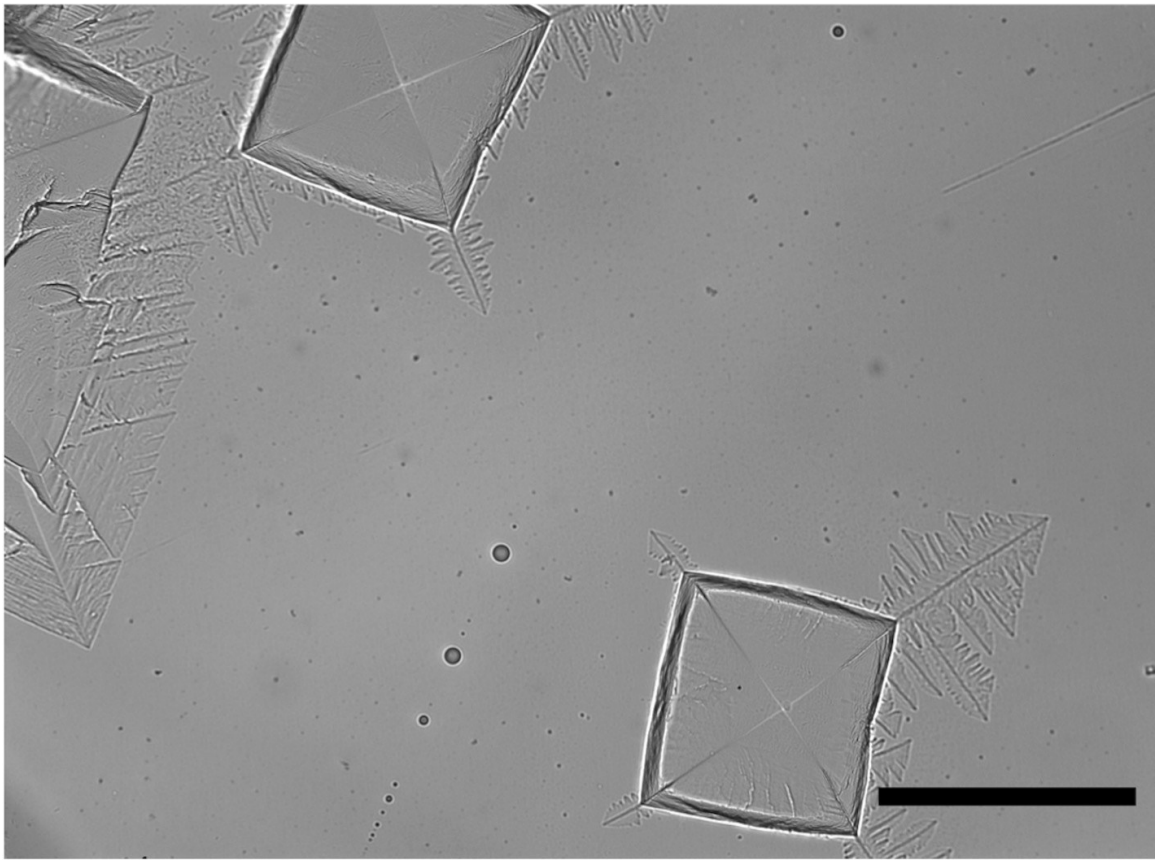

Fig. S2 Microscopy image of crystals of SA-K_6Y and SA-E_6Y. Crystallization solutions consisted of SAs (10 μM each) in 10 mM Tris-HCl (pH 8.0) containing 50 mM NaCl. Scale bar is 200 μm.

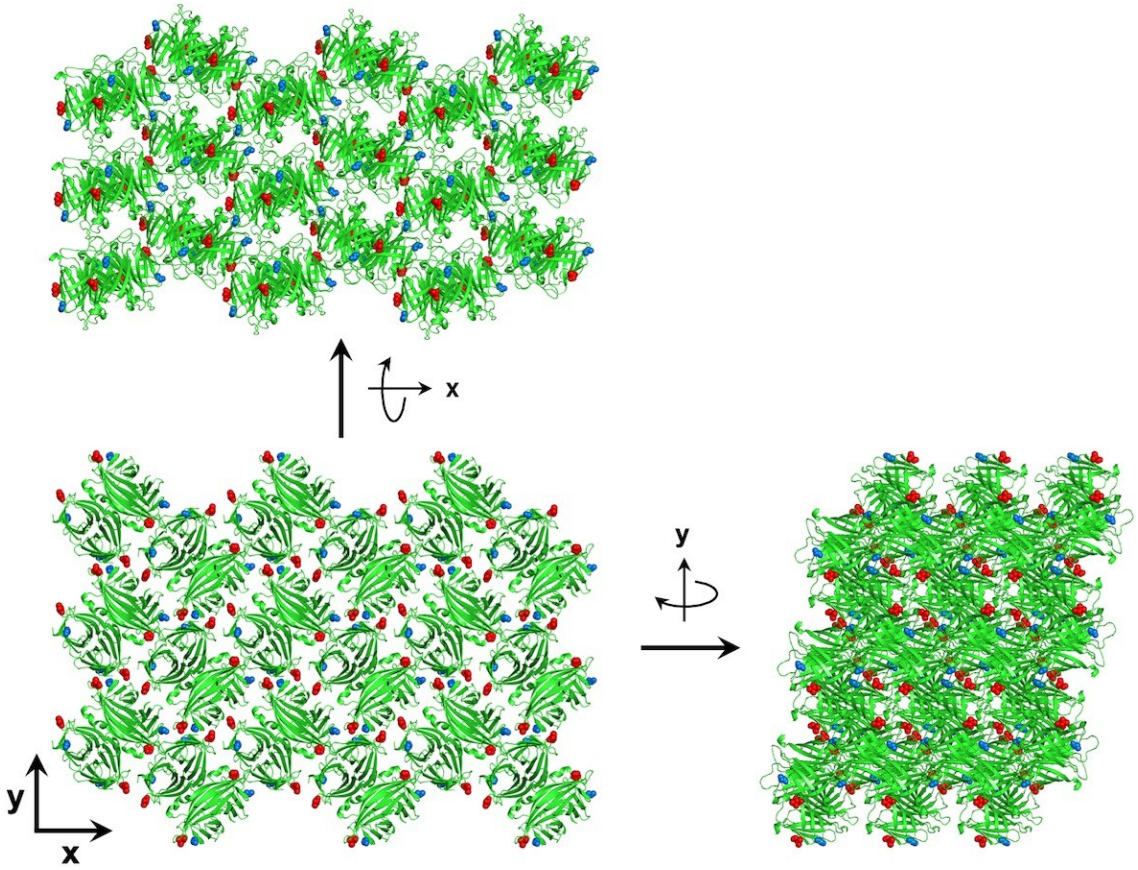

Fig. S3 3D packing images of streptavidin in the crystal prepared by vapor diffusion method. The images were generated by using PDB data of 1SWB, reported as apo-core streptavidin at pH 7.5. The images were in orthoscopic view mode and N- and C-termini of streptavidin subunits were shown in space-filling models of blue and red in color, respectively.

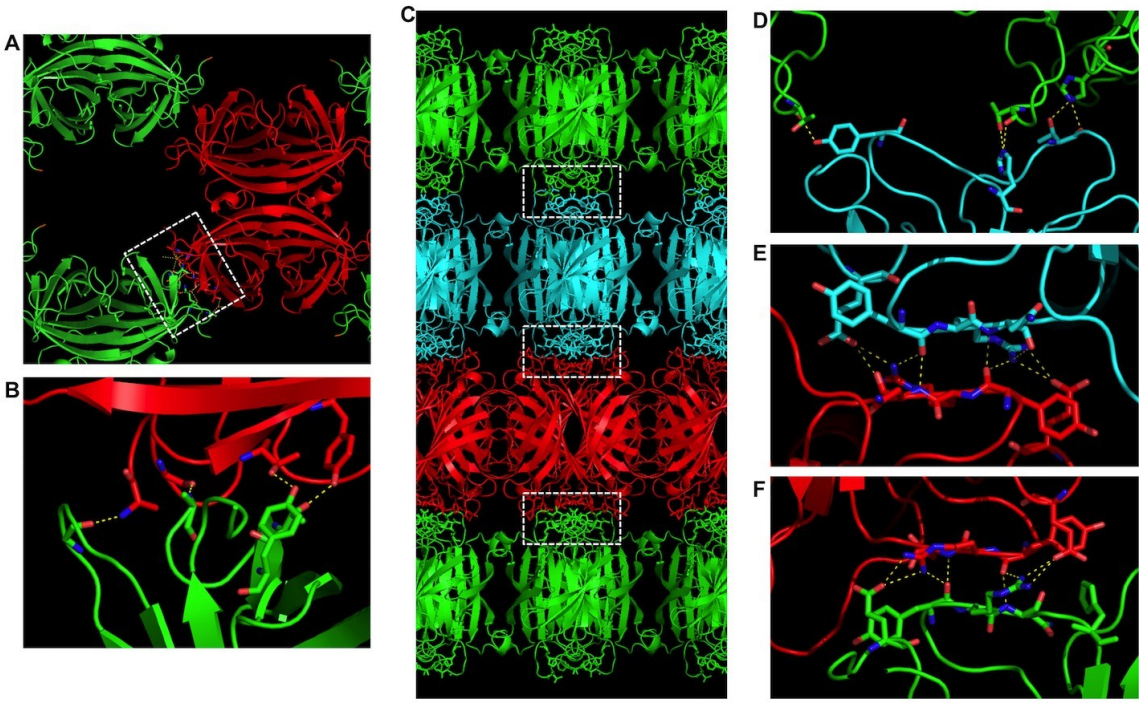

Fig. S4 Interactions other than electrostatic interactions between SA molecules in crystals. (**A**) Interface of neighbouring SA molecules in the SA layer. (**B**) Hydrogen bond formation between SA molecules highlighted in fig. S3A where A4, T9, Y11, and G88 are involved. (**C**) Interface between SA molecules in stacked SA layers. (**D**), (**E**), and (**F**) Hydrogen bond formation between SA molecules highlighted in fig. S3C. Interactions between SA molecules coloured red and blue (fig. S3E), and green and red (fig. S3F) are identical to each other and E40, Y72, R73, and N74 are involved. Interactions between SA molecules coloured blue and green (fig. S3D) are found at T55, Y72, and H76.

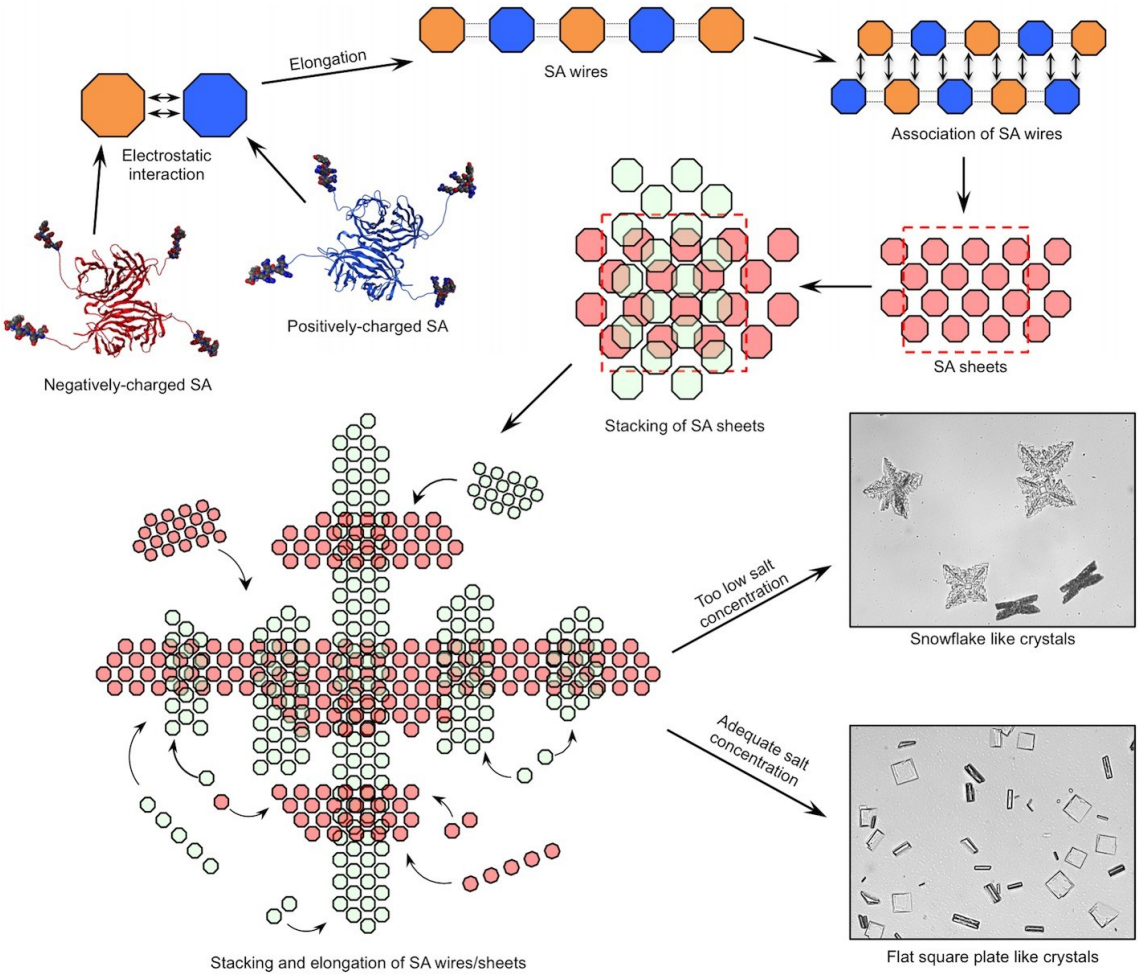

Fig. S5 Expected process of electrostatic interaction-mediated crystallization of SAs. Positively- and negatively-charged SAs, which are illustrated as octagons in blue and orange colors, respectively, associate to form 1D structure, SA wires. As the SA wires extend, they associate each other to form 2D structure of SA sheets. The SA sheets then stack with rotation angles of 90° to form the 3D structure of the SA crystal. The snowflake-like crystals and flat square plate like crystals were obtained using SA-R_4Y and SA-D_4Y (3 μM each) in 10 mM Tris-HCl (pH8.0) containing 0 mM NaCl and 7.5 mM NaCl, respectively.

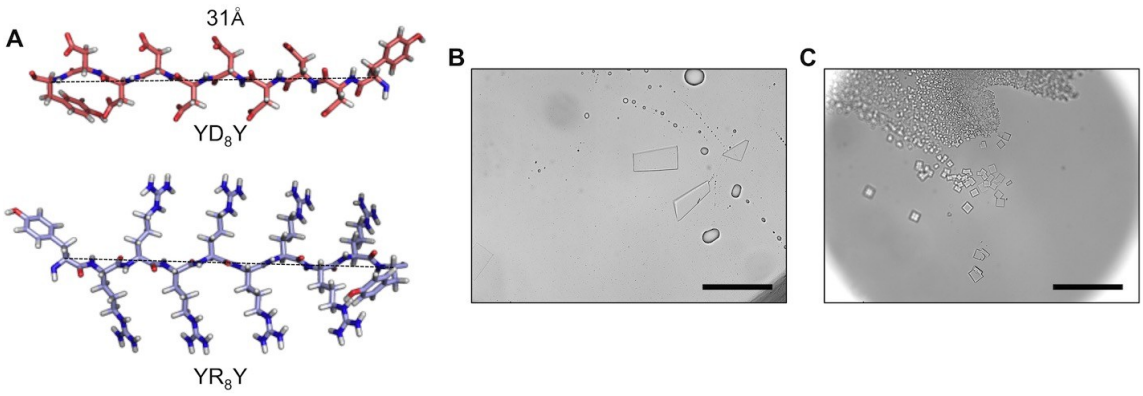

Fig. S6 Crystallization of SAs using charged oligopeptides. (**A**) Modelling images of YD_8Y and YR_8Y peptides. (**B**) Microscopy image of crystals of SA-R_4Y/YD_8Y. (**C**) Microscopy image of crystals of SA-D_4Y/YR_8Y. Scale bars in fig. S5B and S5C are 200 μm.

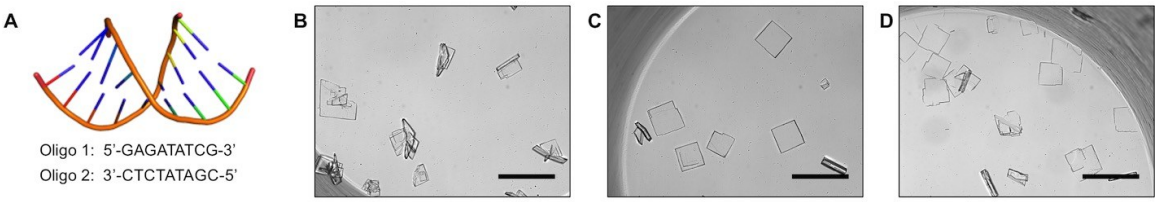

Fig. S7. Crystallization of SAs by addition of oligo-DNA. (**A**) Oligo-DNA sequences used to crystallize SA-R_6Y. (**B**) Crystals of SA-R_6Y with oligo 1. (**C**) Crystals of SA-R_6Y with oligo 2. (**D**) Crystals of SA-R_6Y with oligo 1 and 2. Scale bars are 200 μm.

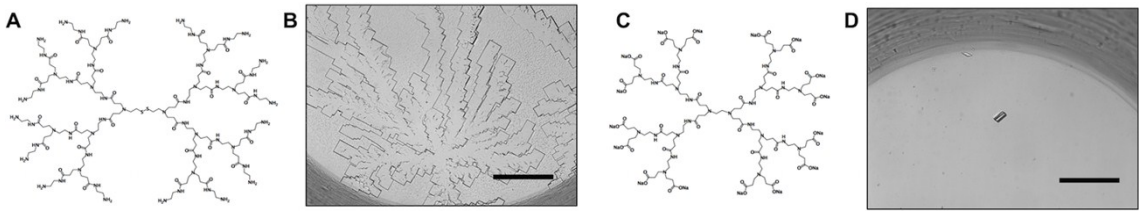

Fig. S8. Crystallization of SAs by addition of dendrimers. (**A**) Structure of PAMAM dendrimer, cystamine core, 2.0 generation (P-Dendrimer). (**B**) Crystals of SA-D_6Y with P-Dendrimer. (**C**) Structure of PAMAM dendrimer, ethylenediamine core, generation 1.5 (N-Dendrimer). (**D**) Crystals of SA-R_6Y with N-Dendrimer.

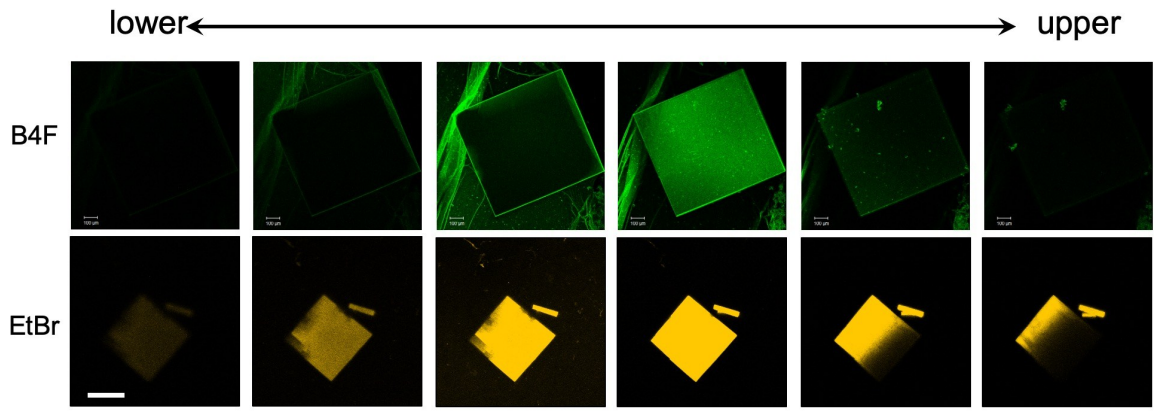

Fig. S9. Cross-sectional images of SA crystals modified with B4F and EtBr. Scale bars:100 μm and 50 μm for crystals modified with B4F and EtBr, respectively.

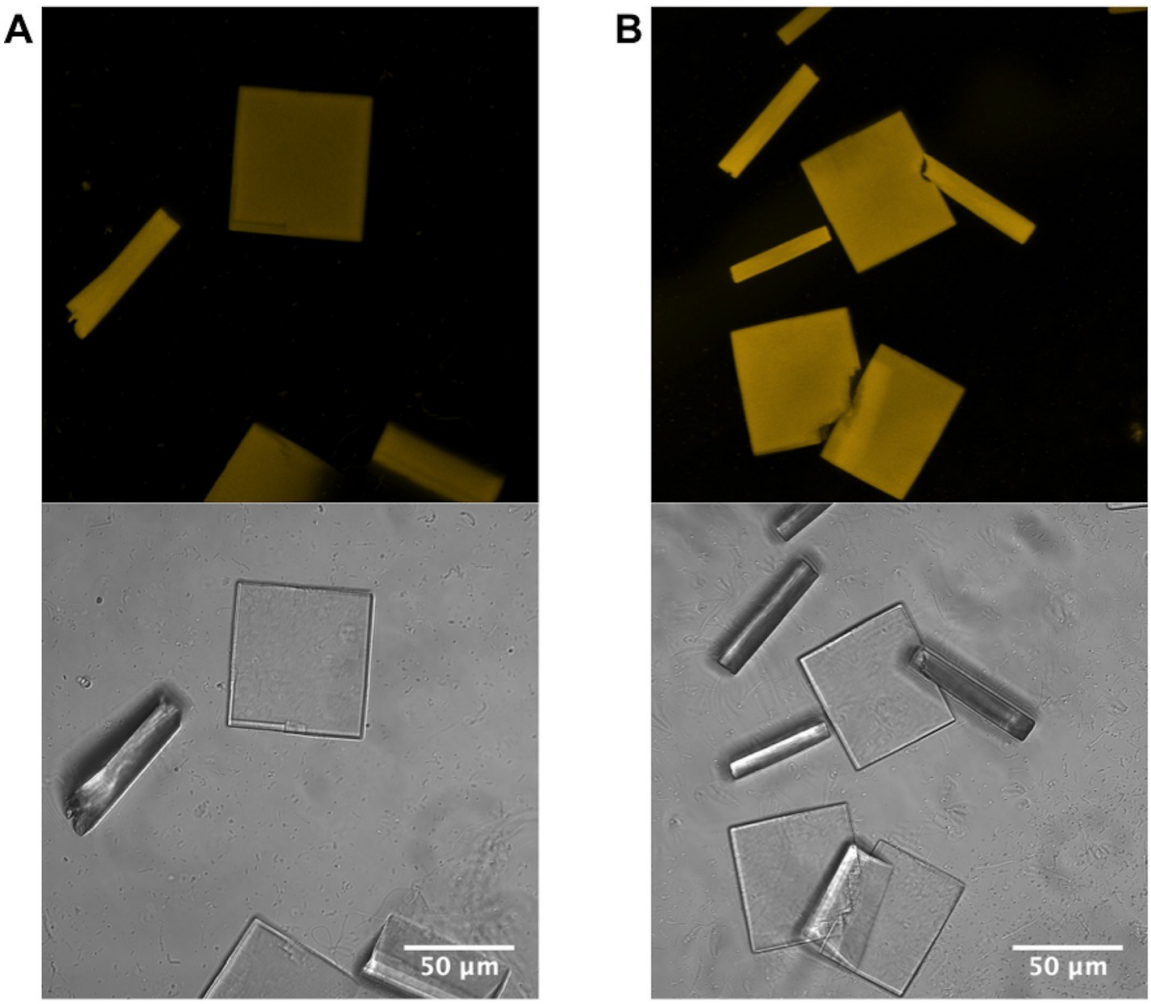

Fig. S10. CLSM observation of SA crystals stained with EtBr. (**A**) SA crystals with EtBr without addition of biotin. (**B**) SA crystals with EtBr with 50 µM biotin in the solution. The crystals were excited with a 543-nm laser.

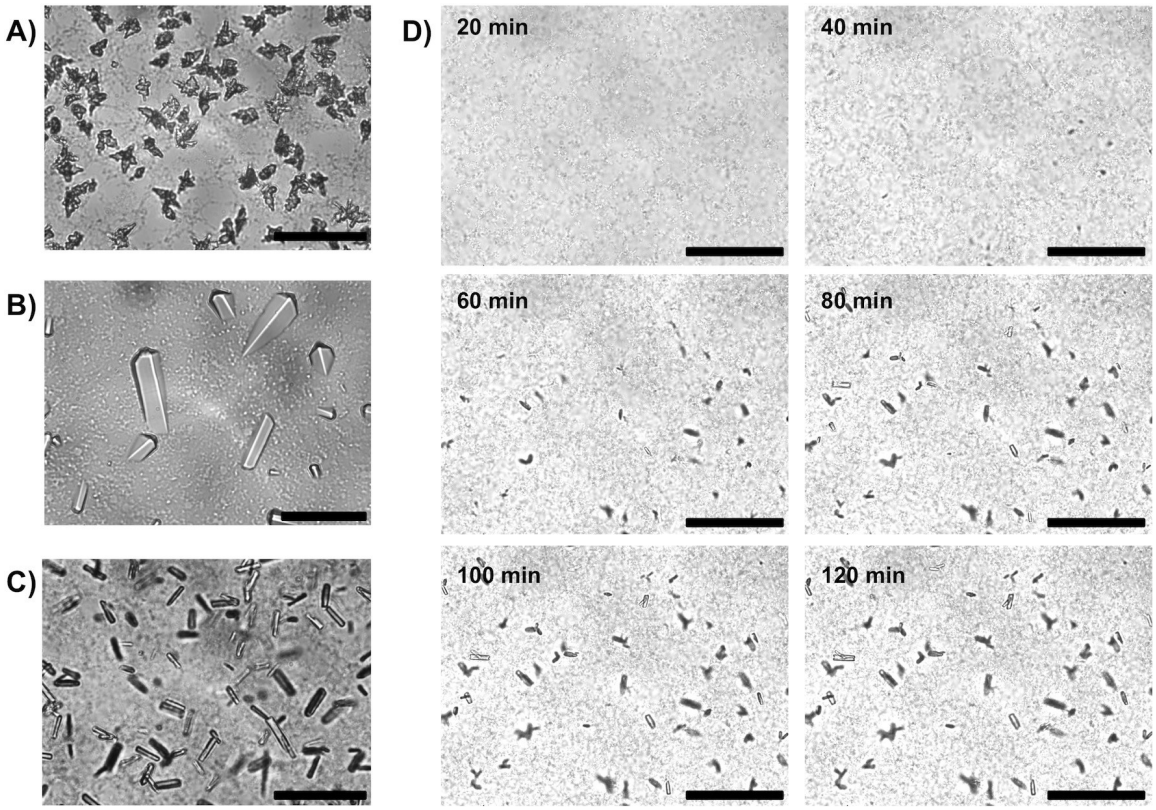

Fig. S11. Microscopy image of crystals of D2 symmetry proteins. A) Co-crystals of D_6-SciDH (Scyllo-inositol dehydrogenase from *Lactobacillus Casei* possessing D_6 sequence at its N-terminus) (2 μM) and YR_8Y oligo peptide (16 μM) in 10 mM Tris-HCl (pH 8.0) containing 60 mM GuHCl; B) Co-crystals of D_6-MazG (MazG protein, nuicletide pyrophosphohydrolase from *Bacillus cereus* possessing D_6 sequence at its N-terminus) (10 μM) and YR_8Y oligo peptide (40 μM) in 10 mM Tris-HCl (pH 8.0) containing 80 mM NaCl; C) Co-crystals of AMPP-D_4 (aminopeptidase P from *E. Coli* possessing D_4 sequence at its C-terminus) (2 μM) and YR_8Y oligo peptide (16 μM) in 50 mM PIPES (pH 6.0) ;D)Time-lapse analysis of crystallization of AMPP-D_4 with YR_8Y peptide. Scale bas are 50 μm.

Table S1 Data collection and refinement statistics (**Molecular replacement**)

	SA-R_6Y/SAD_6Y complex
Data collection	
Space group	*C*2
Cell dimensions	
a, *b*, *c* (Å)	81.3, 80.8, 132.7
α, β, γ (°)	90.0, 99.8, 90.0
Resolution (Å)	37.1-1.80(1.85-1.80)*
R_{merge}	0.069(0.418)
$I/\sigma I$	14.1(3.0)
Completeness (%)	99.6(97.4)
Redundancy	3.67(3.51)
Refinement	
Resolution (Å)	1.80
No. reflections	78143
$R_{work/}$ R_{free}	18.1/21.9
No. atoms	
Protein	5430**
Ligand/ion	36
Water	795
B-factors	
Protein	17.4
Ligand/ion	18.6
Water	29.8
R.m.s deviations	
Bond lengths (Å)	0.007
Bond angles (°)	1.109

One crystal was used for the structural analysis.
*Highest resolution shell is shown in parenthesis.
**Six molecules of SA are included in asymmetric unit of the crystal lattice.

References

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