Self-assembly of coherently dynamic, auxetic, two-dimensional protein crystals

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Two-dimensional (2D) crystalline materials possess unique structural, mechanical and electronic properties1,2 that make them highly attractive in many applications3–5. Although there have been advances in preparing 2D materials that consist of one or a few atomic or molecular layers6,7, bottom-up assembly of 2D crystalline materials remains a challenge and an active area of development8–10. More challenging is the design of dynamic 2D lattices that can undergo large-scale motions without loss of crystallinity. Dynamic behaviour in porous three-dimensional (3D) crystalline solids has been exploited for stimuli-responsive functions and adaptive behaviour11–13. As in such 3D materials, integrating flexibility and adaptiveness into crystalline 2D lattices would greatly broaden the functional scope of 2D materials. Here we report the self-assembly of unsupported, 2D protein lattices with precise spatial arrangements and patterns using a readily accessible design strategy. Three single- or double-point mutants of the C4-symmetric protein RhuA were designed to assemble via different modes of intermolecular interactions (single-disulfide, double-disulfide and metal-coordination) into crystalline 2D arrays. Owing to the flexibility of the single-disulfide interactions, the lattices of one of the variants (C98RhuA) are essentially defect-free and undergo substantial, but fully correlated, changes in molecular arrangement, yielding coherently dynamic 2D molecular lattices. C98RhuA lattices display a Poisson’s ratio of —1—the lowest thermodynamically possible value for an isotropic material—making them auxetic.

Proteins are attractive building blocks for 2D materials because of their structural and chemical diversity, and inherent functions. Examples for natural, protein-based 2D materials include bacterial S-layer proteins and purple-membrane assemblies, which form crystalline arrays in association with cell walls and membranes, respectively, and have been used in diverse technological applications14,15. On the synthetic front, methods for 2D protein crystallization have been developed for the structural characterization of membrane proteins16,17 or functional applications18, and generally rely on lipid layers as supports. Recently, 2D or 3D supramolecular protein arrays have been designed through the symmetric polymerization of protein building blocks via computationally designed protein–protein interactions or fusion of protein components19–22. However, these approaches have engineering-intensive and highly dependent on the accuracy of the design, and the integration of dynamic or adaptive behaviour has yet to be explored.

To address these issues, we used a simple chemical-bonding strategy to control protein self-assembly. We reasoned that both cysteine (Cys)-mediated disulfide bonds and metal-coordination interactions between protein building blocks could produce crystalline and dynamic arrays with minimal design, because these bonds are: (1) strong but reversible (to minimize the surface area to be designed and ensure self-healing); (2) short, yet sufficiently flexible (to simultaneously afford crystallinity and adaptiveness); (3) chemically tunable (to exert external control over self-assembly and enable stimuli-responsiveness); and (4) easily designed and engineered.

The most straightforward route to obtaining 2D lattices is the tessellation of C2-, C4- or C6-symmetric building blocks through appropriately positioned C2-symmetric linkages such as disulfide bonds or many metal-coordination interactions. As a model building block, we chose L-rhamnulose-1-phosphate aldolase (RhuA), a C4-symmetric homotetramer (dimensions, 7 nm × 7 nm × 5 nm) that was previously used as a synthon for supramolecular assemblies23 (Fig. 1a). An inspection of RhuA indicated protrusions in the four corner positions as ideal locations to incorporate one Cys or two histidine (His) residues for disulfide- or metal-directed self-assembly, respectively. We generated two variants, C98RhuA and H63/H98RhuA, with four conditionally self-associating corners for forming square lattices (Fig. 1a, b, top and middle rows). The relative positions of residues 63 and 98 were deemed to be conducive to forming a bis-His metal-coordination motif to afford a tetrahedral or square planar coordination geometry for metal-mediated RhuA pairing interactions. Additionally, we noted that RhuA could be converted into a stable D4-symmetric octamer via a single point mutation (A88F)24. Thus, we prepared a third variant, F88/C98RhuA, which presents eight symmetry-related cysteines at roughly 45° angles in a 2D projection (Fig. 1a, bottom row). We envisioned that the C4-symmetric C98RhuA and H63/H98RhuA variants could yield square lattices with two distinct patterns in terms of the orientation of the building blocks with respect to the 2D plane (Fig. 1b). On the other hand, the D4 symmetry of F88/C98RhuA would dictate a 2D lattice with equivalent faces (Fig. 1b). These RhuA building blocks would also provide three distinct modes of inter-building-block interactions, whose effects on self-assembly we hoped to investigate: single-disulfide, double-disulfide and bis-His-anchored metal-coordination.

For the oxidative self-assembly of C98RhuA and F88/C98RhuA, we tested various strategies including air oxidation, redox buffer systems containing reduced and oxidized glutathione (GSH and GSSG) or low concentrations (≤10 mM) of the reductant β-mercaptoethanol (βME), which slowly decomposes in aqueous solution and results in gradually more oxidizing conditions. In these experiments, solutions of purified C98RhuA and F88/C98RhuA were first rapidly exchanged via repeated centrifugal filtration into solutions that varied in terms of their pH (6–8.5), buffering species (sodium phosphate, CHES, MES, MOPS, Tris; at 5–20 mM), the compositions of the redox buffer systems (1:19 or 19:1 GSH:GSSG; at 1 mM total concentration) or the concentrations of βME (0–10 mM). After adjustment to the desired final protein concentration (25–175 μM), these solutions were monitored for the formation of self-assembled structures by visual inspection for emergence of cloudiness and by transmission electron microscopy (TEM). Likewise, for metal-directed assembly, purified H63/H98RhuA was exchanged into solutions with varying pH and buffer ions (to modulate metal binding kinetics and thermodynamics), with the exception that these solutions did not contain any reductants.

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Self-assembly was initiated by addition of 4–40 molar equivalents of Zn$^{2+}$ and Cu$^{2+}$ (1–10 equiv. per bis-His motif), both of which can accommodate the desired four-coordinate geometries to link H63/H98RhuA building blocks at their corners.

We found that the assembly of all variants into 2D arrays was robust and occurred under a wide range of conditions. As expected, crystalline self-assembly was favoured by conditions that promoted slow, controlled oxidation or slow metal-binding kinetics. For example, uncontrolled air oxidation of C98RhuA solutions or addition of large molar excess of Zn$^{2+}$ and Cu$^{2+}$ to H63/H98RhuA samples resulted largely in amorphous aggregates in addition to some crystalline domains (Extended Data Fig. 1). The following solution conditions yielded 2D assemblies that were optimized in terms of size, crystallinity and yield: C98RhuA and F88/C98RhuA (≥125 μM protein, 10 mM βME, pH 7.5, 10 mM Tris); H63/H98RhuA (25 μM protein, 200 μM ZnCl₂, pH 7, 20 mM MOPS). Under these conditions, C98RhuA reproducibly assembled into straight-edged, single- or few-layered 2D crystals that grew to several micrometres in size over several days (Fig. 2a). Negative-stain TEM, cryo-TEM, scanning electron microscopy (SEM) and atomic force microscopy (AFM) measurements revealed that the C98RhuA crystals were highly ordered and possessed uniform square or rectangular shapes with molecularly sharp boundaries (Fig. 2a, Extended Data Figs 2 and 3); these features have not been previously observed in designed 2D protein crystals 19,21. 2D crystals of H63/H98RhuA (Fig. 2b, Extended Data Fig. 4) and F88/C98RhuA (Fig. 2c, Extended Data Fig. 5) were several hundred nanometres to one micrometre in size, but typically displayed irregular morphologies. H63/H98RhuA lattices were monocry stalline, but tended to grow in three dimensions over time, whereas F88/C98RhuA crystals consisted of polycrystalline domains.

As monitored by TEM and dynamic light scattering (DLS), self-assembly of all variants was reversible upon addition of high concentrations of either βME (>10 mM) in the case of C98RhuA and relative molecular orientations in b. Cys and His residues inserted into positions 98 and 63 are shown in red and cyan, respectively. b, Expected 2D molecular arrangements of C98RhuA, H63/H98RhuA and F88/C98RhuA lattices. M$^{2+}$ refers to Zn$^{2+}$ or Cu$^{2+}$ ions.

**Figure 1** | **RhuA constructs and their disulfide- and metal-mediated self-assembly modes.** a. Schematic representations of the C98RhuA, H63/H98RhuA and F88/C98RhuA structures. The top and bottom halves of RhuA are coloured orange and blue, respectively, to highlight the Cys and His residues inserted into positions 98 and 63 are shown in red and cyan, respectively. b, Expected 2D molecular arrangements of C98RhuA, H63/H98RhuA and F88/C98RhuA lattices. M$^{2+}$ refers to Zn$^{2+}$ or Cu$^{2+}$ ions.
alteration of a single protein building block (Extended Data Fig. 9b). C98RhuA and H63/H98RhuA molecules self-assemble in distinct orientations in the 2D plane, suggesting that the energetic bias stemming from disulfide or metal bond configurations and surrounding protein–protein interactions must be sufficiently large to favour one orientation over others to yield long-range order.

Superior quality and larger sizes of C98RhuA crystals compared to F88/C98RhuA and H63/H98RhuA lattices are readily explained by interactions that direct the self-assembly of the variants. F88/C98RhuA presents a roughly circular distribution of eight cysteines (Fig. 1a, bottom row). This arrangement renders the desired double-disulfide-mediated, side-to-side self-assembly mode non-unique, and permits alternate attachment geometries between F88/C98RhuA molecules (Extended Data Fig. 5). Moreover, the increased Cys-valency of F88/C98RhuA leads to considerably stronger interactions and therefore ‘stiffer’ lattices: the dissolution of F88/C98RhuA polycrystals requires substantially higher amounts of βME and longer incubation periods compared to C98RhuA lattices (Extended Data Fig. 6). Consequently, F88/C98RhuA lattices display vacancies, as well as both high- (>30°) and low-angle (<10°) grain boundaries (Extended Data Fig. 5). H63/H98RhuA crystals possess fewer such defects, owing to the reversibility of Zn2+ coordination interactions. Yet, each H63/H98RhuA building block also contains numerous surface residues that can weakly coordinate Zn2+ ions, promoting crystal growth in the third dimension (Extended Data Fig. 4a).

In contrast to F88/C98RhuA and H63/H98RhuA, self-assembly of C98RhuA is both chemically and orientationally specific. Moreover, the reversibility and inherent flexibility of single Cys–Cys linkages—containing five rotatable bonds—probably allows for the correction of any defects such as vacancies and grain boundaries, which would be difficult to accomplish in a rigid lattice composed of strongly interacting building blocks such as F88/C98RhuA. Indeed, as TEM images of mono-layered C98RhuA crystals illustrate, the outcomes are (a) macroscopic crystal morphologies that reflect the molecular symmetry of the building blocks, (b) molecularly sharp crystal boundaries, and (c) lattices with extremely low defect frequencies. In the hundreds of monocryalline C98RhuA lattices with surface areas of >1 μm² that we examined closely, we rarely found a lattice defect, despite the fact that these crystals grow in 3D space in an unsupported fashion. Even in a rare instance such as that shown in Extended Data Fig. 9d, the defect frequency was one missing C98RhuA molecule within a lattice grid of about 9,000 molecules (about 0.6 μm²).

A more striking consequence of the disulfide bond flexibility is the coherent dynamic behaviour of C98RhuA crystals. Although our initial sample preparations of C98RhuA crystals yielded predominantly open lattices with large pores (Fig. 2a), we noticed that these crystal suspensions developed a dense sediment over a period of 1–3 days at 4 °C (Extended Data Fig. 10a). TEM analysis of these sedimented crystals indicated a close-packed lattice arrangement (Fig. 3a, b, right panels). Upon resuspension of the sedimented crystals by repeated gentle mixing with a pipette and subsequent TEM imaging of the resulting samples, we captured a total of at least seven types of 2D C98RhuA crystals in distinct conformational states (I–VII) (Fig. 3a, Extended Data Fig. 10b). These conformational states were categorized by computational image analysis according to the roundness indices of the lattice pores, ranging from ≥0.85 for state I to ≤0.35 for state VII (see Methods). As evidenced by the retention of p422 symmetry and nearly equal unit-cell dimensions, these seven conformational states are clearly interconnected and implicate a continuous lattice motion between fully open and fully closed states (Supplementary Video 1). These large amplitude motions of the C98RhuA lattices are afforded by a remarkable extent of hinging about the flexible disulfide linkages and their placement at corner locations of RhuA molecules. The transition from the open to the closed state is accompanied by the compression of the inter-C98RhuA hinge angle (α) from >80° to 17°, a decrease of the pore size from approximately 4.4 nm to 1.0 nm (for the passage of a spherical object) and an increase in the relative protein/hole surface density of 170% (Fig. 3b, Table 1).

Conformational dynamics of C98RhuA crystals are fully coherent: in each crystal examined, only one type of conformational state (I–VII) was observed throughout the lattice (Fig. 3b). This observation implies that any mechanical deformation of a C98RhuA crystal is cooperatively propagated along the 2D plane, enabled by both the flexibility and short linker-length of single-disulfide linkages: longer, more flexible linkages would preclude coherent dynamics, whereas inflexible linkages would lead to non-adaptive lattices. Indeed, F88/C98RhuA and H63/H98RhuA lattices do not display any apparent dynamic behaviour...
number for clarity). a, Reconstructed 2D images of seven distinct conformational states (I–VII) of 2D C98RhuA crystals. b, High-magnification views and derived structural models of conformations II, V and VII. Unit cells and hinge angles (\(\alpha\)) between C98RhuA units are highlighted in black and red, respectively (orange and blue colouring as in Fig. 1). c, Population distributions of C98RhuA crystals in different conformational states during repeated resuspension/sedimentation cycles. \(n\) refers to the total number of individual lattices analysed in each panel. d, Schematic representation of the rotating, rigid-square model that describes the 2D C98RhuA lattice dynamics (colouring as in Fig. 1; the building blocks are numbered for clarity). \(\Delta x\) and \(\Delta y\) denote changes in the transverse- and longitudinal-dimension lengths upon lattice opening and closing. e, Digital image-correlation analysis of reconstructed TEM images of C98RhuA crystals for determining auxetic behaviour. Representative volume elements (RVEs) in the lattices of the two extreme states (I and VII) are indicated with red squares, with the vertices of the RVEs numbered 1–4, and the vectors \(\mathbf{m}\), \(\mathbf{n}\) (in state I) and \(\mathbf{m}, \mathbf{n}\) (in state VIII) used to calculate local engineering strains are shown with cyan arrows. The edges of the lattice pores, used for determining the positions of the RVE vertices, are shown as purple lines. f, Calculated Poisson’s ratios (\(\nu\)) of lattice conformational states with respect to state I. The error bars correspond to the uncertainties in pixel selection during digital image processing (see Methods for details).

Table 1 | Structural parameters of C98RhuA crystals in states II, V and VII

| State | Structural Parameter | State II | State V | State VII |
|-------|----------------------|---------|---------|----------|
| Cell (a = b, c) | \(114.4\,\text{Å}, 90^\circ\) | \(110.0\,\text{Å}, 90^\circ\) | \(107.8\,\text{Å}, 90^\circ\) |
| Plane group symmetry | \(p4_2\overline{2}\) | \(p4_2\overline{2}\) | \(p4_2\overline{2}\) |
| Intertetramer hinge angle, \(\alpha\) | \(79^\circ\) | \(49^\circ\) | \(17^\circ\) |
| Protein surface area per unit cell, \(A_{\text{prot}}\) | \(82.7\,\text{nm}^2\) | \(86.2\,\text{nm}^2\) | \(88.0\,\text{nm}^2\) |
| Pore surface area per unit cell, \(A_{\text{pore}}\) | \(44.7\,\text{nm}^2\) | \(34.7\,\text{nm}^2\) | \(28.2\,\text{nm}^2\) |
| Relative protein/pore density, \(A_{\text{prot}}/A_{\text{pore}}\) | 1.9 | 2.5 | 3.1 |

because their double-disulphide- or metal-mediated modes of assembly do not allow rotation of the neighbouring protein molecules with respect to one another.

To establish that the conformational dynamics of C98RhuA crystals are reversible, we subjected them to repeated sedimentation/resuspension cycles within the solutions in which they self-assembled at 4 °C. In each cycle, we obtained TEM images of > 100 individual crystals from the same container, collected from either the sediments that formed overnight or the suspensions immediately after mixing the sediments by repeated pipetting. As shown in Fig. 3c, the conversion between the closed states (VI and VII) and open or intermediate states (I–V) is completely reversible and absolute. Sedimented samples do not contain any open or intermediate states and resuspended samples do not contain any closed states, providing unambiguous evidence that C98RhuA lattices are reversibly dynamic. On the basis of the observed distribution profiles, the opening of the lattices by mechanical agitation and their distribution among conformational states I–V appear to be immediate (at least within the ~5-min timescale of TEM sample preparation), whereas their full closure takes several hours. We posit that the energetic barriers between different C98RhuA lattice conformations (due to different disulphide bond configurations and long-range protein–protein interactions) must be small enough to be overcome by mechanical agitation and internal protein dynamics. The fully closed conformation (state VII) appears to be a kinetically stable conformation that accumulates over time in unagitated solutions at 4 °C. This kinetic stability may be ascribed to the dense protein packing interactions in state VII and the resulting restriction of the dynamics of both the lattice and the individual building blocks.

Geometric considerations based on the retention of \(p4_2\overline{2}\) symmetry (Fig. 3b, d) as well as a strain analysis of the seven conformational states by digital image correlation (Fig. 3e, Extended Data Fig. 10c) indicate that C98RhuA crystals are auxetic and have a Poisson’s ratio of \(\nu = -1.00 \pm 0.01\) (Fig. 3f). Poisson’s ratio is a scale-independent metric that describes the response of a material to strain; it is defined as the ratio between transverse (\(\epsilon_x\)) and longitudinal (\(\epsilon_y\)) strains under uniaxial loading\(^{25}\). The strains \(\epsilon_x\) and \(\epsilon_y\) are, in turn, approximated by changes in material length in the transverse (\(\Delta x\)) and longitudinal (\(\Delta y\)) directions (Fig. 3d)\(^{25}\):

\[
\nu = -\frac{\Delta y}{\Delta x} \approx \frac{\epsilon_y}{\epsilon_x}
\]

where \(-1 \leq \nu \leq 0.5\) for an isotropic 3D material and \(-1 \leq \nu \leq 1\) for an isotropic 2D material\(^{26}\). Most materials have positive \(\nu\); that is, they become thinner in the longitudinal direction when stretched transversely\(^{25}\). By contrast, materials with negative \(\nu\) (that is, auxetic materials) display the counterintuitive behaviour of longitudinal expansion upon transverse stretching. Thus, auxetic materials can be expected to possess enhanced toughness, resistance to indentation and shear stiffness as well as favourable damping and acoustic response, and have been proposed for use in protective armours, smart textiles, actuated filtration, and piezoelectric and biomedical devices\(^{25,27–29}\). Although materials with negative \(\nu\) exist, most fall in the range of \(-0.4 < \nu < 0\), with the lowest reported values of \(-0.7\)
to −0.8 observed in re-entrant foams. It has been postulated that for a 2D lattice of rotating rigid squares with flexible hinges, should be equal to the lowest thermodynamically permissible value of −1 at all rotation angles, RHuA lattices represent a true realization of this theoretical model, and the first isotropic material with = 1 designed and constructed at the molecular scale. Assuming the model of ref. 26, we calculate that RHuA crystals should shrink or expand simultaneously in the x and y dimensions by at least 24% during the conversion between fully open (I) and fully closed (VII) states (Extended Data Fig. 10d).

We envision that upon hierarchical assembly via physical methods or incorporation into polymeric materials using chemical strategies, molecular architectures such as 2D RHuA lattices may be used as feedstocks for adaptive and auxetic macroscopic materials. In general, our study underscores the utility of dynamic covalent bonds in the construction of highly ordered, yet adaptive, protein materials. Specifically, owing to their high structural quality and chemically tunable assembly under ambient conditions, RHuA crystals provide a unique medium for studying molecular self-assembly and crystallization as well as for investigating the energy landscape of lattice dynamics. The resultant understanding of structural dynamics at the nanoscale should greatly aid the fabrication of functional materials.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Methods

Design of RhuA variants and site-directed mutagenesis. RhuA variants were designed as described in the main text on the basis of previously reported crystal structures (Protein Data Bank (PDB) 1GT7 for C98RhuA, H63/H98RhuA, D98RhuA, C133RhuA and 2C66RhuA and PDB 2UYU for F88/C98RhuA). All RhuA variants also contain the mutations E192A and C126S as previously reported.

The gene for C98RhuA, pre-inserted into the pJ414 expression vector optimized for expression in E. coli, was purchased from DNA2.0. F88/C98RhuA, H63/H98RhuA, D98RhuA and RhuA were prepared using QuikChange mutagenesis (Stratagene) with primers obtained from Integrated DNA Technologies (Supplementary Table 1). The mutant plasmids were transformed into XL-1 Blue E. coli cells followed by purification using the QIAprep Spin Miniprep kit (Qiagen). The presence of the mutations was verified by sequencing (Retrogen). Amino acid sequences of RhuA variants are shown in Supplementary Table 2.

Protein expression and purification. Bacterial expression of RhuA variants was performed according to previously published procedures with slight modifications.

Plasmids bearing the variants were transformed into BL21 (DE3) E. coli cells, and the colonies were grown overnight at 37 °C on lysogeny broth (LB) agar plates (pH 7.4) containing 100 mg/l ampicillin. Starter cultures (5 ml with 100 mg/l ampicillin) from single colonies were grown for about 4–6 h at 37 °C (with shaking at 250 rpm) before inoculation into 1-1 L cultures containing 100 mg/l ampicillin. After the cells were grown to an optical density of about 0.8 at 600 nm, protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Gold Biotechnologe) for 12–13 h (at 37 °C with shaking at 250 rpm). Cells were pelleted by centrifugation (5000 r.p.m. for 10 min), and resuspended in a buffer solution containing 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris; pH 7.5), 1 mM ZnCl2 and 10 mM β-mercaptoethanol (β-ME). The cell lysates from RhuA preparations, ZnCl2 was excluded from buffer solutions and β-ME was added to avoid possible protein precipitation during purification. Cell lysis was performed by sonication for 15 min on ice. The lysis solution was centrifuged for 30 min at 12,000 r.p.m. at 4 °C. Polymin-P (Acros) was added to the supernatant at a final concentration of 0.1% (w/v) for nucleic acid precipitation and the resulting mixture was stirred for 30 min before centrifugation at 12,000 r.p.m. at 4 °C. The supernatant was loaded onto a DEAE-Sepharose CL-6B (GE Healthcare) column and eluted using a gradient of 0–300 mM NaCl in Tris buffer at 4 °C. Fractions containing RhuA, which eluted at approximately 200 mM NaCl, were added to 1.7 M ammonium sulphate, and were centrifuged for 45 min at 12,000 r.p.m. at 4 °C. The precipitate was dissolved in a buffer solution containing 5 mM sodium phosphate (NaPi; pH 7.2), 1 mM ZnCl2 and 10 mM β-ME, and then dialysed three times against 51 of the same buffer solution. Further purification was done using either a High Q cartridge column (BioRad; at pH 8.0) using a 0–500 mM NaCl gradient or a Mini CHT Type 1 hydroxyapatite column (BioRad; at pH 7.2) using a 5–500 mM NaPi gradient on a DuoFlow fast protein chromatography workstation (BioRad). Pure protein fractions eluted at 350 mM NaCl and 200 mM NaPi, respectively. These fractions were combined, dialysed three times against a buffer solution of 10 mM Tris (pH 7.5), 1 mM ZnCl2, and 10 mM β-ME, and concentrated in an Amicon stirred cell at 4 °C. The protein was then flash frozen and kept at −80 °C until use. Protein purity was confirmed by SDS-PAGE. The growth of C98RhuA crystals could be accelerated by gentle shaking (Extended Data Fig. 2a, b). In additional experiments, we tested the thermal- and chemo-stability of C98RhuA crystals, as shown in Supplementary Fig. 3.

Electron microscopy. For negative-stain TEM sample preparation, 3–3.5-μl aliquots of crystal suspensions were applied onto negatively glow-discharged carbon-coated Cu grids (Ted Pella, Inc.), washed with Milli-Q water, and stained with 1% uranyl acetate at 4 °C. For cryo-EM sample preparation, 3–3.5-μl aliquots of 25 μM C98RhuA sample (diluted sample) were deposited onto negatively glow-discharged Quantifoil grids, and then plunged into liquid ethane after blotting. The sample was then stored under liquid nitrogen until analysis. Sample screening was performed in an FEI Sphera transmission electron microscope equipped with a LaB6 electron gun at 200 kV and imaged on a Gatan 2K2 CCD. A complete electron crystallographic analysis was done on 43, 50 and 33 micrographs with a scan rate of 0.3–0.5 Hz. The AFM images obtained were processed using NanoScope Analysis (Bruker).

AFM measurements. For AFM sample preparation, 2 μl of crystal suspensions were applied on freshly cleaved mica, washed with deionized water and dried. AFM images were collected using a Dimension Icon microscope (Bruker) using Scan Asyst peak force tapping (in air) mode at a resolution of 512 lines per image and a scan rate of 0.3–0.5 Hz. The AFM images obtained were processed using NanoScope Analysis (Bruker).

DLS measurements. DLS experiments were performed using a Wyatt DynaPro NanoStar instrument. The experiment runs were performed to collect 10 acquisitions with 657-nm excitation at a power setting of 100%. Measurements were plotted using a Rayleigh sphere model. Peak radius cut-offs were fixed according to default settings (0.5–10,000 nm).

Structural modelling and simulations. Projected electron-density maps were used as a reference in the visualization software UCSF Chimera to determine the orientation of the RhuA molecules in each crystal. The atomic coordinates were extracted from PDB entries 1GT7 (C98-RhuA) and 2C66 (F88/C98RhuA) and 2UYU (D98RhuA) using e2pdb2mrc.py and placed manually to fit the position of one subunit in the observed projected density map. The orientation of the molecule was refined in an iterative manner by comparing the experimental density with the density simulated from a model of the unit cell, obtained as explained above. Projected electron-density maps were computed using Boof (http://lbr.niams.nih.gov/boof) and EMAN2 (http://blake.bcm.edu/emanwiki/EMAN2). Starting from the estimated position and orientation of a single subunit, multiple copies of the model were generated to cover one unit cell (bmodeled in Boof) and converted to electron density (e2pdb2mrc.py in EMAN2) with a resolution limit of 30 Å. Finally, the density volume was projected along the vertical direction (bproject in Boof) to generate a 2D density map comparable to the experimental map.

Classification of the conformational states of 2D C98RhuA crystals. TEM micrographs of C98RhuA crystals were analysed computationally to categorize different conformations of the crystals. 2D density maps were computed using the crystallographic density after applying a 3 × 3 mean filter three times, and then converted to a binary image after automatic thresholding to segment out the pores. The mask was then filtered using the opening morphological operator to smooth the shape of the pores and to remove small outliers. Pores that were distorted from potential bending of the crystal in some regions of the field of view were discarded using their size for visual assessment. Micrographs were each processed separately using the 2dx software, which implements a semi-automatic processing pipeline that is mostly based on programs from the MRC suite. The program allows one to determine the 2D plane group lattice symmetry of each crystal, to calculate its unit-cell dimensions and to generate a projection density map. The processing involves estimation of the contrast transfer function (CTF) using CTFIND35, spot-list determination, automatic lattice determination, crystal masking, unbending and generation of a projection map after CTF correction. Within this program, the plane group symmetry is determined using ALLESPACE by comparing the internal phase residual for each symmetry to its expected value. Lattice unit-cell dimensions were determined on a subset of images, screened by the quality of the reflections—12, 14 and 13 for C98RhuA, H63/H98RhuA and F88/C98RhuA, respectively. The estimated resolution limit of each image (15–30 Å) was assessed by both visual inspection of its computed Fourier transform and comparison with simulated images filtered at different levels of resolution.

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to one of the seven identified states using the following criterion: state I, \( >0.85 \); state II, \( 0.75–0.85 \); state III, \( 0.65–0.75 \); state IV, \( 0.55–0.65 \); state V, \( 0.45–0.55 \); state VI, \( 0.35–0.45 \); state VII, \( <0.35 \).

Generation of video simulating the lattice motions. Supplementary Video 1 was generated to illustrate the dynamics of 2D RhuA crystals starting from the TEM snapshots of the seven conformational states (I–VII, Fig. 3a). All projected density images were rescaled to include the same field of view (hint and bing from Bsoft)). Twenty-four intermediate frames were created between each pair in the sequence by pseudo-morphing, using the program convert from the software suite ImageMagick. Finally, the video was generated with ffmpeg (http://www.ffmpeg.org) by combining the images as frames.

Digital image correlation for determining Poisson’s value. Using Matlab, we performed digital image processing of the reconstructed 2D TEM images of dynamic C98 RhuA crystals for evaluating Poisson’s ratios. As a first step, histogram normalization was performed, taking the image of conformational state I as a reference to normalize the intensity of the images (Extended Data Fig. 10c). This normalization guarantees that the thresholds used for pixel selection have the same meaning for all the images. After normalizing, we proceeded to select the representative volume element (RVE) in each of the images. The RVE considered has a rectangular section whose vertices correspond to the centroid of the lattice pores (shown as green rectangles in Extended Data Fig. 10c and red rectangles in Fig. 3e). To find the position of the centroids, we determined the edges of the pores using the Sobel edge-detection method. The edges of the pores are shown as purple lines in Extended Data Fig. 10c and Fig. 3e. Then, centroid calculation was performed by taking the mean of the coordinates in \( x \) and \( y \) for each pixel in the border. After the selection of the RVE, the size of the RhuA building blocks was measured on each image by defining the circle that is circumscribed to each square (shown as blue circles in Extended Data Fig. 10c). Because the square shape of the RhuA building blocks can be assumed to remain rigid in each conformational state, we determined that the images were at slightly different scales; thus, appropriate magnification factors were calculated to normalize the intensity of the images (Extended Data Fig. 10c). This normalization was performed, taking the image of conformational state I as a reference to one of the seven identified states using the following criterion: state I, \( >0.85 \); state II, \( 0.75–0.85 \); state III, \( 0.65–0.75 \); state IV, \( 0.55–0.65 \); state V, \( 0.45–0.55 \); state VI, \( 0.35–0.45 \); state VII, \( <0.35 \).

The deformed and undeformed configurations are related through the deformation gradient \( F \):

\[
\begin{bmatrix}
M_x \\
M_y \\
M_z
\end{bmatrix} = F \begin{bmatrix}
M_x' \\
M_y' \\
M_z'
\end{bmatrix} ; \\
\begin{bmatrix}
m_x \\
m_y \\
m_z
\end{bmatrix} = F \begin{bmatrix}
n_x \\
n_y \\
n_z
\end{bmatrix}
\]

where:

\[
F = \begin{bmatrix} 1 + e_x & e_{xy} & e_{xz} \\ e_{yx} & 1 + e_y & e_{yz} \\ e_{zx} & e_{zy} & 1 + e_z \end{bmatrix}
\]

Homogenized values of the engineering strains for the RVE are calculated as:

\[
\begin{bmatrix}
e_{xx} \\
e_{yy} \\
e_{zz}
\end{bmatrix} = \begin{bmatrix}
n_x & n_y & n_z \\ m_x & m_y & m_z \\ n_x & n_y & n_z
\end{bmatrix} \begin{bmatrix}
N_x \\
N_y \\
N_z
\end{bmatrix} M^{-1}
\]

Finally, Poisson’s ratio is calculated as:

\[
\nu = -\frac{e_y}{e_x}
\]

To determine the uncertainties in \( \nu \) (error bars in Fig. 3f), we calculated the local standard deviation given the distribution of pixel intensity of each Fourier transform image (using the command stdfilt in Matlab). From this local standard deviation, we obtained the uncertainty in the intensity of each pixel that forms the edges of the pores. These uncertainties were propagated in calculating the positions of RVE vertices and finally in calculating the Poisson’s ratios.

32. Kroemer, M. & Schulz, G. E. The structure of L-rhamnulose-1-phosphate aldolase (class II) solved by low-resolution SIRT phasing and 20-fold NCS averaging. Acta Crystallogr. D58, 824–832 (2002).
33. Edelhoch, H. Spectroscopic determination of tryptophan and tyrosine in proteins. Biochemistry 6, 1948–1954 (1967).
34. Crowther, R., Henderson, R. & Smith, J. MRC image processing programs. J. Struct. Biol. 116, 9–16 (1996).
35. Mindell, J. A. & Grigorieff, N. Accurate determination of local defocus and specimen tilt in electron microscopy. J. Struct. Biol. 142, 334–347 (2003).
36. Valpuesta, J. M., Carrascosa, J. L. & Henderson, R. Analysis of electron microscope images and electron diffraction patterns of thin crystals of O29 connectors in ice. J. Mol. Biol. 240, 281–287 (1994).
37. Petersen, E. F. et al. UCSF Chimera—a visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605–1612 (2004).
38. Heymann, J. B. Bsoft: image and molecular processing in electron microscopy. J. Struct. Biol. 133, 156–169 (2001).
39. Tang, G. et al. EMAN2: an extensible image processing suite for electron microscopy. J. Struct. Biol. 157, 38–46 (2007).
40. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. Nature Methods 9, 676–682 (2012).
41. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. Nature Methods 9, 671–675 (2012).
42. Henderson, R., Baldwin, J. M., Downing, K. H., Lepault, J. & Zemlin, F. Structure of purple membrane from halobacterium halobium: recording, measurement and evaluation of electron micrographs at 3.5Å resolution. Ultramicroscopy 19, 147–178 (1986).
Extended Data Figure 1 | TEM characterization of C98RhuA and H63/H98RhuA self-assembly under non-optimal conditions. a, 25μM C98RhuA was incubated at 4 °C under air exposure for 1 day; the solution contained 20 mM Tris (pH 8) and no reductants or oxidants. b, 25μM H63/H98RhuA was incubated in the presence of 1 mM ZnCl₂ at 4 °C for 1 day; the solution contained 20 mM MOPS (pH 7).
Extended Data Figure 2 | TEM characterization of optimized 2D C98 RhuA crystals. 

**a**. 125 μM C98RhuA was incubated in the presence of 10 mM βME at 4 °C in a standing solution for 3 days.

**b**. 125 μM C98RhuA was incubated in the presence of 10 mM βME at 4 °C with gentle shaking for 3 days, followed by 2 days at rest, during which a dense precipitate of crystals formed.
Extended Data Figure 3 | Additional structural characterization of 2D C98RhuA crystals. a, AFM; b, SEM; c, Cryo-TEM. The middle column in a shows the profiles along the arrows in the left column.
Extended Data Figure 4 | TEM characterization of 2D réSeArCHRhuA crystals. a, b. 25 μM H63/H98RhuA was incubated with 200 μM ZnCl₂ (a) or CuCl₂ (b) in a 20 mM MOPS buffer solution (pH 7.0) at 4 °C for 1 day.
Extended Data Figure 5 | TEM characterization of 2D F88/C98 RhuA crystals. 

**a**, 125 μM F88/C98 RhuA was incubated in the presence of 10 mM βME at 4 °C with gentle shaking for 2 days.  

**b**, Schematic representations of various types of defects observed in F88/C98 RhuA crystals, corresponding to boxed areas (I–IV) in **a** (colouring as in Fig. 1).
Extended Data Figure 6 | Reversibility of oxidative or metal-mediated self-assembly of RhuA variants. a, b, C98RhuA (a) and F88/C98RhuA (b) crystals. Left panels, the crystals were incubated in the presence of 20 mM or 30 mM βME (as indicated) and imaged by TEM at the indicated times after addition of βME. Right panels, 125 μM C98RhuA (a) or F88/C98RhuA (b) were incubated under oxidative self-assembly conditions (in the presence of 10 mM βME at 4 °C with gentle shaking) and self-assembly was monitored by DLS at t = 0 (top), t = 3 days (centre) and upon addition of 100 mM βME after self-assembly (bottom). D_h refers to the hydrodynamic diameter. c, Left panel, H63/H98RhuA crystals were incubated in the presence of 10 mM EDTA and imaged by TEM at 2 h after addition of EDTA. Right panels, 25 μM H63/H98RhuA was incubated under metal-mediated self-assembly conditions (in the presence of 200 μM ZnCl₂ at 4 °C) and self-assembly was monitored by DLS at t = 0 (top), t = 3 days (centre) and upon addition of 10 mM EDTA (bottom). Scale bars are 5 μm in all panels.
Extended Data Figure 7 | Characterization of the self-assembly of D98 RhuA, C133 RhuA and C266 RhuA variants by TEM and DLS. a, TEM (right panels) and DLS (left panels) characterization of D98 RhuA self-assembly under oxidative or metal-mediated self-assembly conditions that were optimized for C98 RhuA; see main text or Extended Data Figs 2 and 4 for details. b, Possible mode of disulfide-mediated self-assembly of C133 RhuA (top panels) and TEM characterization of the self-assembly products obtained under conditions that were optimized for C98 RhuA (bottom panels). c, Possible mode of disulfide-mediated self-assembly of C266 RhuA (top panels) and TEM characterization of the self-assembly products obtained under conditions that were optimized for C98 RhuA (bottom panels). No crystalline assemblies were detected under these conditions for any of these three variants (or other conditions that were used for screening C98 RhuA self-assembly). Colouring in the top panels of b and c as in Fig. 1.
Extended Data Figure 8 | Crystallographic analysis of the 2D lattices of RhuA variants. a, (i) Representative Fourier transforms calculated from the full field of view of crystal images, displayed up to 10 Å. Reciprocal lattice axes are indicated with H and K. The reflections are consistent with the plane group symmetries estimated from the analysis of the phase residuals (see Supplementary Table 4). (ii) Integer quality (IQ) plots calculated from the spectra in column (i). The size of the boxes around the reflections reflects their IQ value, defined as the ratio of the reflection-peak amplitude and the amplitude of the background signal around each peak. The most significant reflections are labelled with their IQ values, 1–4. Rings are displayed at resolutions of 30 Å, 15 Å and 10 Å. (iii) Overlap of fast Fourier transform and IQ plots. b, Crystallographic data for the 2D lattices of RhuA variants. Reported are the numbers of TEM images used for the analysis of plane group symmetry. The numbers in parentheses are the number of images used for determining the statistics for the unit-cell dimensions of each lattice. The plane group symmetry was determined by consensus from all the available images. c, Electron diffraction patterns of C98RhuA and H63/H98RhuA lattices.
Extended Data Figure 9 | 2D TEM reconstruction and structural modelling of the 2D lattices of RhuA variants. a, Comparison of the observed (experimental) and simulated projection maps of the 2D lattices of RhuA variants. b, Molecular arrangements in C98RhuA, H63/H98RhuA and F88/C98RhuA lattices viewed from different angles (colouring as in Fig. 1). c, Representative images of 2D C98RhuA crystals containing no defects. d, Representative images of 2D C98RhuA crystals containing a single lattice vacancy. The stain artefact images are shown to highlight areas that may appear to contain lattice vacancies in low-magnification views, but actually do not.
Extended Data Figure 10 | Conformational analysis of C98RhuA lattices. a. Photographs of sedimented and resuspended C98RhuA crystals (alternative views with different backgrounds are shown for clarity). b. Representative TEM images for different conformational states (I–VII) of the C98RhuA crystals shown in Fig. 3. Scale bars are 25 nm in all panels. c. Digital image processing of reconstructed images of each conformational state. Red lines represent the border of the pores in the lattice structure. Selected RVEs are shown with green lines, with the vertices of the RVEs numbered 1–4. Blue lines show the circles circumscribed to the square RhuA building block. Values of the scaling between these and the TEM images (with conformational state I as a reference) are shown below each image. d. Changes in the dimensions of a C98RhuA lattice of arbitrary size assuming a rotating-rigid-squares model.