Electronic Supplementary Material (ESI) for CrystEngComm.

Supplementary information

Loading Luminescent Lanthanide Complexes into Crosslinked Protein Crystal Matrices

Yu Zhang[a], Xiaoting Zhang[a], Jianguo Tang*[a], Christopher D. Snow*[bc], Guotao Sun[a], Ann E. Kowalski[b], Luke F. Hartje[c], Ning Zhao[c], Yao Wang[a], Laurence A. Belfiore[ab]

[a]Institute of Hybrid Materials, National Center of International Research for Hybrid Materials Technology, National Base of International Science & Technology Cooperation, College of Materials Science and Engineering, Qingdao University, Qingdao, 266071, P. R. China. Email: tang@qdu.edu.cn
[b]Department of Chemical and Biological Engineering, Colorado State University, Fort Collins, Colorado 80523 USA. Email: Christopher.Snow@colostate.edu
[c]Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, Colorado 80523 USA. Email: Christopher.Snow@colostate.edu

Figure S1

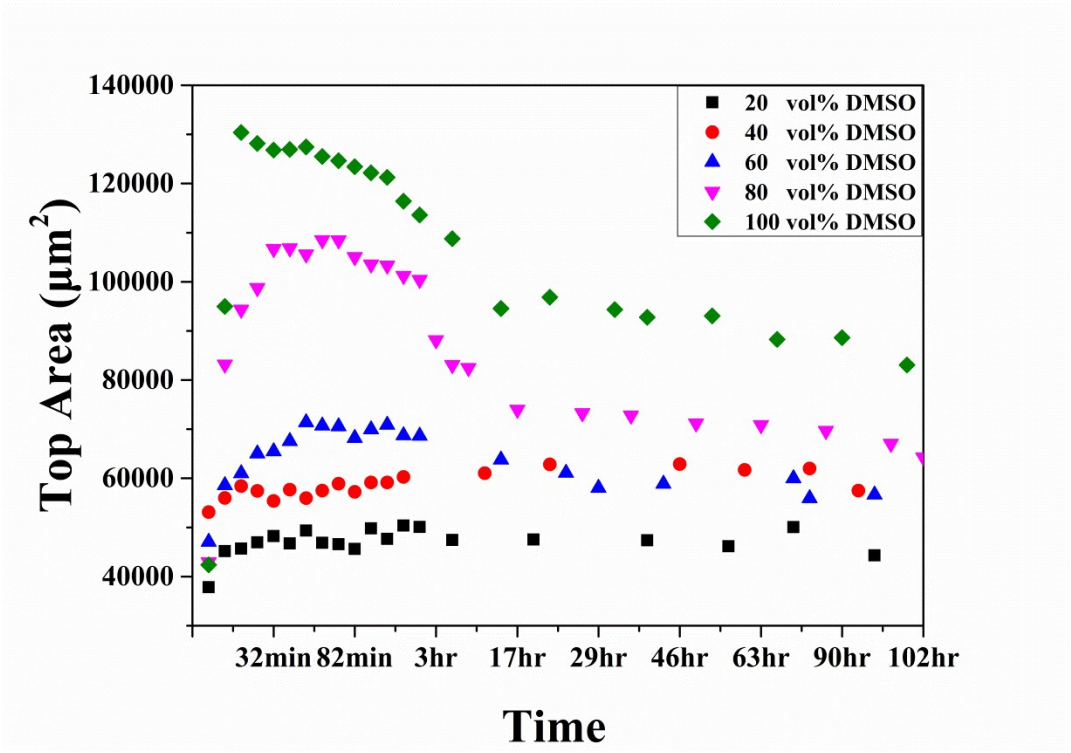

Figure S1. Top-view crystal area change versus time for CLLC soaked in varying aqueous DMSO aqueous solutions.

These HEWL crystals were crosslinked in 4 vol% aqueous glutaraldehyde solution for 24 hours at room temperature. CLLCs were only incubated in DMSO aqueous solution of one concentration. As shown in Fig. S1, CLLCs reached the first equilibrated state after being 30-minute soaking in corresponding DMSO aqueous solution and maintained a relatively stable value for top-view area for 5 hours for 20-60 vol%. For 80 and 100 vol%, their first equilibrated state seemed changed from 2-3 hours after soaking.

The required length of time for CLLC denaturation to equilibrate to a new solvent was estimated on the basis of the empirical data from denaturation experiments were carried out at room temperature (Fig. S1). We assume because of the strong hydroscopicity of DMSO and incomplete sealing, the actual value of concentration possibly decreased over time, which causing the second equilibrated state. Therefore, for renaturation, we provided a longer time period (long enough for CLLCs reaching the second equilibrated state) at each DMSO concentration to maximize the chances for the lysozyme proteins within the CLLC to successfully refold.

Figure S2

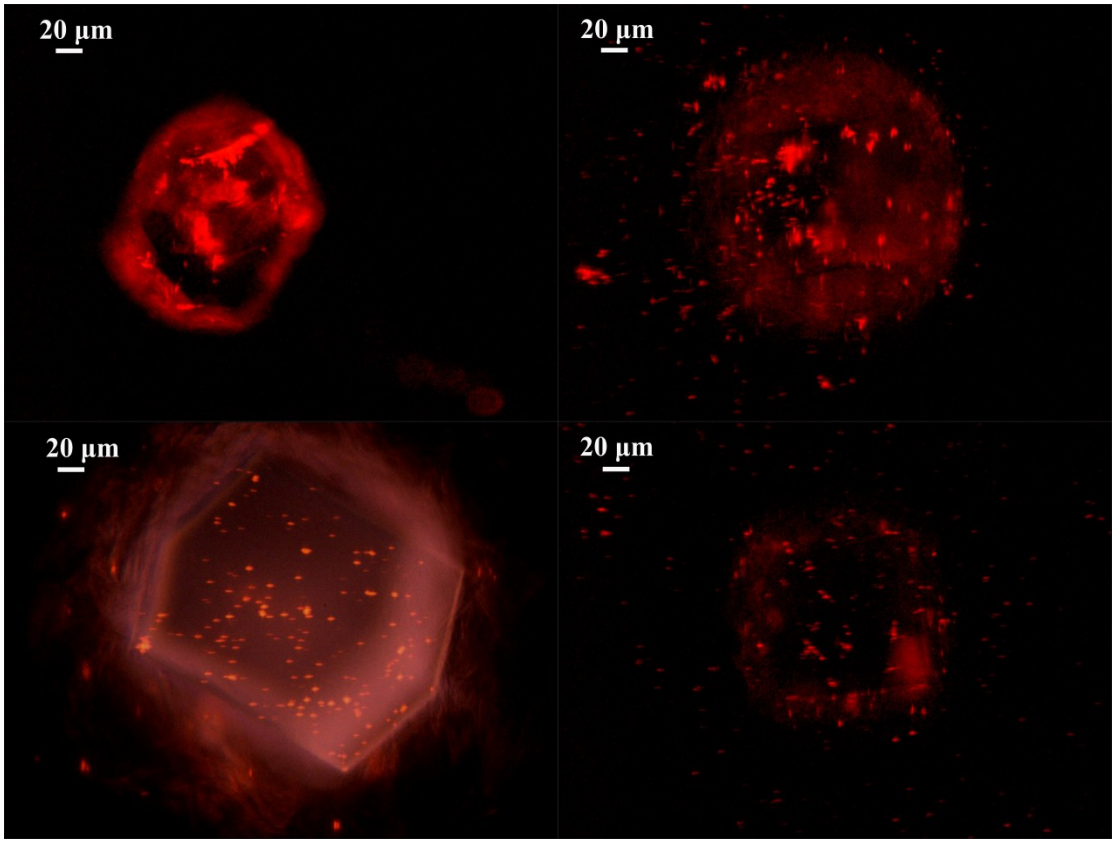

Figure S2. Fluorescence microscope images of loading pre-assembled $Eu(TTA)_3phen$ via anhydrous ethanol into CLLCs. Excitation wavelength is about 365 nm.

The images were collected a couple of days into the incubation and revealed significant aggregation of LC outside of the crystals and on the crystal surfaces.

Figure S3

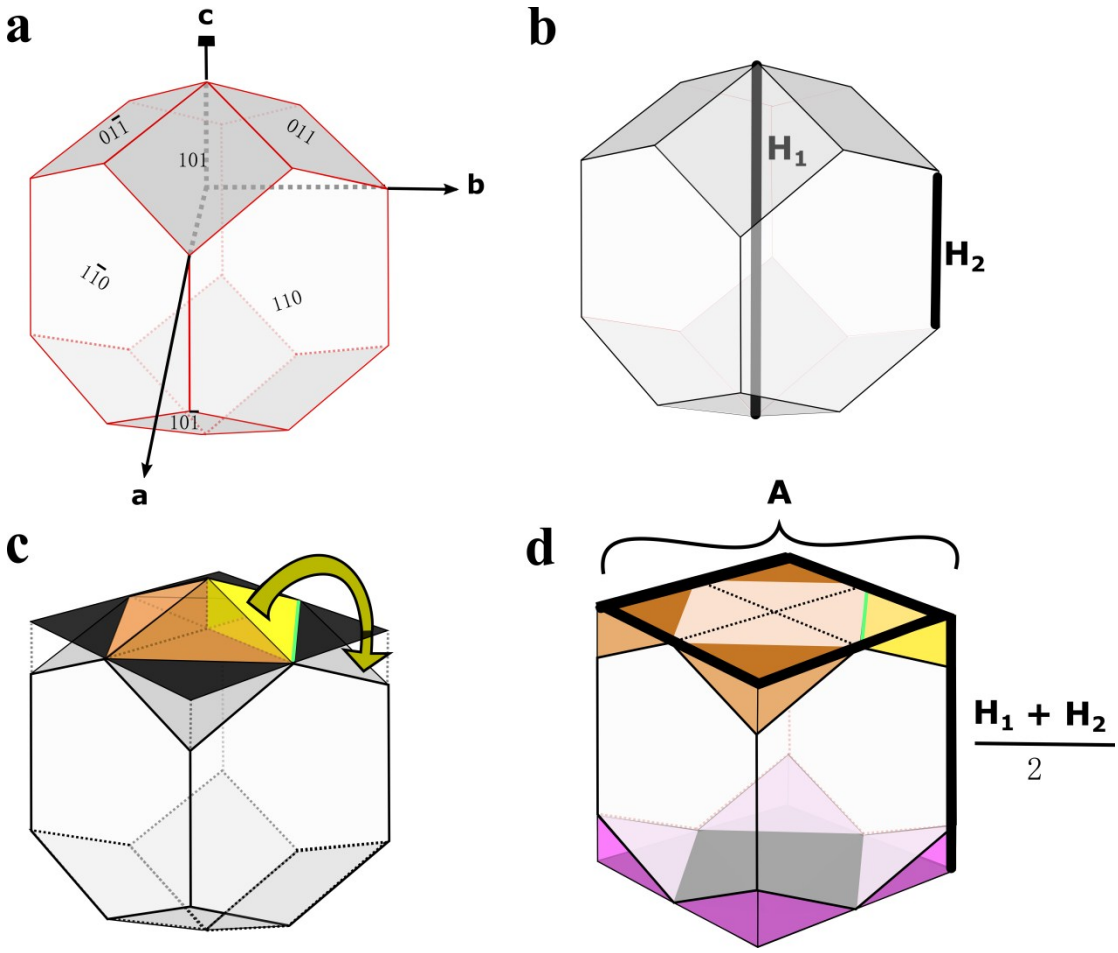

Figure S3. Graphic illustrations motivating our scheme for measuring CLLC volume during denaturation-renaturation experiments.

Specifically, the apex of a tetragonal lysozyme crystal could be divided into four components, these components could be pivoted around axes that bisect the 101, 01, 01 and 011 facets to generate a rectangular cuboid. The value of H in the main text Fig. 5 (and Fig. S8) was the average of the value of each CLLC's H_1 and H_2.

Figure S4

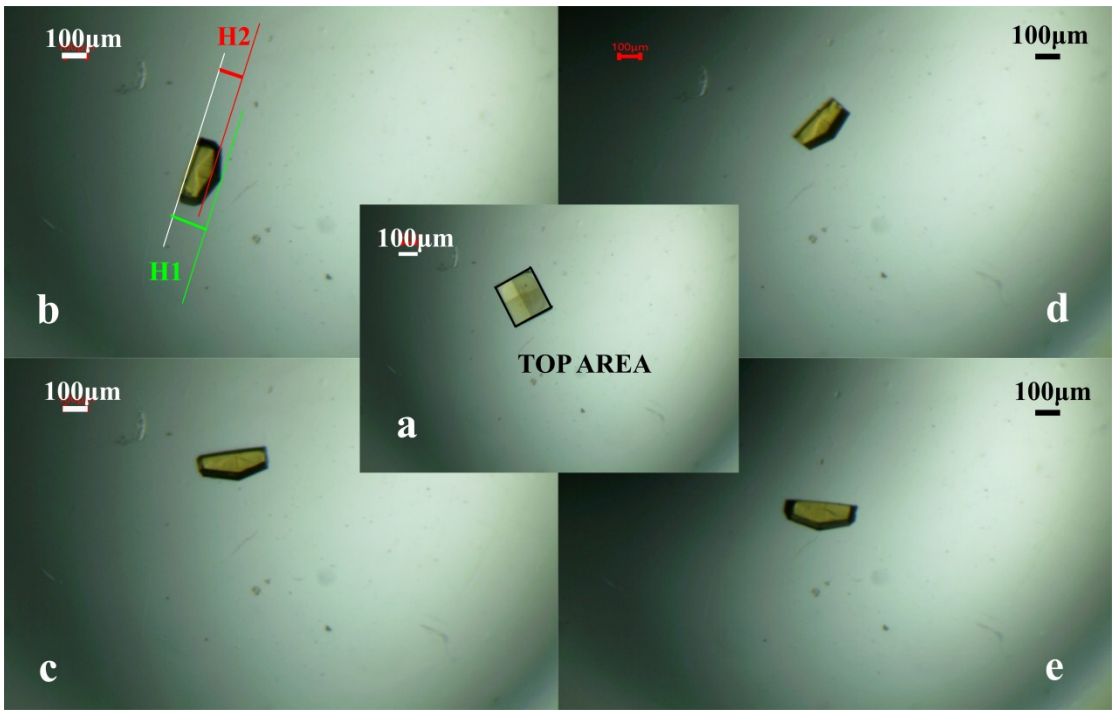

Figure S4. Stereo-zoom microscope images of (**a**) top view of one CLLC. (**b-c**) Height (H) of the CLLC in (a) with its 10 110 10 1 facets facing the camera.

Figure S5

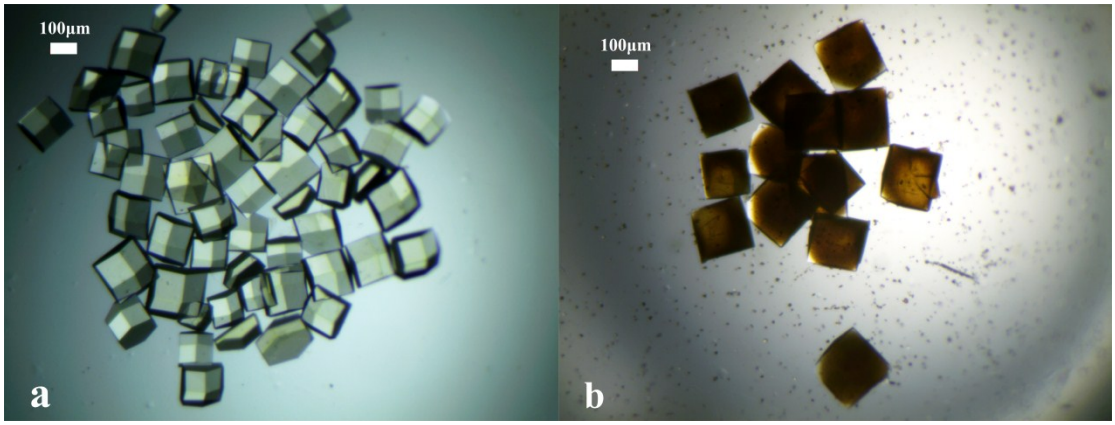

Figure S5. Stereo-zoom microscope images of (**a**) CLLCs before ligand loading (**b**) CLLCs after soaking in ligand anhydrous ethanol solution for 7 days. Ligand anhydrous ethanol solution was prepared by mixing equal volumes of 30mM TTA and 10 mM phen anhydrous ethanol solution, with pH adjusted to 7.4 by 2M ammonia.

Figure S6

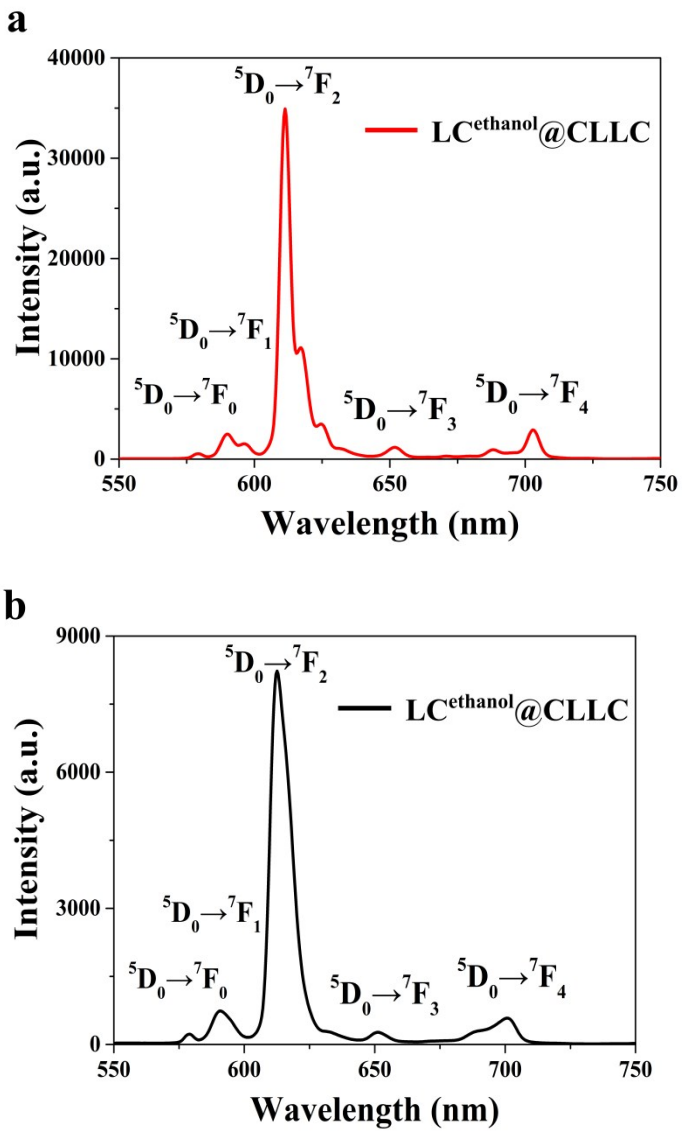

Figure S6 Emission spectra of LC$^{DMSO\&ethanol}$@CLLC (**a**) and LC^{ethanol}@CLLC (**b**) under illumination at 365 nm.

Figure S7

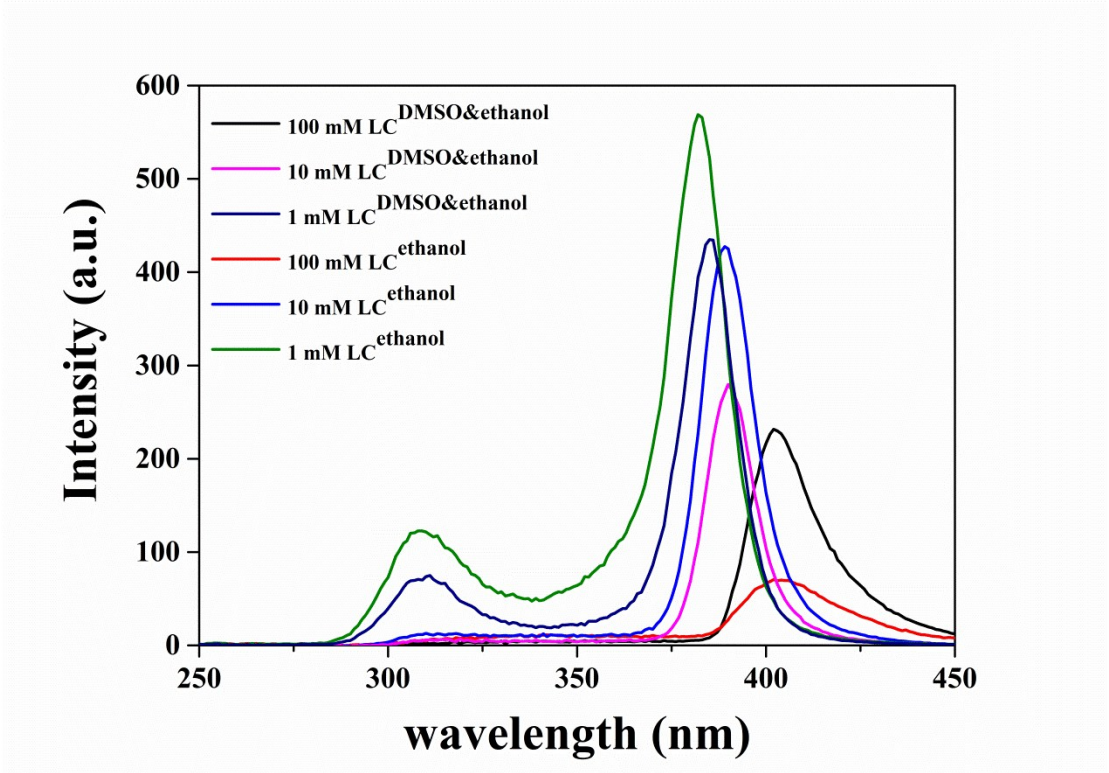

Figure S7. Excitation spectra of $LC^{DMSO\&ethanol}$ and $LC^{ethanol}$ were obtained by detecting the characteristic emission wavelength of europium ion from $^5D_0 \rightarrow {}^7F_2$ transtion. Emission and excitation slits are all 5 nm.

All LC solutions were synthesized by mixing corresponding ligand solution (Ligand solution was prepared by mixing equal volumes of 3/30/300mM TTA and 1/10/100 mM phen solution, with pH adjusted to about 7.4 by 2M ammonia.) and 1/10/100mM europium chloride anhydrous ethanol solution. From Fig. S7, we could speculate that LC synthesized inside CLLCs probably resembles 1 mM $LC^{ethanol}$/$LC^{DMSO\&ethanol}$, for $LC^{DMSO\&ethanol}$@CLLC and $LC^{ethanol}$@CLLC could both be excited by 365 nm and 405 nm.

Figure S8

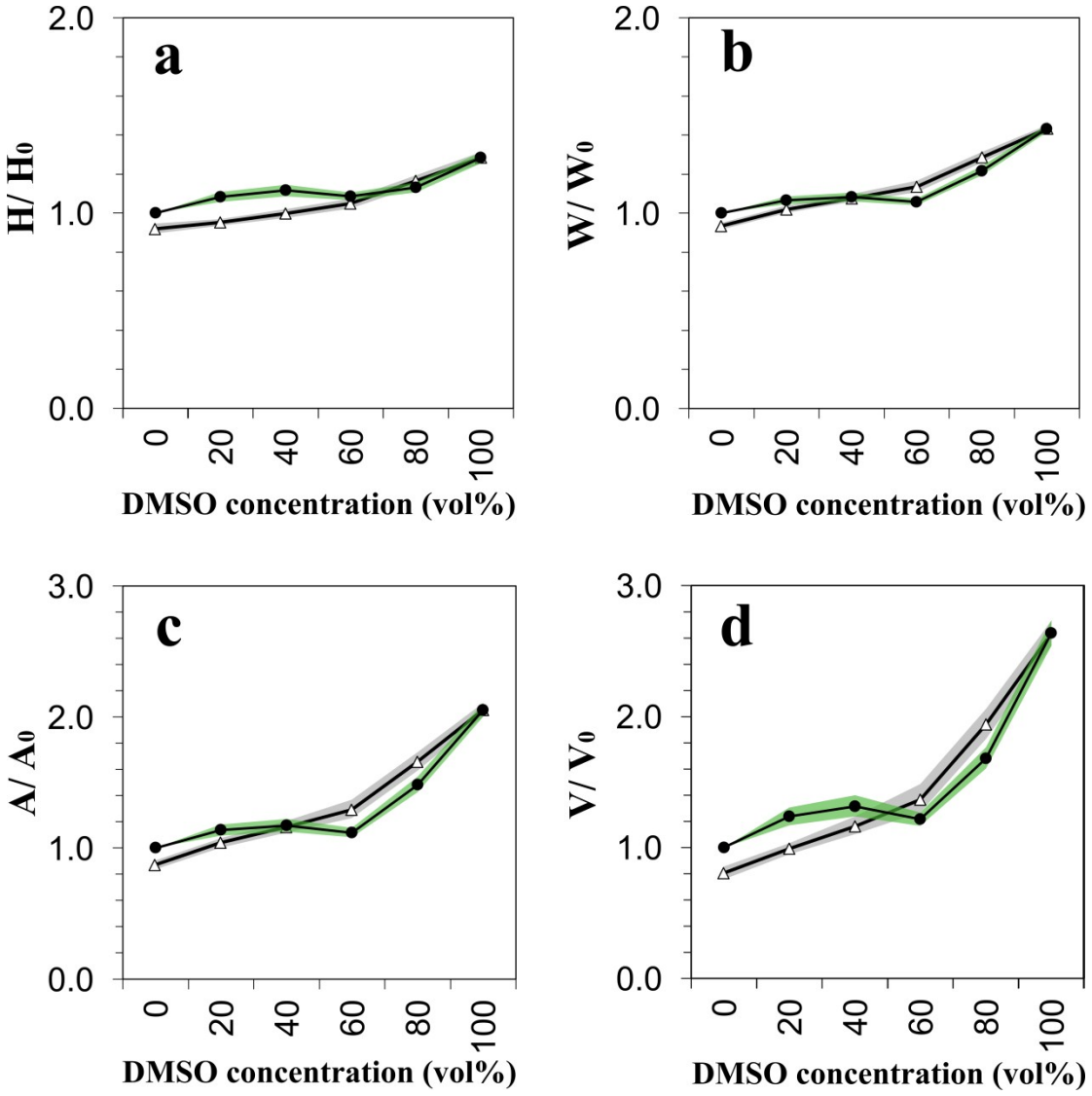

Figure S8. The mean relative value of height (a), width (b), top area (c) and volume (d) for the crystals during expansion (black circle) and during renaturation (white triangle) is displayed, along with the 90% confidence interval for the mean obtained via bootstrap analysis (shaded regions). Abscissa axes represent DMSO concentration. The initial (H_0, W_0, A_0 and V_0) and final values for each panel (were measured when CLLC were soaked in crystallization solution. All data came from 20 individual CLLC which experience the entire continuous denaturation-renaturation process.

Figure S9

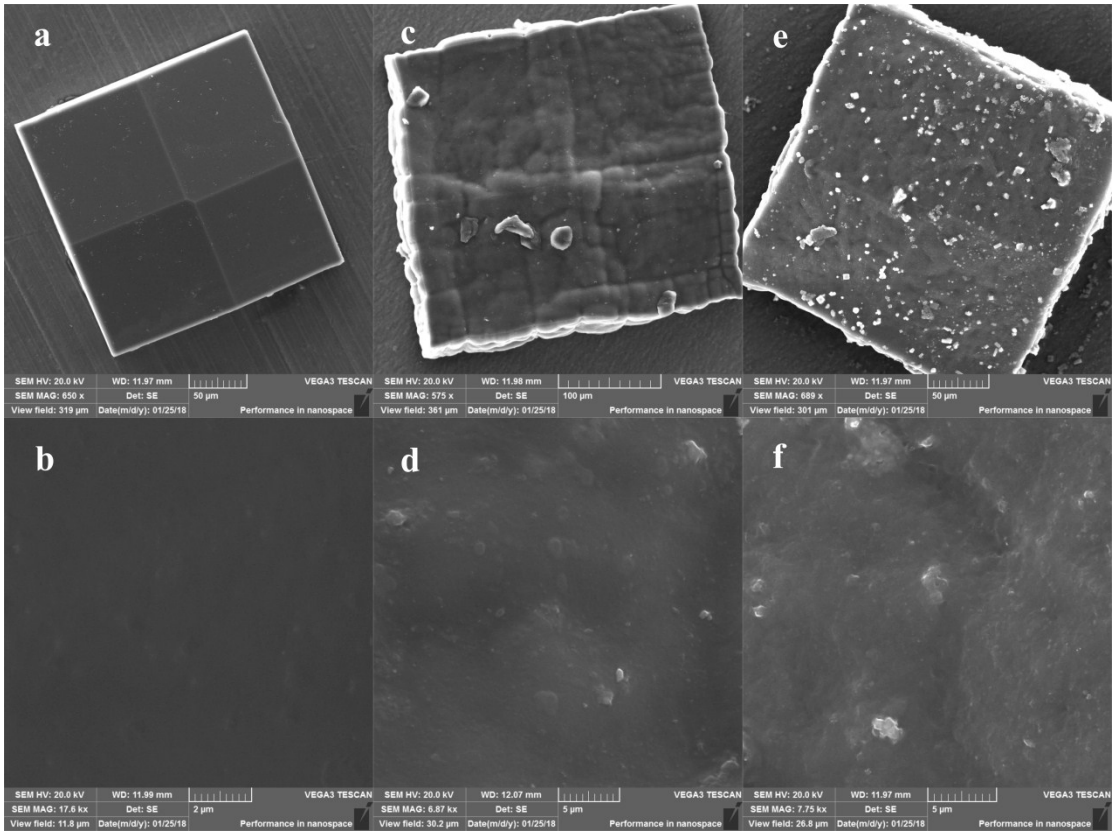

Figure S9. Scanning electron microscopy images: apex area of CLLC (where the 101, 01, 01 and 011 facets meet) for (a) CLLC soaked in anhydrous ethanol for 5 days, (c) CLLC denatured in DMSO, (e) denatured CLLC soaked in anhydrous ethanol for 2 hours; Surface details of apex area of (b) surface close-ups from panel a, (d) surface close-ups from panel c, (f) close-ups from panel e. All samples were dried in vacuum at 20℃ overnight and treated by gold sputtering.

Figure S10

The ability of glutaraldehyde cross-linked HEWL crystals to retain molecular order after 48-hour incubation in 100% ethanol at 20° C was assessed using X-ray diffraction (XRD). Prior to cross-linking, HEWL crystals were washed by loop transferring them into 2.5 M NaCl, 50 mM sodium acetate pH 4.6 for 15 minutes to remove residual HEWL monomers. Crystals were then transferred into a fresh mixture of 2.5 M NaCl, 50 mM sodium acetate pH 4.6 containing 10% glutaraldehyde and cross-linked for 2 hours. The cross-linking reaction was quenched by transferring HEWL crystals into a solution of 0.3 M hydroxylamine, 25 mM DMAB, 0.15 M NaCl, and 0.1 M citric acid at pH 5.0 for 1 hour.

Cross-linked HEWL crystals were then transferred into a vacuum sealed glass well containing 100% EtOH and allowed to incubate for 48 hours at 20° C. After 48 hours, without using cryo-protectant, the diffraction quality was assessed using a Rigaku Compact HomeLab with a micro-focus X-ray generator and a Pilatus 200K detector. Crystals were flash frozen in a cryogenic nitrogen stream at 100 K and exposed to X-rays for 60 seconds at a detector distance of 60 cm—the resulting images (Fig. S10) revealed poor diffraction (< 6.0 Å), indicating moderate destabilization of molecular order.

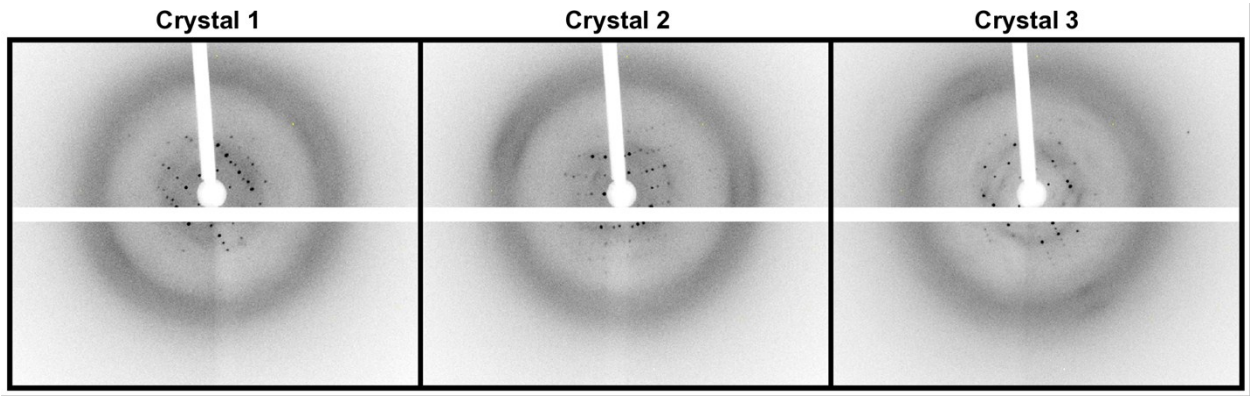

Figure S10. Representative diffraction images of glutaraldehyde cross-linked HEWL crystals after incubation in 100% ethanol for 48 hours at 20 ° C.

Figure S11

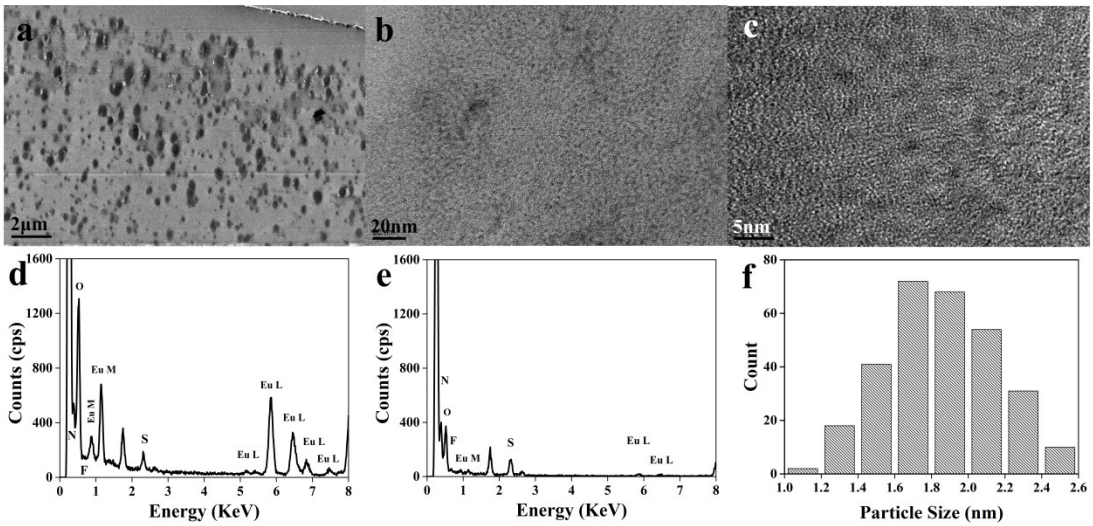

Figure S11. HRTEM images of an internal microtome slice of $LC^{ethanol}$@CLLC: **(a)**, **(b)** and **(c)**. **(b)** A zoomed-in image of one dark dot in panel a. **(c)** A further magnified image of an area in panel b. **(d)** EDX spectrum of a dark dot area in panel a. **(e)** EDX spectra of an area with weaker contrast in panel a. **(f)** Size distribution of electron rich features found within panel b. $LC^{ethanol}$@CLLCs were dried at 50℃ for 2 days and embedded in epoxy resin and left to cure at 50℃ for over 3 days. The thickness of all sections was 100 nm.

We were not able to observe the lattice structure of the HEWL crystal via HRTEM, either due to the lack of contrast between the protein and the resin, or due to the loss of the crystal periodicity during crystal preparation or handling, or due to the microtome plane not being aligned with a recognizable crystal plane. However, the EDX data obtained for two areas suggests the presence of LC. If we assume that the electron dense features found in panel b correspond to particles, then the histogram of particle size distribution (panel f) shows that the particle size is in the range of 1.4-2.4 nm. Compared to maximum diameter of $Eu(TTA)_3phen$ (around 1.3 nm), these dark dots seem oversized.

We hypothetically attribute the non-uniform or aggregated distribution of suspected $Eu(TTA)_3phen$ particles to the possible reasons below: (1) The cross-linking process may disorder the natural structure of HEWL crystals. Ligands penetrating the internal solvent channels of CLLCs may stop at certain locations and block the further transport of following ligands, which might form a multi-ligand aggregate in situ. (2)

After the entrance of Eu^{3+} into ligand-laden CLLCs, these trivalent ions will not only be induced into where the multi-ligand aggregates are, but be attracted to carboxylic acids present on the surface of HEWL monomers, which would enlarge the contour of those dark dots. (3) In the process of embedding $LC^{ethanol}$@CLLCs in epoxy resin, resin infiltration or drying at 50℃ over five days could damage the internal structure and alter the location of guest LCs. Taking all the probable reasons above, and the EDX spectra (Fig. S11d, 11e) we speculate those dark dots are aggregates of $Eu(TTA)_3phen$ with probable Eu-lysozyme complex surrounding.