Skin-deep Surface Patterning of Calcite

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This Supplementary Information contains:

Experimental details of the cp20k expression, materials, stock solution preparation, example crystal growth protocols, cp20k fluorescent tagging, image analysis and modelling techniques

Supplementary Figures S1-S9

Supplementary Table S1-S2

Experimental

1. Protein expression studies

The expression of the 20 kDa cement protein (cp20k) from *Megabalanus rosa* in *Escherichia coli* was carried out as described previously with some modification.[S1] The DNA fragment encoding mature cp20k (from 20th Histidine to 183rd glutamate) was synthesized with codon optimization to *E. coli*. NheI site including start codon and XhoI site were attached to 5'- and 3'- side of DNA fragment, respectively. The plasmid vector harbouring synthesized cp20K was treated and NheI – XhoI fragment was excised. This fragment was cloned into the NheI – XhoI site of the expression vector pET22b (+) (Merck Millipore, MA, USA) to add 6 x His-tag to the C-terminal of the expressed protein. *E. coli* strain Origami™ 2 (DE3) (Merck Millipore, MA, USA) was transformed by the resulting plasmid, pETcp20k. Transformed *E. coli* was cultivated at 37˚C on the LB medium (1% peptone, 0.5% yeast extract, 1% NaCl) with ampicillin and tetracycline as antibiotics. Culture fluid was transferred to LB-medium so that OD_{600} was 0.1 and this medium was cultivated at 37˚C. When OD_{600} became 0.6, isopropyl ß-D-1-thiogalactopyranoside was added and its final concentration was adjusted as 0.1 mM. Thereafter, cultivation was carried out for 6 hours at 37˚C. *E. coli* cells were harvested by centrifugation and cell pellet was resuspended in xTractor buffer (TakaraBio, Shiga, JP). This suspension was centrifuged and supernatant was subjected to TALON® metal affinity resin (Takarabio, Shiga, JP) and purification of His-tagged cp20k was carried out as described by manufacturer's instruction. Purified cp-20k was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure S9).[S2] Purified cp20k was concentrated by ultrafiltration and solvent was exchanged for distilled water. cp20k Primary sequence (with His-tag attached): (190 amino acids MW: 21.343 kDa)

MHEEDGVCNSNAPCYHCDANGENCSCNCELFDCEAKKPDGSYAHPCRRCDANNICKCSCTAIPCNEDHPCHHCH
EEDDGDTHCHCSCEHSHDHHDDDTHGECTKKAPCWRCEYNADLKHDVCGCECSKLPCNDEHPCYRKEGGVVSC
DCKTITCNEDHPCYHSYEEDGVTKSDCDCEHSPGPSEHHHHHH

2. Crystal growth studies

2.1. Materials

Poly(acrylic acid sodium salt)(MW 5100) (PAA), Poly(styrene sulfonate sodium salt)(MW 70000)(PSS), Poly(styrenesulfonate-*alt*-maleic acid) 13 wt% in water (MW 510000) (PSS-MA), Calcium chloride dihydrate, magnesium chloride hexahydrate, sodium carbonate anhydrous, ammonium carbonate, fluorescein isothiocyanate, glycine, sodium hydrogen carbonate and 99.5 wt% formamide aqueous solution were used as purchased from Sigma Aldrich, UK without further purification. Sulfuric acid was used as purchased from Fluka, UK. 30% hydrogen peroxide solution were used as purchased from Fisher, UK without further purification. DI water was used as obtained from in-house Millipore^{TM} Q system (<2 ppm TOC and 18.2 MΩ).

Additive influenced calcite growth studies were conducted 3x4 clear polypropylene well plates (Nunc). Each well was charged with an individual and removable clean glass substrate for reproducible sample preparation for characterisation. Glass substrates were cleaned with Piranha solution (3:1 H_2SO_4:30% H_2O_2 solution) for 16 h before extensive rinsing with DI water. (Warning: Piranha solution is highly oxidising and corrosive, and becomes extremely hot upon mixing, thus care must be taken upon addition of 30% H_2O_2 solution to ice-cold H_2SO_4).

2.2. Stock solutions

200 mM $CaCl_2.2H_2O$ (7.3505 g in 250 mL DI water), 200 mM $MgCl_2.6H_2O$ (10.1655 g in 250 mL DI water) and 200 mM Na_2CO_3 (5.2994 g in 250 mL DI water) aqueous solutions were prepared in 250 mL volumetric flasks and stored in separate glass bottles. 0.1 mg/ml cp20k stock solution was prepared by dilution of received cp20k (i.e. 0.97 mg/mL in DI water) with DI water (i.e. 1.03 mL 0.97 mg/mL stock into 8.97 mL DI water) and stored at 4 °C until required. 100 mM carbonate buffer (pH 9.2) was prepared by mixing 10 mL 200 mM Na_2CO_3, 90 mL 200 mM $NaHCO_3$ and 100 mL DI water in a glass bottle. 4 mg FITC was suspended in 4 mL 100 mM carbonate buffer (pH 9.2) in a foil-covered glass vial and dissolved by vortexing for 5 min before storage at 4 °C. 100 mM glycine (0.075 g in 10 mL DI water)

aqueous solution was prepared in a 10 mL volumetric flask and stored in a sealed glass vial. For PAA and PSS, 0.4 g was dissolved in 20 mL DI water under stirring to yield a 20 mg/mL 20 mL stock. For PSS-MA, 3.08 mL stock solution was added to 16.92 mL DI water under stirring to yield a 20 mg/mL 20 mL stock. For formamide, 12 mL formamide 99.5 % was added to 8 mL DI water under stirring to yield a 60% (v/v) 20 mL stock. All stocks (included DI water stock) were filtered after preparation immediately before use through a syringe-driven Millipore 0.22 μm pore size polycarbonate membrane.

2.3. Example crystal growth experiments

Carbonate Direct Method (CDM). In an example CDM experiment where [cp20k] = 0.015 mg/mL, a well was charged with 50 μL filtered 200 mM $CaCl_2.2H_2O$ aqueous solution and 300 μL 0.1 mg/mL filtered cp20k aqueous solution, followed by 1.6 mL filtered DI water, resulting in 1.95 mL of total volume within the well. In order to achieve reaction initiation and a final volume of 2 mL, 50 μL filtered 200 mM Na_2CO_3 aqueous solution was added followed by stirring to ensure complete mixing. Well plates were then covered with well plate covers and sealed with Parafilm for 5 days. After this time, the glass substrate was washed with water and ethanol and allowed to dry under a stream of air.

Ammonia Diffusion Method (ADM). In an example ADM experiment, a well was charged with 50 μL filtered 200 mM $CaCl_2.2H_2O$ aqueous solution and 300 μL 0.1 mg/mL filtered cp20k aqueous solution, followed by 1.65 mL filtered DI water, resulting in 2 mL of total volume within the well. This was then covered with Parafilm and added to the incubator for 5 days. Where different [cp20k] was required, volumes of cp20k 0.1 mg/mL stock solution and DI water were adjusted accordingly. After this time, the glass substrate was washed with water and ethanol and allowed to dry under a stream of air.

Calcite seed preparation. Dissolution and restructuring studies were conducted on calcite seeds. These were precipitated onto clean, glass substrates over 5 days by ADM (see Supporting Information

section 1.2.5 for more details) from a solution containing CaCl2 solution, where [Ca^{2+}] = 10 mM. Seeds were washed briefly with DI water and ethanol before drying under flowing air and used immediately.

Dissolution and restructuring studies. In an example dissolution study ([PSS] = 10 mg/mL), a dissolution solution was prepared in a glass vial by adding 2 mL PSS stock solution (20 mg/mL) to 2 mL DI water to yield 4 mL [PSS] = 10 mg/mL aqueous solution. 2 mL of this was then transferred to a well on a well-plate, equilibrated at 65 °C for 30 min, then charged with a glass slide loaded with calcite seeds. The well plate was then sealed with a well plate lid and Parafilm, then placed back into the oven and left for 65 °C for 16 h. After this time, glass slides with treated seeds were then rinsed with DI water and ethanol, then dried under a stream of air. In an example restructuring study, the same protocol as above was followed except the well plate was left exposed.

Mixed Additive Studies. In an example CDM experiment where [cp20k] = 0.015 mg/mL and [Mg^{2+}]/[Ca^{2+}] = 0.5, a well was charged with 50 µL filtered 200 mM $CaCl_2.2H_2O$ aqueous solution, 25 µL filtered 200 mM $MgCl_2.6H_2O$ aqueous solution and 300 µL 0.1 mg/mL filtered cp20k aqueous solution, followed by 1.575 mL filtered DI water, resulting in 1.95 mL of total volume within the well. In order to achieve reaction initiation and a final volume of 2 mL, 50 µL filtered 200 mM Na_2CO_3 aqueous solution was added followed by stirring to ensure complete mixing. Well plates were then covered with well plate covers and sealed with Parafilm for 5 days. In an example ADM experiment with the same [cp20k] and [Mg^{2+}]/[Ca^{2+}], a well was charged with 50 µL filtered 200 mM $CaCl_2.2H_2O$ aqueous solution, 25 µL filtered 200 mM $MgCl_2.6H_2O$ aqueous solution and 300 µL 0.1 mg/mL filtered cp20k aqueous solution, followed by 1.625 mL filtered DI water, resulting in 2 mL of total volume within the well. This was then covered and added to the incubator for 5 days.

The influence of alternative additives. In an example ADM experiment with [PSS] = 10 mg/mL, a well was charged with 50 µL filtered 200 mM $CaCl_2.2H_2O$ aqueous solution and 1000 µL 20 mg/mL filtered

PSS aqueous solution, followed by 1300 µL filtered DI water, resulting in 2 mL of total volume within the well. This was then covered with Parafilm and added to the incubator for 5 days. After this time, the glass substrate was washed with water and ethanol and allowed to dry under a stream of air.

Fluorescein isothiocyanate (FITC) Tagging of cp20k. A small proportion (≈ 1%) of cp20k stock solution was tagged to enable CLFM imaging without affecting the protein function. In a typical experiment, 2 mL of filtered cp20k ([cp20k] = 0.1 mg/mL) solution was set aside for tagging in a clean glass vial. 50 µL of this was transferred to a clean, foil-wrapped centrifuge tube. 1 µL 1 mg/mL FITC in 100 mM carbonate buffer (pH 9.2) was added to the cp20k solution in the centrifuge tube and allowed to react for 30 min at room temperature. After, 49 µL of 100 mM aqueous glycine solution was added to render all remaining FITC hydrophilic and water-soluble. The total 100 µL of solution was added back into remaining 1.95 mL of apportioned cp20k stock to yield 2.05 mL. This was transferred to a centrifugal protein concentrator (Millipore Amicon Ultra-15 10kDa cutoff) and centrifuged at 5000 x g for 20 min. Washing by addition 10 mL filtered DI water to the concentrated protein solution was conducted 3 times by centrifugation at 5000 x g for 20 min. The volume of the concentrate was accurately determined by mass and made up to 2 mL with DI water, then calculated as 0.1 mg/mL. This stock was then used in studies as described above.

3. Characterisation

Image analysis. For all confocal fluorescence, optical, polarised light, TEM and SEM micrographs; rendering and analysis was conducted in ImageJ. For confocal fluorescence microscope *z*-stacks, optical images were taken from the central most plane. Fluorescence confocal micrographs were obtained by forming a *z*-projection for *z*-stacks. Surface images, as viewed from various angles, were obtained by rendering confocal fluorescence *z*-stacks into 3D, and rotating the rendered image manually. Orthogonal views images were obtained using orthogonal views option, where each plane

(XY, ZY, ZX) was selected to best represent the surface and internal structure of each sample. Aspect ratio measurements were obtained from low magnification SEM.

Calcite morphology modelling. Calcite models, such as those appearing in Figure 8, were drawn using WinXMorph software.[S3, S4]

Supplementary References

S1. Mori, Y.; Urushida, Y.; Nakano, M.; Uchiyama, S.; Kamino, K., Calcite-specific coupling protein in barnacle underwater cement. *Febs J* **2007,** 274, (24), 6436-6446

S2. Laemmli, U. K., Cleavage of Structural Proteins during Assembly of Head of Bacteriophage-T4. *Nature* **1970,** 227, (5259), 680-&.

S3. Kaminsky, W., WinXMorph: a computer program to draw crystal morphology, growth sectors and cross sections with export files in VRML V2.0 utf8-virtual reality format. *J Appl Crystallogr* **2005,** 38, 566-567.

S4. Kaminsky, W., From CIF to virtual morphology using the WinXMorph program. *J Appl Crystallogr* **2007,** 40, 382-385.

Supplementary Tables

Amino acid Symbol	Amino acid Name	Amino acid Molar mass	No. Amino acid in cp20k	Total Mr of amino acid in cp20k	Final %mass amino acid in cp20k
A	Alanine	89.1	8	712.8	2.88
C	Cysteine	121.2	32	3878.4	15.66
D	Aspartic Acid	133.1	21	2795.1	11.29
E	Glutamic Acid	147.1	19	2794.9	11.29
F	Phenylalanine	165.2	1	165.2	0.67
G	Glycine	75.1	10	751	3.03
H	Histidine	155.2	25	3880	15.67
I	Isoleucine	131.2	3	393.6	1.59
K	Lysine	146.2	10	1462	5.90
L	Leucine	131.2	3	393.6	1.59
M	Methionine	149.2	1	149.2	0.60
N	Asparagine	132.1	11	1453.1	5.87
P	Proline	115.1	11	1266.1	5.11
Q	Glutamine	146.1	0	0	0
R	Arginine	174.2	4	696.8	2.81
S	Serine	105.1	12	1261.2	5.09
T	Threonine	119.1	7	833.7	3.37
V	Valine	117.1	5	585.5	2.36
W	Tryptophan	204.2	1	204.2	0.82
Y	Tyrosine	181.2	6	1087.2	4.39

Table S1: Details of the cp20k protein and constituent amino acids.

	ADM	CDM	Dissolution	Restructuring
PAA	1 mg/mL	1 mg/mL	1 mg/mL	1 mg/mL
PSS	10 mg/mL	10 mg/mL	10 mg/mL	10 mg/mL
PSS-MA	10 mg/mL	10 mg/mL	10 mg/mL	10 mg/mL
Mg^{2+}	5 mM	5 mM	2.5 mM	5 mM
Formamide	30% (v/v)	30% (v/v)	1% (v/v)	NA

Table S2: Concentrations of additives used for surface roughness studies with common calcite-modifying additives. NA: Not applicable.

Supplementary Figures

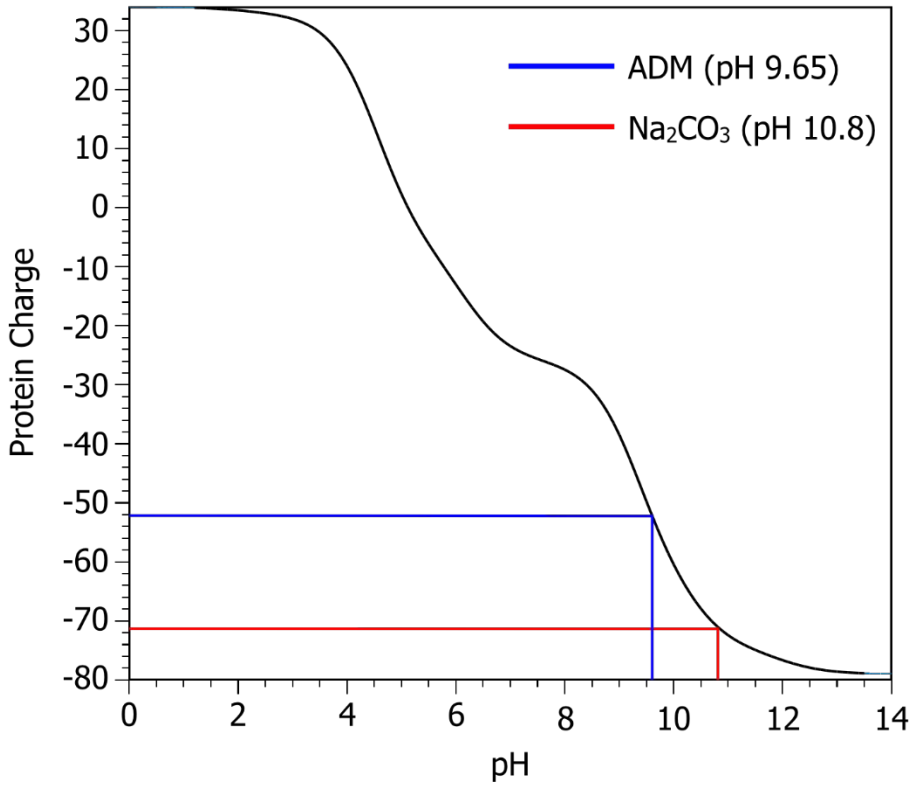

Figure S1: Net charge of cp20k vs. solution pH plot. pH of individual experiments ADM (pH 9.65, blue) and CDM (pH 10.8, red) are given to show the contrast protein charge in each experiment.

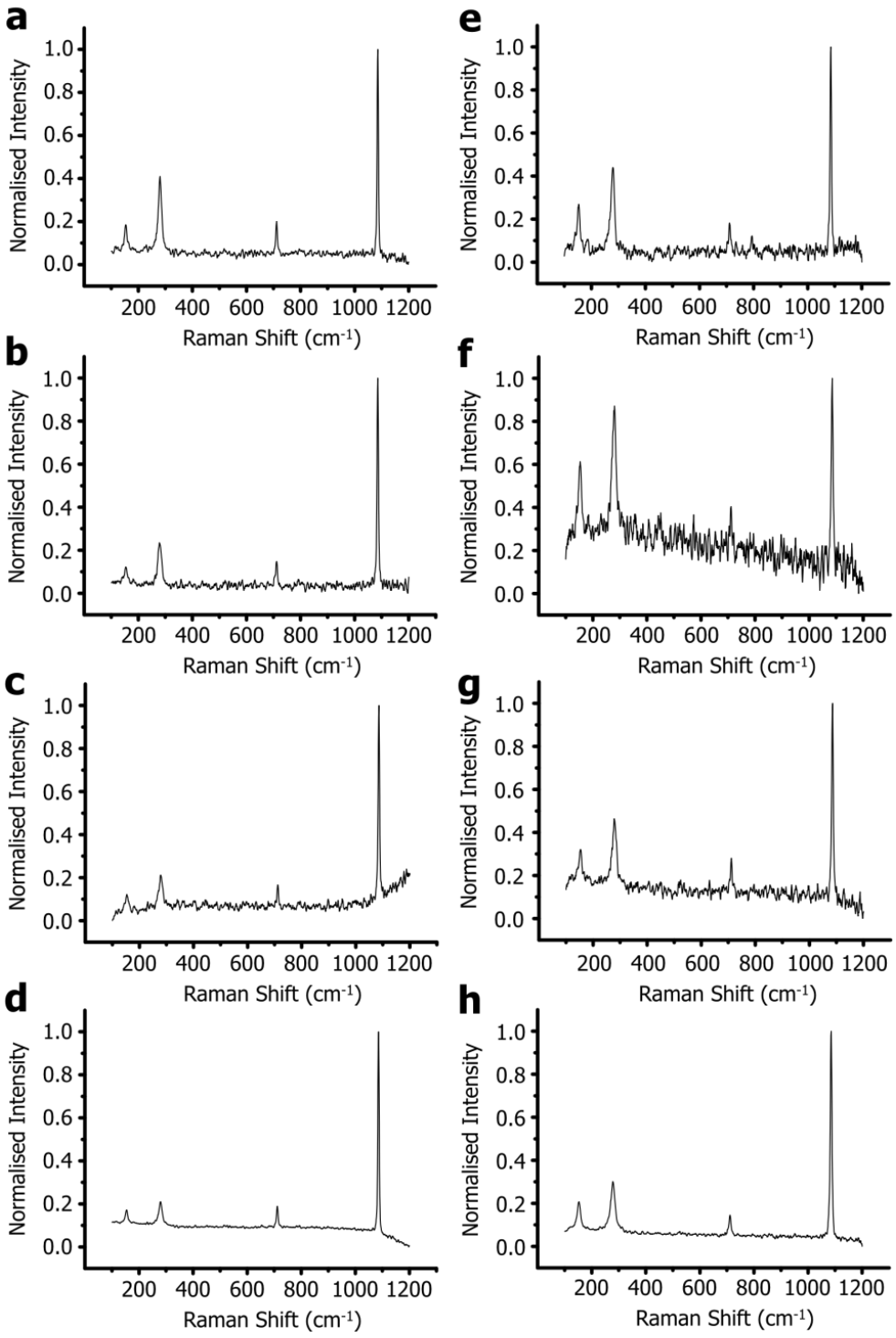

Figure S2: Raman spectra taken from calcite grown in the presence of different concentrations of cp20k (0 (**a**), 0.001 (**b**), 0.005 (**c**) 0.015 (**d**), 0.025 (**e**) 0.05 (**f**), 0.075 (**g**) and 0.095 (**h**) mg/mL) by CDM ($[Ca^{2+}] = [CO_3^{2-}] = 5$ mM). Calcite was the only observed polymorph in all samples.

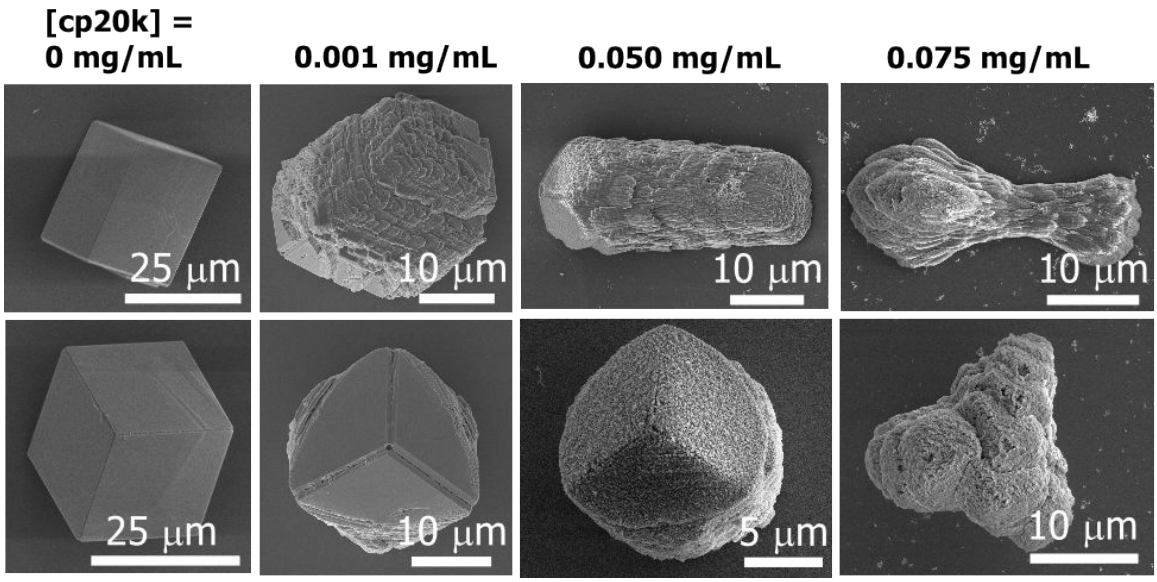

Figure S3: SEM micrographs of calcite grown in the presence of different concentrations of cp20k by CDM ($[Ca^{2+}] = [CO_3^{2-}] = 5$ mM). Micrographs show crystals approximately orientated (104)-up (**above**) and (001)-up (**below**).

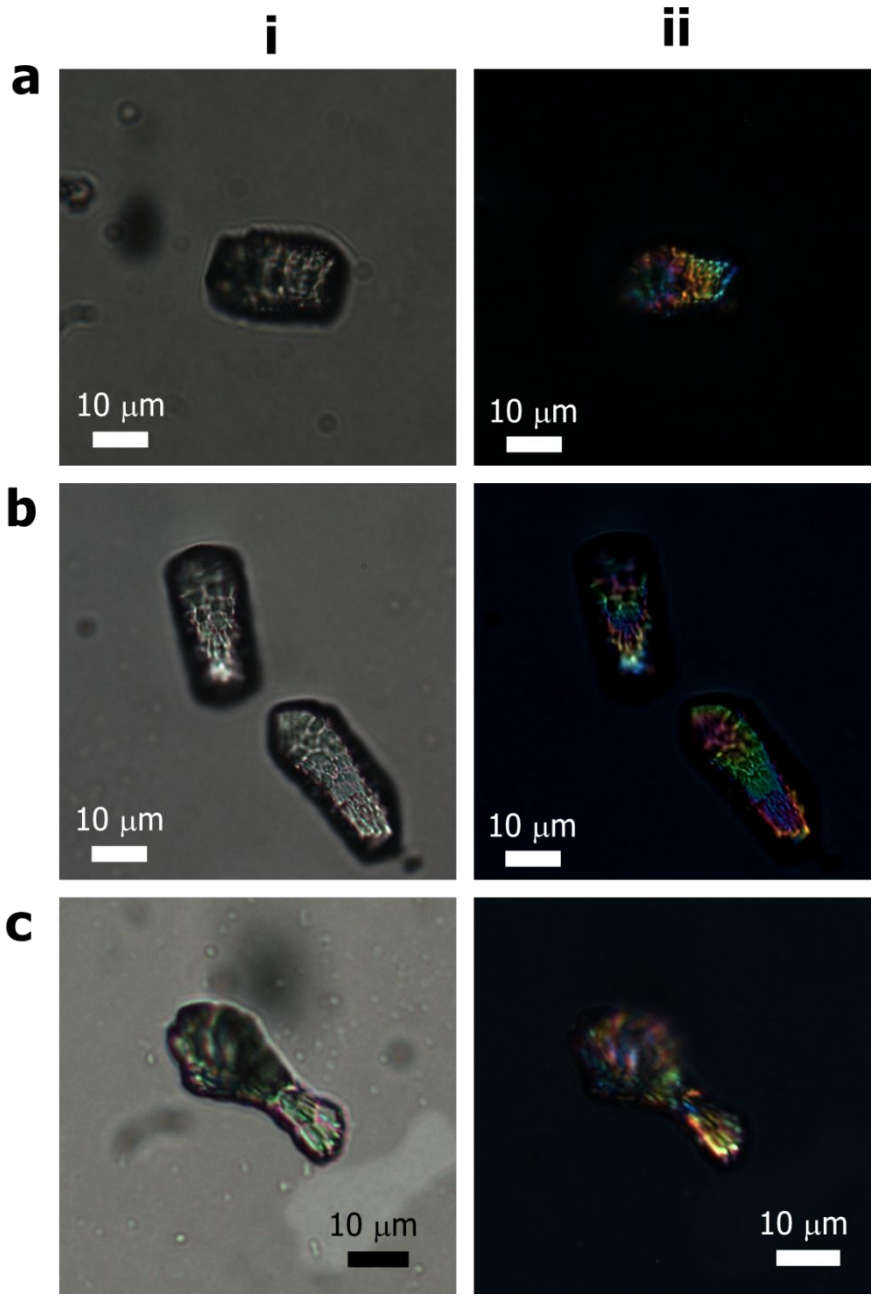

Figure S4: Optical (**i**) and PLM (**ii**) micrographs of calcite grown in the presence of cp20k ([cp20k] = 0.025 (**a**), 0.050 (**b**) and 0.075 (**c**) mg/mL) by CBM ($[Ca^{2+}] = [CO_3^{2-}]$ = 5 mM) showing a difference between single crystal modified calcite and polycrystalline aggregates.

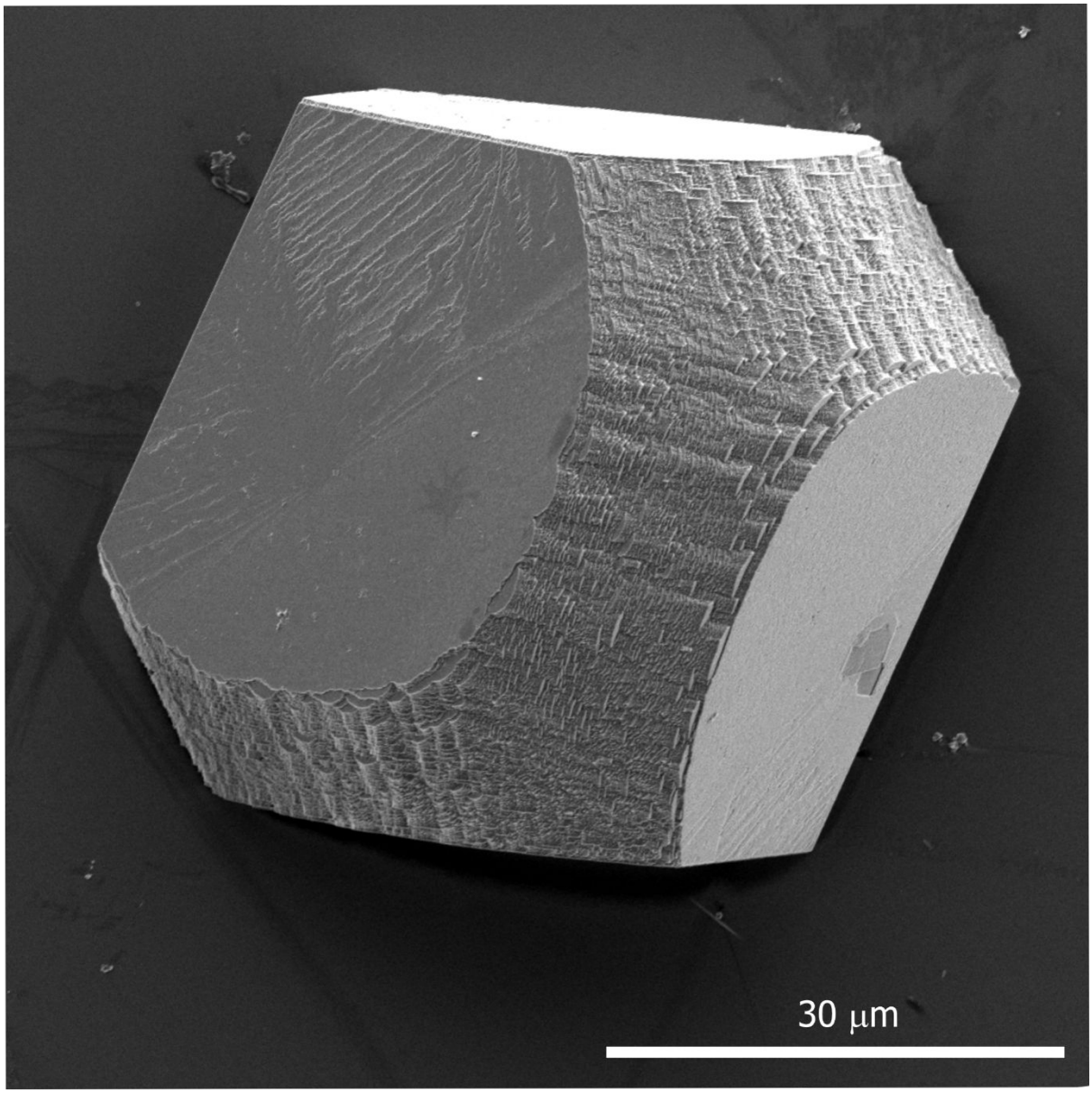

Figure S5: SEM micrograph of ADM-prepared calcite precipitated in the presence of [cp20k] = 0.015 mg/mL.

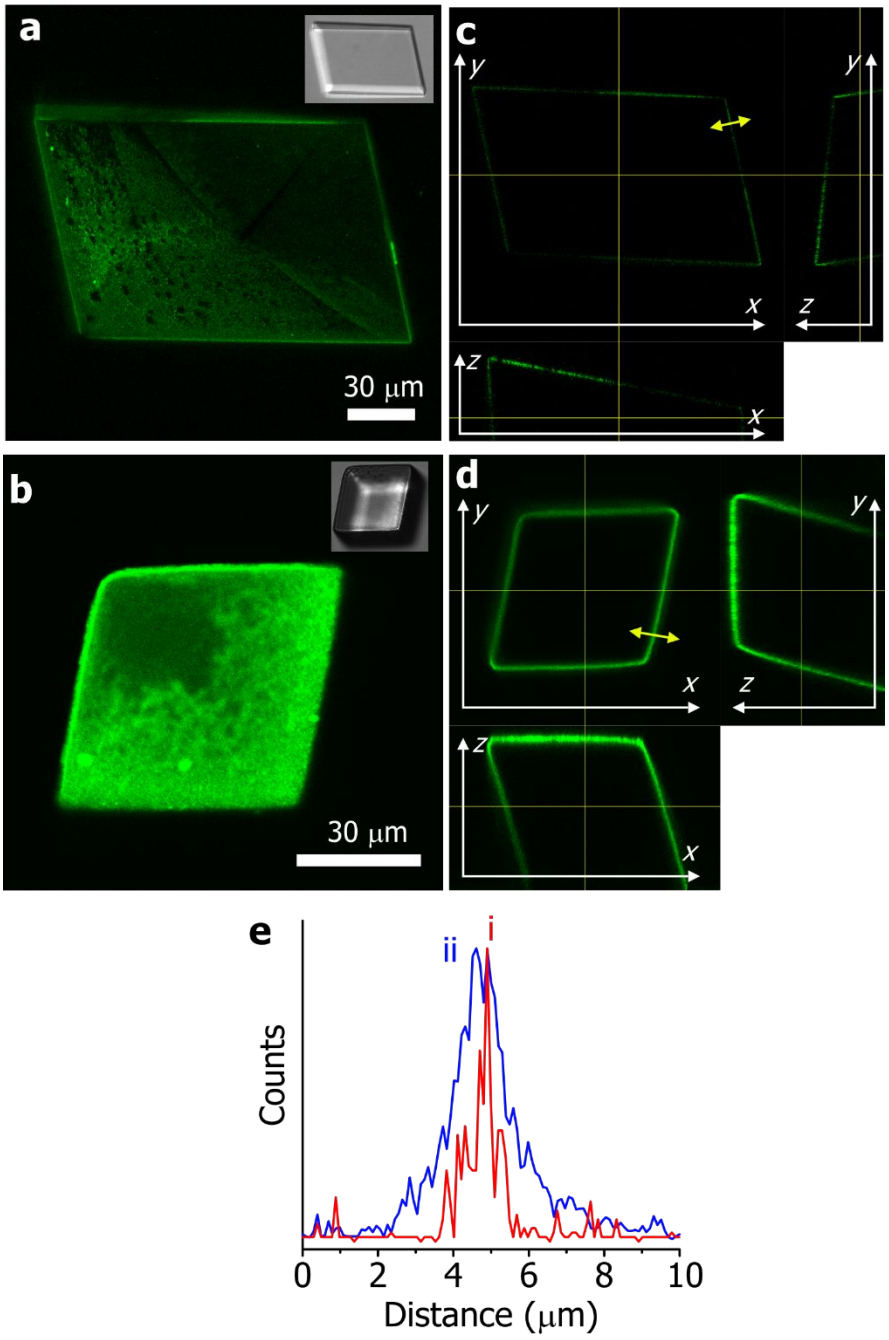

Figure S6: Corresponding CLFM analysis of samples from **Figure 4**. **a**) CFM micrograph of the dissolution sample (optical micrograph as inset). **b**) orthogonal views micrographs of the dissolution sample. **c**) CFM micrograph of the restructuring sample (optical micrograph as inset). **d**) orthogonal views micrograph of the restructuring sample. **e**) Line profiles obtained from c (i, red line) and d (ii, blue line) showing the large difference in depth of the region where cp20k is incorporated.

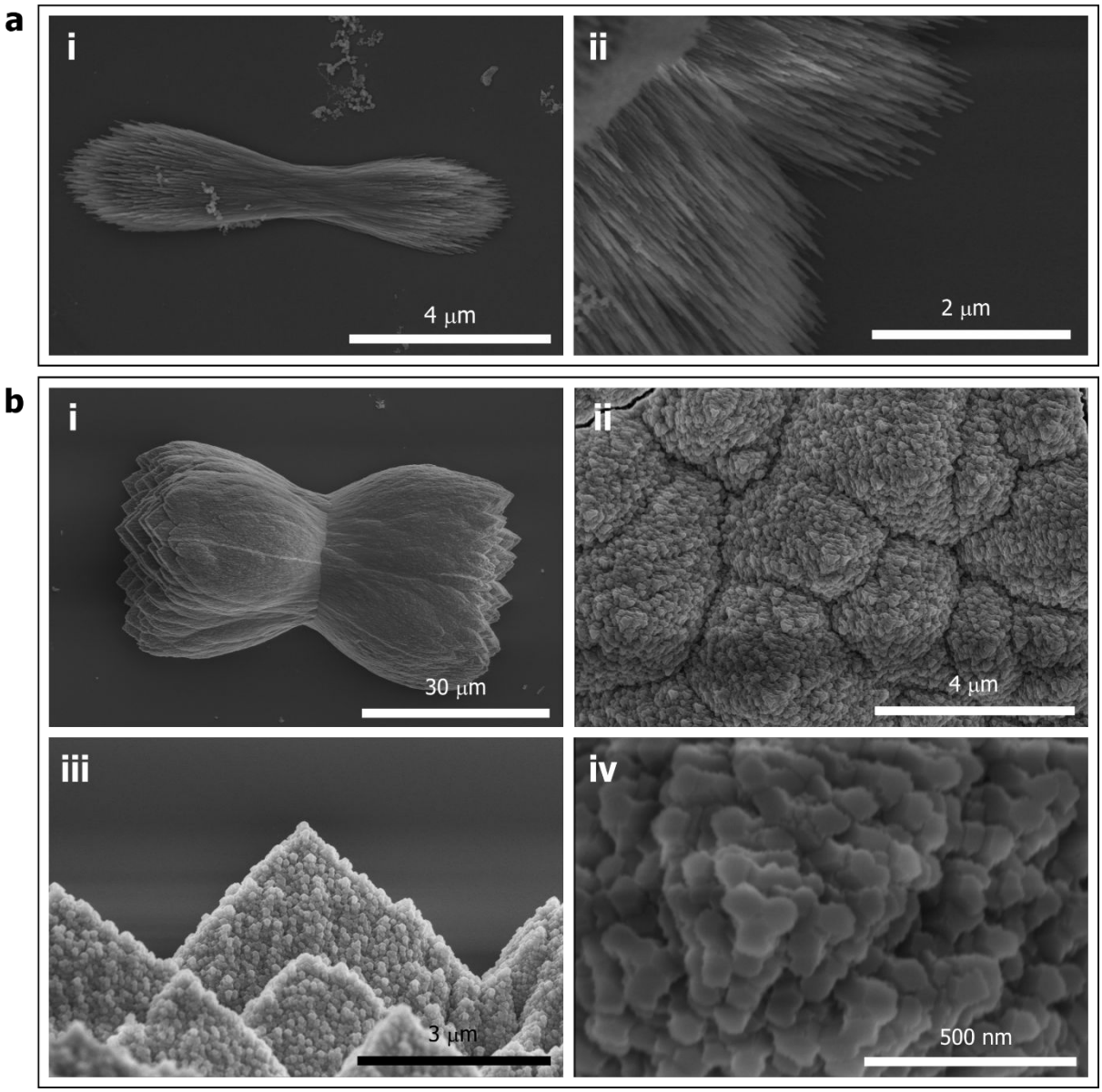

Figure S7: SEM micrographs of calcite grown in the presence of cp20k ([cp20k] = 0.015 mg/mL) and Mg^{2+} ($[Mg^{2+}]/[Ca^{2+}]$ = 1.0) by CDM ($[Ca^{2+}] = [CO_3^{2-}]$ = 5 mM) (**a**) and ADM (**b**). Low magnification micrographs (**ai** and **bi**) show overall morphology, whereas higher magnification micrographs (**aii**, **bii-iv**) show nanoshoot or surface structure in greater detail.

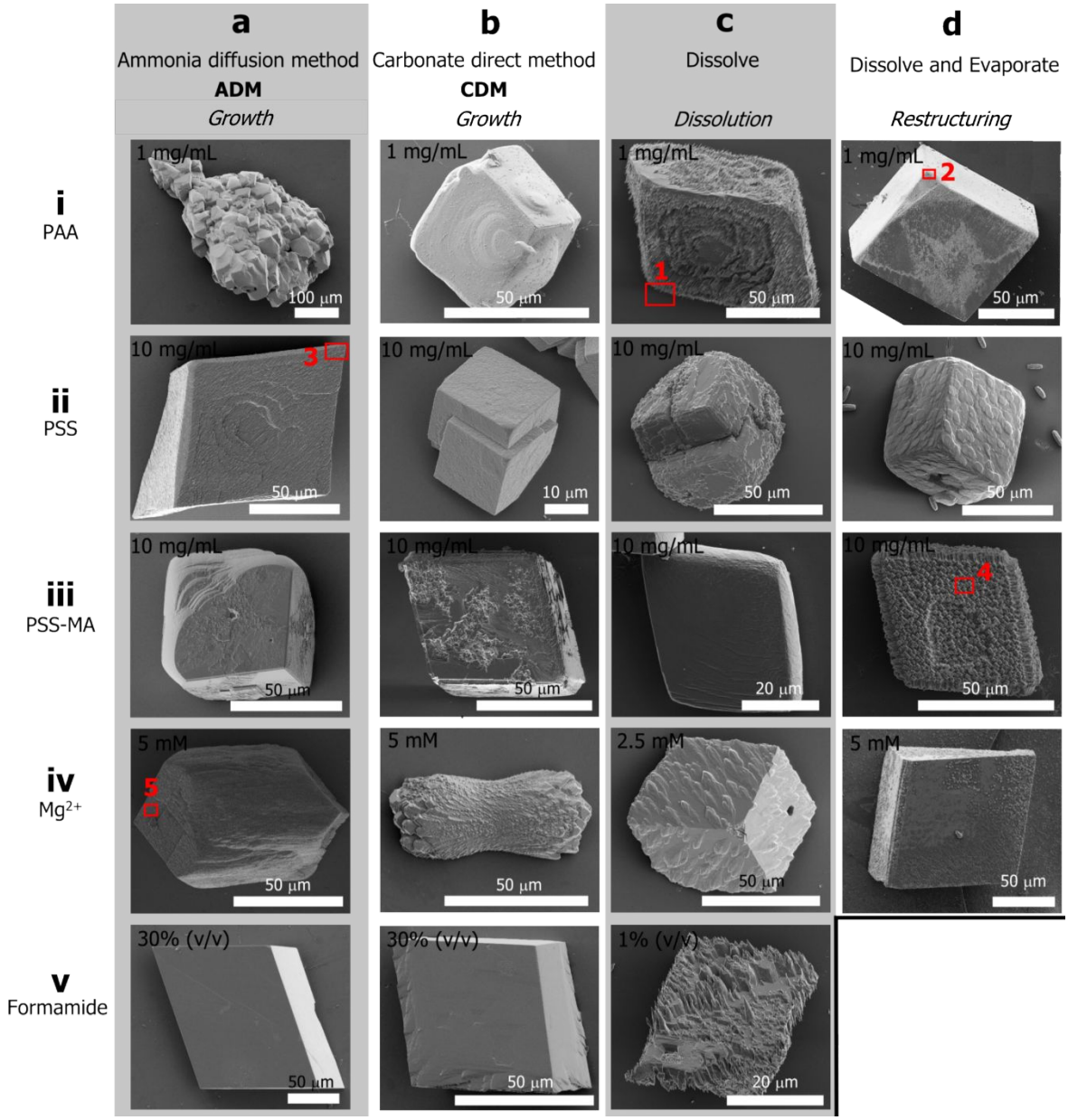

Figure S8: SEM micrographs of surface-roughened calcite prepared with various methods calcite was precipitated de novo using ammonia diffucsion method (ADM, **a**) or the carbonate direct method (CDM, **b**); or calcite seeds were dissolved (**c**) or surface restructured (**d**) in the presence of additives PAA (**i**), PSS (**ii**), PSS-MA (**iii**), Mg^{2+} (**iv**) or formamide (**v**). Restructuring studies with formamide were not possible due to high boiling point. Higher magnification images of boxes 1-5 are shown in **Figure 7**.

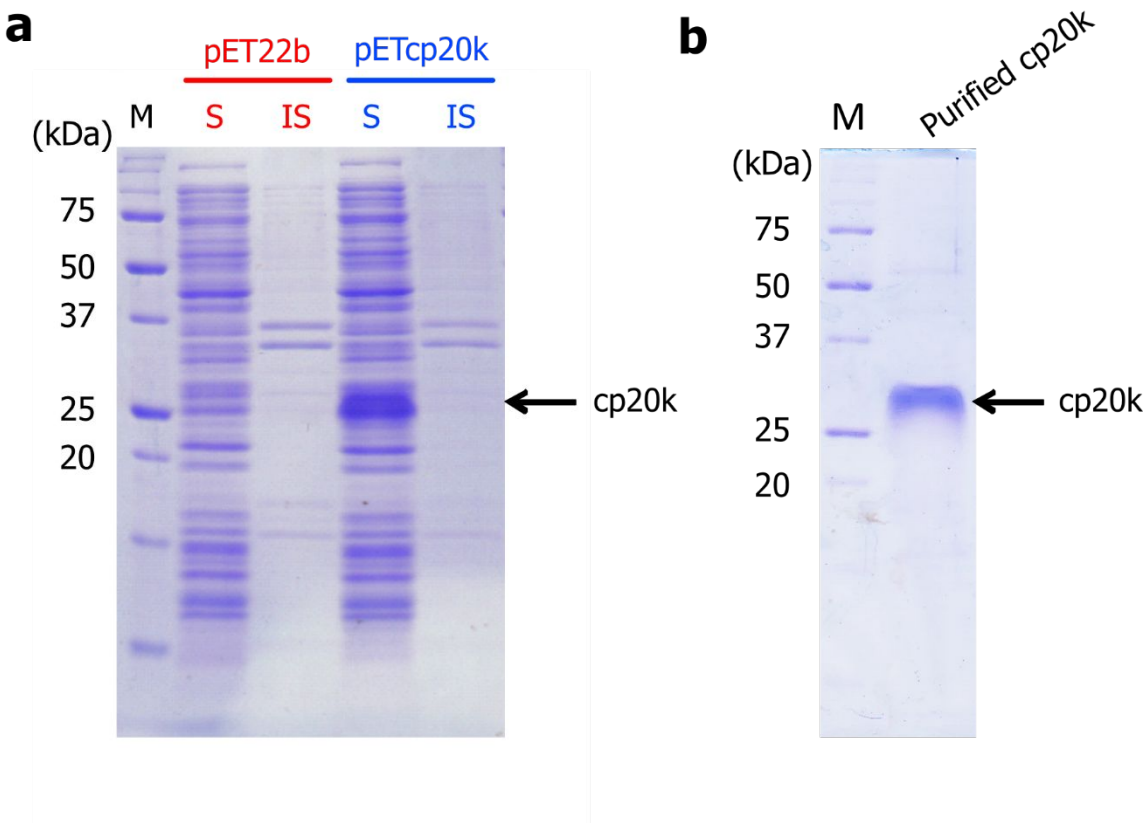

Figure S9: **a**) SDS-PAGE of purified cp20k from *E. coli* cell lysate. Cell lysate from *E. coli* harbouring pET22b (control) and pETcp20k. The expression of cp20k was able to be confirmed. M: molecular weight marker, S: soluble fraction of cell lysate, IS: insoluble fraction of cell lysate. Arrow represents the position of cp20k. **b**) Electrophoresis of purified cp20K. Single protein band was confirmed.