Supporting information

Ubiquitin Designer Proteins as a New Additive Generation toward Controlling Crystallization

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1. Protein expression

1.1 Expression of Ub N25M D32M D58M (UbMet3)

The cDNA encoding human N25M D32M D58M Ub was cloned into the pGEX2TK vector (Figure S20) by BamH I / EcoR I double digest and T4 DNA ligase (NEB) ligation. Methionine (Met) auxotrophic E. coli B834 (DE3) transformed with pGEX2TK-N25M D32M D58M Ub was cultured in LB medium containing 100 mg L⁻¹ carbenicillin at 37 °C overnight. The pre-culture was diluted with LB medium containing 100 mg L⁻¹ carbenicillin to an OD₆₀₀ value of 0.1. Cells were grown at 37 °C until they reached an OD₆₀₀ of 0.6, before expression of GST fusion protein was induced by addition of 1 mM IPTG. Cells were cultured at 37 °C for 6h before they were harvested. The pellet was kept at -20 °C. Then, cells were resuspended in 1xPBS buffer containing 1% Triton X-100, and lysed by sonication. The lysate was clarified by high speed centrifugation and the supernatant was incubated with glutathione agarose beads at 4 °C for 5 h. The beads were packed into a column, washed three times with 1xPBS, and incubated with 10 units of thrombin (Sigma) at room temperature overnight. The released N25M D32M D58M Ub was eluted with (10x0.3 ml) of 1xPBS. The purity of proteins was analysed by 12.5 % SDS-PAGE followed by Coomassie blue staining, and the concentration was measured by BCA protein assay (ThermoFisher).

1.2 Expression of Ub N25Aha D32Aha D58Aha (UbAha3)

The cDNA encoding human N25M D32M D58M Ub was cloned into the pGEX2TK vector by BamH I / EcoR I double digest and T4 DNA ligase (NEB) ligation. Methionine (Met) auxotrophic E. coli B834 (DE3) transformed with pGEX2TK-N25M D32M D58M Ub was cultured in LB medium containing 100 mg L⁻¹ carbenicillin at 37 °C overnight. The pre-culture was diluted with NMM medium containing 0.04 mM Met and 100 mg L⁻¹ carbenicillin to an OD₆₀₀ value of 0.1. Cells were grown at 37 °C until they reached an OD₆₀₀ of approximately 1.3, harvested and resuspended in the same volume of fresh NMM supplemented with 0.5 mM Aha.
After incubation at 37 °C for 30 min, expression of GST-fusion protein was induced by addition of 1 mM IPTG and cells were further incubated overnight at 25 °C. Then, cells were pelleted, resuspended in 1xPBS buffer containing 1% Triton X-100, and lysed by sonication. The lysate was clarified by high speed centrifugation and the supernatant was incubated with glutathione agarose beads at 4 °C for 5 h. The beads were packed into a column, washed three times with 1xPBS, and incubated with 10 units of thrombin (Sigma) at room temperature overnight. The released N25Aha D32Aha D58Aha Ub was eluted with (10x0.3 ml) of 1xPBS. The purity of proteins was analysed by 12.5% SDS-PAGE followed by Coomassie blue staining, and the concentration was measured through BCA protein assay (ThermoFisher).

1.3 Modification of UbAha3 with propargyl phosphate (UbP3)

Generation of phosphate modified Ub (Figure S1a) was performed by CuAAC in 20 mM Tris-HCl (pH 8) supplemented with 0.5 mM SDS under argon atmosphere. N25Aha D32Aha D58Aha Ub (45 µM) was mixed with 100-fold excess of propargyl phosphate (4.5 mM) and 6 mM THPTA before the reaction mixture was flushed with argon. The reaction was initiated by adding 3 mM Cu(MeCN)₄BF₄ and the reaction mixture was flushed with argon again. After incubation on ice for 3 h, the reaction was quenched with 15 mM EDTA and dialyzed at 4 °C overnight 25 mM HEPES buffer (pH 7.5). Analysis of the reaction was performed via MS measurement (Figure S1b).

2. Gas Diffusion Mineralization experiments

Calcium carbonate precipitation was induced by using the gas/vapour diffusion method.¹ CaCl₂ droplets with three different types of genetically engineered Ubiquitin protein (Figure S3) were set in a sealed desiccator in the presence of ammonium carbonate. Two types of protein were used as reference (UbMet3 and UbAha3, Figure S3a and b) to differentiate between alterations caused by the ubiquitin protein (UbMet3), the unnatural aminoacid Aha (UbAha3) and the incorporation of mineral interacting functions (Phosphate groups) in UbP3.
CaCO₃ mineralization was induced by the decomposition of (NH₄)₂CO₃ and the consequent diffusion of NH₃ and CO₂ molecules into calcium chloride droplet. This resulted in an increased of the pH of the solution and in the reaction of aqueous CO₂ to form carbonic acid, which in turn deprotonated into both carbonate and bicarbonate ions. As a consequence, supersaturation with respect to calcium carbonate was reached. During the gas diffusion experiments, the initial and final pH were approx. 6.0 and 9.5, respectively.

Experimental runs were carried out in 24 cell culture microwell plates placed on top of the porcelain plate in the hermetically-sealed desiccator (Duran DN 150 with 2.4 dm³ internal volume). Plates were covered with a lid in which holes of a diameter of 0.5 mm were made to allow the in-diffusion of ammonia and carbon dioxide. In each well, 20 µl drops of solution were deposited on top of 5 x 5 mm Si wafer to facilitate their ex-situ analysis. A wide range of CaCl₂ and protein concentrations were studied in every experimental run (Figure S21).

A petri dish with 0.5 mg of freshly pestled ammonium carbonate was covered with a slid, with three pinholes (diameter of 1 mm), and was placed at the bottom of the desiccator. Aqueous solutions were prepared utilizing calcium chloride (Sigma Aldrich, C4901) and doubly deionized water (Milli-Q, resistivity >18.2 MΩ·cm). The concentrations of calcium and Ub variants were varied within 10-20 mM and 0.01-2.5 mg mL⁻¹, respectively. The experiments were carried out for 5 min to 1 day as indicated. The mineralizations were stopped by removing the well plate from the desiccator. Drops were removed from the wafer using filter paper and then the wafers were dried in air and characterized.

Note that CD-spectroscopy experiments showed that typical calcium concentrations (around 10 mM at the start of experiments) do not affect the protein structure of Ub (Figure S22), whereas the pH level upon NH₃ in-diffusion before precipitation is moderate (pH 9.0-9.5), and does not affect Ub structures either. The protonation probability of protonatable amino acids in Ubiquitin was calculated using the pKa-values obtained by PropKa3.1². The pdb file of Ubiquitin 1UBQ³ was used. The protonation probability for
pH 7.0 (starting pH of our gas diffusion experiments) and 9.4 (approx. maximum pH during gas diffusion experiments) was calculated using the following equation:

\[
\text{probability} = \frac{1}{1 + 10^{(\text{pH}\text{–}pK_a)}}
\]

The change in protonation probability between the two pH is shown in Figure S23. It can be seen that there are not large differences between the probabilities of protonation at both pH. The only significant difference correspond to the protonation state of N-terminal.

3. CO₂ free experiments

Complementary experiments in a CO₂-free environment were carried out to check the interaction between NH₃, calcium ions and UbP3 protein. In these experiments, a 20mM calcium chloride drop with a concentration of 1 mg mL⁻¹ of UbP3 was deposited onto a Si wafer (similar as in the gas diffusion experiments in the desiccator described in the section above). CaCl₂ and NH₃ solutions were freshly prepared in CO₂-free water as follows. Briefly, Milli-Q water was boiled for 4 hours to remove the aqueous CO₂ prior to storage under N₂ atmosphere. 20 mM CaCl₂ solution was prepared by dissolving CaCl₂ dihydrate (3.895 g, 20 mmol, MW 147.01 g/mol, Sigma Aldrich) in one liter of the CO₂-free water under N₂ atmosphere. Saturated aqueous NH₃ solution was prepared by bubbling NH₃ gas through CO₂-free Milli-Q for 1 hour and stored under N₂ atmosphere. Then, N₂ atmosphere was created within a rounded bottom flask in which a glass vial with the Si wafer was previously introduced. Following, 10 ml of NH₃ solution were introduced in the flask and a 20 µl calcium chloride (20mM) drop containing 1 mg mL⁻¹ UbP3 was deposited onto the Si wafer. Experiments were run for 1-5 h and no film was detected afterwards. The drops were dried with a filter paper still under N₂ atmosphere. SEM analysis of the Si wafer are shown in Figure S16.
4. Characterization of the samples

4.1 SEM observations

Scanning electron microscopy (SEM) imaging of the dried Si wafer was performed on TM3000 (Hitachi), CrossBeam 1540 XB and FESEM Auriga (Zeiss). Si wafer were Au or C coated (10-15 nm thick layer) prior to analysis which allowed better imaging of the resulting precipitates.

4.2 TEM observations

In the course of selected experimental runs, samples were taken by dipping carbon film coated copper TEM grids into the CaCl$_2$ drop. TEM and HRTEM analysis of grids was carried out using a Philips CM20 and JEOL both operated at 200 kV. TEM observations were performed using a 40 μm (CM20) or a 30 μm (JEOL) objective aperture. SAED patterns were collected using a 10 μm aperture, which allowed collection of diffraction data from a circular area of 0.5 μm in diameter.

4.3 ESEM observations

In-situ gas diffusion experiments were carried out in an Environmental Scanning Electron Microscope (ESEM) in which wet samples can be imaged by means of varying partial water vapour pressure in the microscope specimen chamber. The WET-STEM system allows transmission observations of suspensions dispersed in a TEM grid if the film is thin enough.$^4$ The integrated STEM detector allows detection of electrons transmitted through the sample. The ESEM was operated at 30 kV and the samples could be observed simultaneously in Bright field (BF) and Dark field (DF) transmission mode and in SEM mode.

A CaCl$_2$ drop (1 μl) was set onto a TEM grid and 1 mg of ammonium carbonate was placed next to it. The diffusion of NH$_3$ and CO$_2$ molecules into the drop yields to CaCO$_3$ precipitation which could be imaged in situ in SEM and STEM mode (Figure S11 and S12). Two types experiments were carried out: only with CaCl$_2$ (20mM) in the drop and another with CaCl$_2$ (20mM) and UbP3 (1 mg mL$^{-1}$).
4.4 ATR-FTIR measurements of CaCO$_3$ on Si wafer

Attenuated-total-reflection Fourier-transform infrared (ATR-FTIR) spectra were recorded in the region 4000-650 cm$^{-1}$ with a Perkin Elmer spectrometer 100, equipped with a diamond ATR crystal (universal ATR-sampling accessory). 16 scans were performed for each spectrum with a spectral resolution of 4 cm$^{-1}$. After gas diffusion experiments the Si wafers were placed on the diamond crystal and force was applied on the samples before a measurement was performed. For all sample measurements, the Si wafer was used as background reference. Measurements were performed at least twice to confirm reproducibility.
5. Figures

Figure S1. a) Reaction scheme of click reaction to modify UbAha3 (N25Aha D32Aha D58Aha) with phosphate linker propargyl phosphate. b) MS analysis of the modification of three proteins used in this study UbMet3, Ub3Aha and UbAha3 with propargyl phosphate (UbP3).
Figure S2. Scheme for the incorporation of the unnatural amino acid Aha in a nascent protein by selective pressure incorporation. The growth medium (NMM) contains Azidohomoalanine (Aha) instead of methionine. The endogenous MetRS and tRNA\textsuperscript{Met} utilize Aha instead of Met to be incorporated into the protein.

a) \(\text{UbMet3} \quad (\text{Ub N25M D32M D58M})\);
b) \(\text{UbAha3} \quad (\text{Ub N25Aha D32Aha D58Aha})\);
c) \(\text{UbP3} \quad (\text{Ub N25Aha+Phos. D32Aha+ Phos. D58Aha+ Phos})\).

Figure S3. Scheme of the three types of Ubiquitin used in this study. a) UbMet3 (Ub N25M D32M D58M); b) UbAha3 (Ub N25Aha D32Aha D58Aha); c) UbP3 (Ub N25Aha+Phos. D32Aha+ Phos. D58Aha+ Phos).
Figure S4. FESEM images of the obtained CaCO$_3$ precipitates from gas diffusion experiments at different calcium and UbP3 concentration. All scale bars 10 µm.

Figure S5. Elemental analysis of a) CaCO3 film deposited onto TEM grid (inset) carried out in SEM. EDX maps of b) Ca, c) C, d) O, e) Cl and f) EDX point analysis of the film shown in Figure 1a in the main manuscript.
Figure S6. ATR-FTIR spectra of samples precipitated in 20mM CaCl₂ and in the absence of protein (control) and in the presence of 1 mg mL⁻¹ of UbMet3, UbAha3 and UbP3 (1 h). a) 2500-650 cm⁻¹ region with labelled CO₃²⁻ IR vibrational modes and b) 4000-650 cm⁻¹ showing traces of structural water at ~ 3300 cm⁻¹ and 1640 cm⁻¹.

Figure S7. ATR-FTIR spectra of samples precipitated in 40mM CaCl₂ and increasing amounts of UbP3 as indicated. a) 2500-650 cm⁻¹ region with labelled CO₃²⁻ IR vibrational modes and b) 4000-650 cm⁻¹ showing traces of structural water at ~ 3300 cm⁻¹ and ~ 1640 cm⁻¹.
Figure S8. FESEM images of the obtained precipitates from gas diffusion experiments at different calcium and UbMet3 concentrations. All scale bars 10 µm.

Figure S9. FESEM images of the obtained CaCO₃ precipitates from gas diffusion experiments at different calcium and UbAha3 concentration. All unlabelled scale bars are 10 µm.
**Figure S10.** Morphological changes induced by UbMet3 and UbAha3 on the CaCO₃ precipitates formed during gas diffusion experiments. a) Calcite rhombohedra formed in the presence of 1 mg mL⁻¹ of UbMet3 characterized by rounded edges and terraces in some of the surfaces (inset). b) Platonic shape calcite crystal with truncated edges obtained in the presence of 1 mg mL⁻¹ UbAha3. Macro steps of approximately 100 nm height were clearly distinguished (inset). c) Vaterite particle formed in the presence of 1 mg mL⁻¹ UbMet3. 100 nm sized nanoparticles subunits can be observed on the surface. Scale bars in the insets are 1 µm.

**Figure S11.** SEM image of the complete film formed in the presence of 1 mg/ml of UBP3 and 20 mM CaCl₂ concentration.
Figure S12. a) Scheme of the WET-STEM device used in an ESEM for annular dark field imaging. b) Representation of the experimental design of our experiments. Ammonium carbonate powder was placed next to a TEM grid in which a CaCl$_2$ drop (1 µl) was set and precipitation process in the presence and in the absence of protein (UbP3, 1 mg mL$^{-1}$) could be imaged in SEM and STEM mode.

Figure S13. Bright field STEM and secondary electron images of CaCO$_3$ precipitates obtained from gas diffusion experiments inside of the ESEM chamber. a) Precipitates obtained in protein-free experiments after 30 minutes. b) SEM image of vaterite precipitates after 45 minutes in the absence of protein. c) Wavy structures (film) with some rounded shapes (50 minutes). d) Fractal-like precipitates formed probably from the film after water loss.
Figure S14. Photographs of the Si wafers after 1h gas diffusion experiments. On the left side, the effect of 2.5 mg mL$^{-1}$ of UbP3 protein was investigated. The film could be seen by naked eye. On the right, a protein-free experiment is shown as a control.

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Figure S15. Temporal evolution of the obtained CaCO$_3$ precipitates during gas diffusion experiments carried out at fixed calcium (20 mM) and protein concentration (1 mg mL$^{-1}$). The three types of engineered proteins were tested (UbMet3, UbAha3, UbP3). In the upper side the identified phases by IR spectra (c=calcite, v=vaterite and acc=amorphous CaCO$_3$). The scale bar is 5 µm if unlabelled.
Figure S16. a) TEM image of the CaCO3 film formed in 20 mM CaCl2 solution and 1 mg mL⁻¹ UbP3; b) HRTEM image of the irradiated area showing lattice fringes due to calcite and vaterite (FFT in the inset shows d-spacings corresponding to 220cat, 110cal and 006cal); c) low magnification TEM image of the film in which the irradiated area (marked by blue circle) can be clearly distinguished from the part of the film that was not irradiated by the e-beam.

Figure S17. SEM images of the experiments carried out in a CO2 free environment but with NH3 diffusion into a 20mM CaCl2 drop with and without UbP3 (1 mg mL⁻¹). The drop was carefully dried with filter paper under N₂ atmosphere to avoid any contact with CO₂. No film was observed on the droplet’s surface by naked eye in either case. a) SEM image of CaCl2 precipitates formed on the Si wafer after 1h in the absence of UbP3 and b) Ca and b) Cl EDX maps. When UbP3 was present in the 20mM CaCl2 drop, apart from CaCl2 precipitates, d) the protein was found on the wafer as confirmed by EDX e) C and f) N EDX analysis.
Figure S18. A) TEM micrograph of the film and the nanoparticles formed. EDX analysis (inset) confirm the presence of C, O and Ca. b) Isolated spherical particle with film in the background. EDX analysis (inset in left corner) confirm the presence of C, O and Ca and SAED (inset in right corner) confirms its amorphous nature.

Figure S19. SEM images of the precipitates obtained in presence of the of 3mM propargyl phosphate (linker molecule) on CaCO₃ precipitation after 1h. a) 20 Mm CaCl₂ and b) 40 Mm CaCl₂. No clear effects were observed when an equivalent concentration of propargyl phosphate was present in the precipitation media.
Figure S20. SEM images of CaCO₃ precipitates formed between 5 and 24 h in the presence of 1 mg mL⁻¹ UbP3. Complex a) spherical and b) rhombohedral morphologies of CaCO₃ were found after the PILP film vanished. c) Higher magnification of the surface of spherical particles in a) shows elongated CaCO₃ rod bundles. d) The surface of the rhombohedral crystals also show rod bundles and prismatic sub-micrometer units.
**Figure S21.** Vector map of the plasmid used for the expression of Ub (N25M D32M D58M) and the generation of Ub (N25Aha D32Aha D58Aha). Scheme of Ub (N25M D32M D58M) as GST fusion protein and the cleavage of the fusion protein by thrombin.

**Figure S22.** Well plate ‘map’ showing the experimental design of the plates used for gas diffusion experiments. Colour coding of the wells is used for the different Ubiquitin protein tested (pink= UbMet3, purple=UbAha3, green=UbP3 and blank=no protein). Inside each well, the upper row corresponds to CaCl₂ concentration (10, 20 and 40 mM) and the row below denotes the protein concentration tested in this well (in mg mL⁻¹).
Figure S23. a) CD (circular dichroism) spectroscopy of Ub wild type, UbMet3 UbAha3 in standard buffer HEPES buffer. b) Comparison of CD spectra of the UBP3 in the presence and in the absence of 20 mM CaCl₂ (dashed lines).

Figure S24. Change in the protonation probability of ubiquitin from pH = 7 to pH = 9.4.
References


