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# Supporting Information

# Ceramophilic chitin and biohybrid materials enabled by a genetically engineered bifunctional protein

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## Materials and Methods

### Gene construct

The aspein gene DNA sequence from *Pinctada fucata* was codon optimized and sequence repetitiveness reduced for *E. coli* expression. A synthetic gene was ordered from GeneArt (Life Technologies) where the sequence for the chitin binding domain[1] was fused to the optimized aspein *via* a linker encoding GGSGGS amino acid sequence. The gene was flanked with BsaI restriction sites for "Golden gate" cloning into the plasmid vector pGBtacLacZ.[2] The insertion replaces the lacZ gene with the synthetic gene under the control of the tac promoter and downstream of pelB signal sequence for periplasmic expression. The sequence of the resulting plasmid pGG-ChBD-aspein20-77 was verified by sequencing (Macrogen, Netherlands). Protein expression levels in various *E. coli* strains were low. Thus, the construct without the pelB signal sequence was inserted as an NcoI/HindIII fragment into pET-28b(+) (Novagen) resulting in plasmid pET-ChBD-asp20-77 where the construct is under the *lac* repressor controlled T7*lac* promoter. The plasmid was then transformed into XL1Blue strain, sequenced and finally transformed into BL21Star(DE3) strain (Invitrogen)[3] that contains the T7 RNA polymerase needed for the expression. The amino acid sequence of ChBD-aspein is shown in Figure S1.

Figure S1. Amino acid sequence of ChBD-aspein. The chitin binding domain, linker and aspein fragment are indicated. Residues that have a negative or positive charge at pH 8 are colored red and blue, respectively.

### Protein production and purification

The protein ChBD-aspein was expressed from the plasmid pET-ChBD-asp20-77 by induction using 0.5 mM isopropyl β-D-1-thiogalactopyranoside at 37°C. Pelleted cells were resuspended in 20 mM bis-Tris buffer at pH 5.5 containing protease inhibitors (Complete EDTA-free, Roche). The cells were lysed by passing through a French press and the nucleic acids were removed by addition of nucleases (New England Biolabs). After centrifugation to remove cell debris the solution was applied to a HiTrap DEAE

FF anion exchange column (GE Healthcare) equilibrated to the sample buffer at pH 5.5 and eluted using a linear NaCl-gradient (20 mM bis-Tris buffer at pH 5.5 containing 1 M NaCl). Fractions containing the protein were pooled and further purified with reversed phase chromatography using a Vydac C4 column (Grace). A linear acetonitrile gradient (10 – 65 %) was used to fractionate the sample. Fractions containing the protein were pooled and freeze-dried. The yield of pure protein was about 50 mg per L of culture.

### Chitin binding assay

Protein at different concentrations were added to washed chitin beads (New England biolabs) in 20 mM Hepes buffer at pH 8.5 containing 500 mM NaCl and incubated 1 h at room temperature. The unbound protein was separated from the chitin beads by centrifugation and the amount of unbound protein was determined using the BCA-assay (Pierce) and compared to a standard curve.

### Chitin production and isolation

Fresh frozen lobsters were obtained from the market in Stockholm (CoopExtra, Sweden) as the starting materials for preparation of chitin nanofibres. The lobsters were washed in water to remove tissues and salts. The exoskeleton shells were freeze dried and crushed to powder in order to increase surface area for further chemical and mechanical treatments.

Chitin nanofibres were disintegrated from lobster exoskeleton. The powder from the lobster exoskeleton shells was demineralized against 2 M HCl for 2 hr. The demineralized powder was soaked overnight in 96% ethanol to remove pigments. Then, treatment to remove protein was performed with 20% concentration of NaOH for 2 weeks. All treatments were carried out at room temperature. The colloidal suspension was blended at pH 3 in the presence of acetic acid by a powerful kitchen blender (VM0105E, USA) and thereafter homogenized by passing 10 times through the microfluidizer (Microfluidics, USA); five times through 400 and 200 µm chambers and then, five times through 200 and 100 µm chambers.

### Biomimetic mineralization in solution

Mineral precursors $CaCl_2$ (0.5 M, aq) and $Na_2CO_3$ (99.5%) as well as NaOH were obtained from Sigma Aldrich and used without purification. Purified MilliQ water (18.2 mΩ) (Millipore) was used for dilutions. The mineralization of $CaCO_3$ was carried out in a glass beaker at room temperature. Aqueous solutions of 0.05 M $CaCl_2$ and 0.05 M $Na_2CO_3$ were prepared from feedstock. ChBD-aspein proteins were diluted into water with total volume of 45 ml and the pH of the solution was adjusted to pH 8 by using 0.1 M NaOH. $CaCl_2$ and $Na_2CO_3$ solutions were added into the protein solution simultaneously and slowly by two syringe pumps for measuring accurate volume dosage and to maintain even 40 ml/h flow. The solution was mixed with magnetic stirrer while adding precursor solutions. After addition of precursors the solution was stirred for ten minutes. One droplet of the solution was taken on the microscope glass and analysed using light microscope (Olympus BH-2). Crystals were centrifuged and washed with water three times. Few droplets of washed crystals in water were air dried on carbon tape for SEM measurements. Rest of the washed crystals were air dried overnight for XRD measurements.

### Film preparation

For preparing self-standing films CaCO3 mineralization was performed the same way as the mineralization in solution (above), but performed in chitin nanofibre suspension with or without

protein. Chitin nanofibres and proteins were diluted into water with total volume of 5 ml in a glass beaker, where the pH was adjusted to 8. Then 500 Joules was applied to the chitin nanofibre dispersions via tip sonicator (Vibra-Cell VCX 750, Sonics & Materials Inc.) to enhance the dispersity of the nanofibres. The used power was 40 % of the full output power. Varying the concentrations of $CaCl_2$ and $Na_2CO_3$, the solutions were measured out via syringe pumps keeping the flow at 10 ml/h. The dispersion was kept under magnetic stirring during the salt solution dosing; furthermore the dispersions were allowed to stabilize 10 minutes under magnetic stirring. Vacuum filtration was used to create the films from 5 ml of dispersions by the removal of water and buffer solution. The dispersions were filtrated using a Durapore membrane (GVWP, 0.22 µm, millipore, U.S.A.) and an O-ring, wherein the O-ring was used to determine the diameter of the films. After filtration the films were pressed gently with a 300 g load for 10 min to prevent wrinkling. Films were dried overnight in oven at +40°C.
Preparation of the $Ca^{2+}$-films was performed as explained above (biomineralized films) with the exception that only $CaCl_2$ was added by pipetting it to the chitin and chitin-protein dispersions after sonication. After addition of the $CaCl_2$ the dispersion was allowed to stabilize before vacuum filtration.

Scanning electron microscopy (SEM)

SEM (JEOL JSM7500FA field emission microscope, Japan) was carried out to image the cross-sections of the films and dried dispersion of $CaCO_3$ crystals using acceleration voltages of 2-15 kV depending on the sample. A thin Pd or Au-Pd layer was sputtered on top of the samples (Emitech K950X/K350, Quorum Technologies Ltd., Kent, UK). JSM-7500FA is also equipped with a JEOL energy-dispersive X-ray (EDX) analysis. Spectra were taken over 2 min using 15 keV electron energy to analyse the composition of samples.

Wide angle X-ray scattering (WAXS)

The measurements were done with the wide angle X-ray scattering (WAXS) set-up of the Laboratory of Electronic structure, University of Helsinki. The X-rays were produced with a conventional copper anode X-ray tube with point focus. Monochromatization was done with collimating Montel optics (Incoatec, Geesthacht, Germany) to obtain Cu-$K_\alpha$ radiation (wavelength $\lambda$=1.541 Å). The experiments were carried out in perpendicular transmission geometry with MAR345 image plate as detector (Marresearch, Norderstedt, Germany). The detector-to-sample distance was 8 cm.

The samples were layered two-fold on a sample holder and measured for 45 minutes. The angular calibration was done with lanthanum hexaboride and silver behenate samples. Intensities were corrected for absorption, geometry of the detector and air scattering.

The minimum crystal size *s* was calculated from the Scherrer formula:

$$s = \frac{0.9\lambda}{\sqrt{(\Delta 2\theta)^2-(\Delta 2\theta_{inst})^2}\cos\theta} \qquad (1)$$

where $\lambda$ is the wavelength of the radiation, $\Delta 2\theta$ is the full width at half maximum (FWHM) for the diffraction peak, $\Delta 2\theta_{inst}$ is the instrumental broadening determined from the FWHM of the (110) reflection of a thin $LaB_6$ sample, and $\theta$ is half of the scattering angle.

## Tensile testing

Tensile testing was performed on 5 kN Tensile/compression module (Kammrath & Weiss GmbH, Germany) using 100 N load cell with a nominal strain rate of 8.35 µm/sec (0.5 mm/min). The gauge length was 10 mm for all of the samples. At least 4 specimens were measured from each sample, with the exception of one sample (1x $CaCO_3$ + chitin), where the absence of protein ChBD-aspein degraded the film forming properties significantly. Thus only two samples could be prepared and measured with precision. Specimen sizes were 2 cm x 2 mm x 3-7 µm, length, width and thickness, respectively. Sample thicknesses were measured using linear gage (LGF-01100L-B transmission-type photoelectric linear encoder with EF-12PRH counter, Mitutoyo), herein at least 6 measurements from each sample was measured to calculate average value for thickness. The widths were measured with digital slide gauge (Digimatic, Mitutoyo). Samples were taken directly from oven into a desiccator from where the samples were transferred and attached to the tensile tester. Desiccator was kept in the relative humidity of ~30% and the tensile tester was held under humidity controlled box, where relative humidity was adjusted to ~30%.

## Cryo-Transmission Electron microscopy (CryoTEM)

Chitin nanofibre dispersions were characterized with JEOLS JEM-3200FSC Cryo- Transmission Electron Microscope operating at liquid nitrogen temperature. Specimens were blotted and subsequently vitrified in a mixture of liquid ethane and propane (-180°C) for cryo-imaging using a vitrobot (FEI, U.S.A.). Zero-loss imaging of vitrified samples was carried out with JEOLS JEM-3200FSC 300 keV TEM with an energy filter using slit size of 20 eV.

## Figures

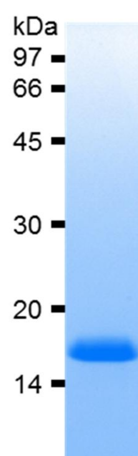


Figure S2. Purity of the ChBD-aspein protein. Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis of cytosolically expressed, ion-exchange and reversed-phase purified ChBD-aspein. The identity of the single band was verified by western blot using an anti-ChBD antibody (NEB). The apparent molecular weight of ChBD-aspein is higher than the calculated 11 kDa based on the amino acid sequence. The very hydrophilic and charged nature of aspein may bind less SDS and thus have a non-standard electrophoretic mobility as has been reported before.[4] Molecular weight markers in kDa are indicated on left.

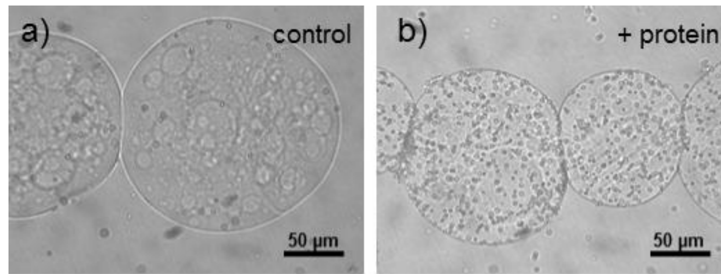


Figure S3. Optical microscopy shows that in solution the formed crystals are preferentially bound to the ChBD-aspein functionalized beads (b) and not to the unmodified beads (a).

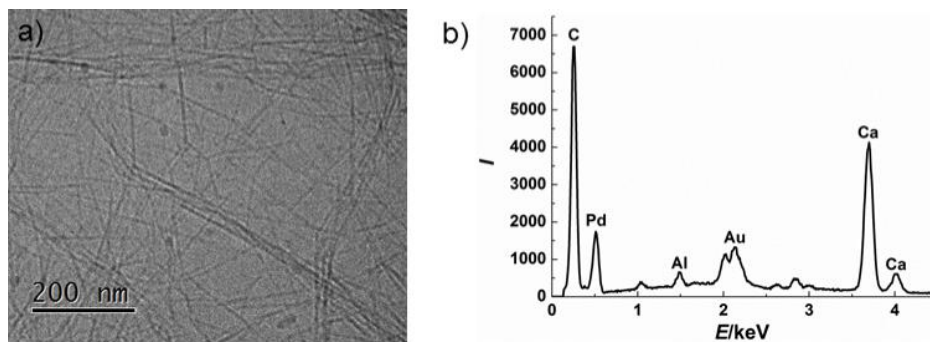


Figure S4. a) Cryo-TEM image of a vitrified dispersion of plain native chitin nanofibres. b) An EDX spectrum showing the presence of $Ca^{2+}$-ions in a ChBD-aspein modified chitin film formed from a suspension containing $CaCl_2$. X-axis is the energy in keV and y-axis is the intensity.

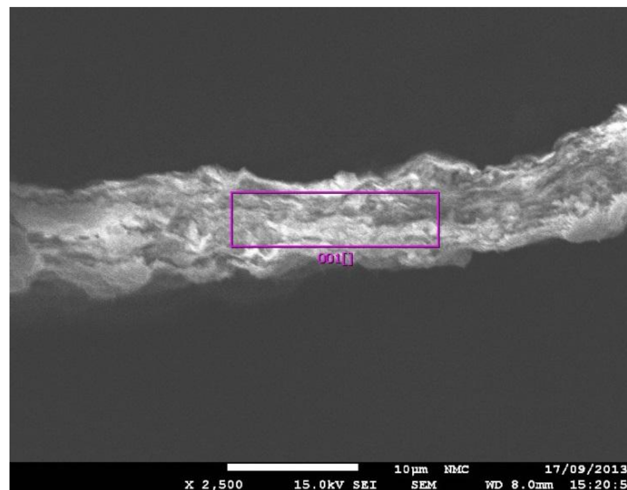


Figure S5. SEM-image of a cross-section from a 10x$CaCO_3$-Chitin-ChBD-aspein-film. EDX-spectrum was recorded from the squared area.

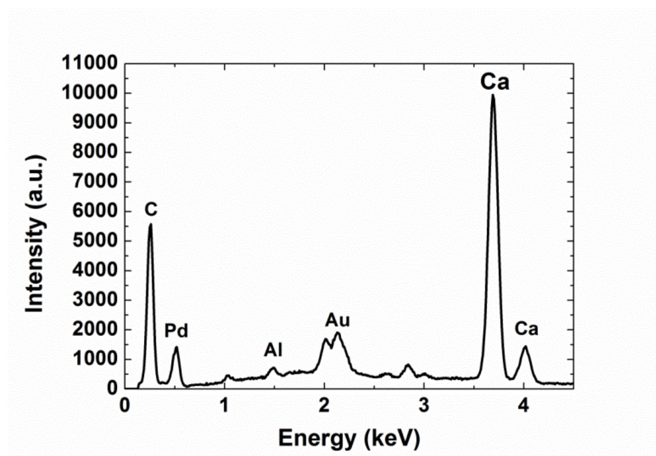


Figure S6. EDX-spectrum from the squared area in Figure S5.

## References

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