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Bioinspired Functionally Graded Composite Assembled Using Cellulose Nanocrystals and Genetically Engineered Proteins with Controlled Biomineralization

Pezhman Mohammadi,* Julie-Anne Gandier, Nonappa, Wolfgang Wagermaier, Ali Miserez, and Merja Penttilä
Methods and materials

1.1 Cloning, protein production, and purification

All DNA sequences were codon-optimized and synthesized by GeneArt gene synthesis (Thermo Fisher Scientific) for expression in *Escherichia coli*. These include sequences encoding the following: bacterial type three CBM from *Ruminiclostridium thermocellum* (Uniprot ID: Q06851), aspartic-rich cluster residues 131-293 from the sequenced fragment of *Odontodactylus scyllarus* club mineralizing protein 1 (CMP1) named eCMP1\(^{\text{Asp}}\), 6 repeats of tandem repeat motif 1 from the sequenced fragment of *Eumeta variegate* fibroin named BGW6 \(^{[1]}\), 80 repeats of tandem repeats of \((VPGVG)_{80}\) name ELP\(^{[2]}\), 4 repeats of tandem repeats of \(((A)_{25}K(VPGVG)_{15}GD)_{4}\) named eSilEl\(^{[2]}\) and 4 repeats of tandem repeats of \(((YKYKYKY(VPGVG)_{5})_{3}GD(A)_{25}K)_{4}\) named eSilEl\(^{(+)}\) and 4 repeats of tandem repeats of \(((YKYKYKY(VPGVG)_{5})_{3}GD(A)_{25}K(A)_{12}KSV VYV)_{4}\) by combining parts of BGW6 and eSilEl and named eBGWSilEl\(^{(+)}\). All constructs were made using seamless golden gate cloning assembly of synthetic fragments in pEt-28a (+) (kanR) protein expression vector (Novagen) in frame with the C-terminal 6×His-tag coding sequence for facilitating the purification \(^{[3]}\). Resulting 3-block protein architectures were named CBM-BGW6-CBM (subsequently denoted as reinforcing protein 1 or RP1 in the text and the figures), CBM-ELP-CBM (described in the text and the figures as reinforcing protein 2 or RP2), CBM-eSilEl-CBM (described in the text and the figures as reinforcing protein 3 or RP3) and CBM-eBGWSilEl\(^{(+)}\)-CBM (described in the text and the figures as reinforcing protein 4 or RP4).

Finally made a mineralizing protein. This was a modified version of RP4 whereby the C-terminus CBM was replaced with the Asp-rich cluster residues 131-293 from CMP1\(^{[4]}\) (called eCMP1\(^{\text{Asp}}\)), and all the poly-Lys stretches of eBGWSilEl\(^{(+)}\) were mutated to glutamic acid (Glu) thus forming CBM-eBGWSilEl\(^{(+)}\)-eCMP1\(^{\text{Asp}}\) (subsequently denoted as mineralizing protein 1 or MP1 in the text and the figures). For cloning purposes 10-beta (New England Biolabs) competent E. coli (Δ(arα1eu) 7697 araD139 fhuA ΔlacX74 galK16 galE15 e14 φ80dlacZΔM15 recA1 relA1 endA1 nupG rpsL (Str\(^{R}\)) rph spoT1 Δ(mrr hsdRMSmcrBC)) was used. Unless otherwise stated Luria-Bertani (LB) medium and LB-agar plates were used for growth at 37 °C overnight with the following antibiotics when appropriate: kanamycin (50 μg/ml), ampicillin (100 μg/ml). The resulting constructs were either transformed into E. coli strain BL21 (FompT hsdSB (rB'mB') gal dcm (DE3)) (ThermoFisher Scientific), BL21 T7 express™ (fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr' 73::miniTn10 TetS)2 [dcm] R(zgb 210::Tn10 TetS) endA1 Δ(mcrC mrr)114::IS10) (New England Biolabs) or BL21 Star™ (FompT hsdSB(rB'mB) gal dcm rne131 (DE3)) for the expression. For the expression, one colony from a freshly grown overnight LB-plate was picked and cultured in 5 mL LB-media supplemented with kanamycin (50μg/ml) for 6 hours at 37 °C-250 rpm which was then used to inoculate 500 mL fresh LB media in
a 2 L Erlenmeyer flask that was then grown (37 °C-250 rpm) until mid-log phase (OD600 ~0.4; 2 to 3 hours) at which point recombinant expression was induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (Sigma-Aldrich) and the temperature decreased to 20 °C. Cultures were then harvested 15-20 hours after induction by centrifugation at 16,000 xg, 15min, 4°C. Cell pellets were resuspended with 5 mL of Lysis Buffer containing: 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5, 5 mM Mgcl2, 200 mM NaCl, 20 mM Imidazole (Sigma-Aldrich), 0.5 mg ml-lysozyme (Chicken egg white, Sigma-Aldrich), 0.01 mg ml−1DNase I (Bovine pancreas, Sigma-Aldrich) and 1 × SIGMA FAST protease inhibitor cocktail (EDTA). Sonication (Qsonica 500) was used to physically break the cells using 20% amplitude input, for 2 minutes with 2-second intervals with the cells on ice. Cell debris was collected by centrifugation at 16,000 xg, 60 min, 4°C. This was followed by purification of the soluble fraction using HisTrap FF immobilized metal affinity chromatography (IMAC) connected to an ÄKTA-Pure fast protein liquid chromatography system with the following binding and elution buffers: binding buffer contains 500mM NaCl, 20mM imidazole, pH 7.4 and elution buffer contains 500 mM NaCl, 500 mM imidazole, pH 7.4. Buffer exchange was carried out with the Econo-Pac10 DG desalting prepacked gravity-flow columns (Bio-Rad) against 30 mM Tris-HCl pH 7.4. Proteins were analyzed by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4%-20% gradient gels (Bio-Rad). SDS-PAGEs were stained with Coomassie Brilliant Blue stain. For storage, samples were frozen with liquid nitrogen and kept at -80 °C until their use.

1.2. Preparation of Cellulose nanocrystals (CNC)

The cellulose nanocrystals were purchased from the USDA’s Forest Products Laboratory (FPL, Madison, WI) through the Process Development Center at the University of Maine. CNCs were prepared by sulfuric acid hydrolysis of dissolving-grade wood fibers followed by dialysis against Milli-Q water (Millipore Synergy UV). The resulting CNC showed average dimensions of 5–20 nm in width and 150–200 nm in length after characterization with TEM and dynamic light scattering (DLS).

1.3. Coacervation of the proteins

After the protein purification, coacervation of the reinforcing proteins as well as mineralizing protein (unless otherwise stated) were triggered by the addition of the common Hofmeister salt ion, potassium phosphate (K₂HPO₄) with a purity grade of 99.5% (Sigma-Aldrich). We prepared coacervation inducing solution containing 2 M potassium, 10 mM Tris-HCL buffer and the pH was adjusted to 7.4. Coacervation was induced by intermixing 1 M coacervation inducing solution with 100 μM protein solutions. Salt-protein solutions were
incubated for 4 hours. Coacervates were collected using centrifugal force 16,000 ×g for 20 min at room temperature (RT), followed by three washing steps using Milli-Q water before use. All coacervate solutions were prepared fresh without any intermediate storage step.

1.4. Composite film preparation

Composite films were prepared by mixing CNC (in MW water) at a concentration of 3% w/v and the reinforcing proteins in their coacervate form (in MQ water) at desired concentrations ranging from 10-40 % with respect to the concentration of CNC. Under controlled conditions (50% relative humidity), CNC-coacervate solutions were concentrated by evaporation to the critical concentration of CNC (6% w/v), after which they were allowed to fully dry at ambient temperature and 20% relative humidity (RH) for a period of 2 days. To make the bulk samples, instead of concentrating the CNC-coacervate solution to 6% w/v, solutions were concentrated by evaporation to 10 % w/v after which they were allowed to dry under similar condition except for the period of 4 days to form highly expanded chiral nematic superstructure.

1.5. Biomineralization of the composite films

Biomineralization solution (modified simulated body fluid (SBF)) was prepared according to the earlier protocol. All chemicals were purchased from Sigma-Aldrich with purities of 99.5 % or higher. The solution contained 138.3 mM NaCl, 3.02 mM KCl, 1.32 mM K2HPO4, 3.26 mM MgCl2, 2.65 mM CaCl2, 4.22 mM NaHCO3, 0.50 mM Na2SO3 and 50 mM NH2C(CH2OH)3 its pH adjusted to 7.4 using 1M HCl. Biomineralization for all the samples including the controls (samples without mineralizing proteins) was initiated by placing the dried specimens on the bottom of the vacuum manifold filter plate and rehydrating them overnight by submerging them under 0.5 ml Milli-Q water without disturbance of the self-assembled chiral nematic superstructure. Milli-Q water was then removed using vacuum suction (0.3 mBar). To make the composite with mineralization proteins, specimens were infiltrated by mineralizing proteins in the coacervate form at the concentration of 10 % with respect to the concentration of CNC. Unbound and excess coacervate solutions were removed by vacuum suction. All the samples including the controls were submerged under 3 mL biomineralization solution and incubated at RT for a period of 16 days. Samples were rinsed 3 times once per day with Milli-Q water followed by the addition of freshly prepared biomineralization solution until fluorapatite crystals were formed on the CNC surfaces. Samples were allowed to completely dry at RT for 4 days under 20% RH after day 16.

1.6. Atomic force microscopy
A Veeco dimension 5000 AFM instrument was used and images were recorded in tapping mode in the air with scan rates of 0.8–1 Hz with a FASTSCAN-B cantilever.

1.7. Fluorescence recovery after photobleaching (FRAP)

FRAP was performed using Leica TCS SP5 confocal microscope with FRAP booster (DM5000) and a DD488/561 dichroid beam splitter at 63x/1.2 (water objective). Samples labelled with Oregon Green 488 (carboxylic acid, succinimidyl ester, 6-isomer (Thermo Fisher)) were bleached using a laser at 488 nm with spot diameters of 2.5 to 5 µm. The intensity traces were collected using Leica AF Lite–TCS MP5, upon passing the emission through the 88/561 dichroid and detected by a standard photomultiplier. Fitting of the data and calculation of the diffusion coefficient were carried out according to equation (1).[^5]

\[
D = \left(\frac{V_0^2\gamma_0}{4\gamma_2}\right) \left[\frac{\tau_c}{\tau_{1/2}}\right]^2
\]

1.8. Optical, fluorescence and polarized microscopy

Multiple microscopy platforms were used for the visualization. These include (1) Olympus IX81 Microscope equipped with a motorized stage, DP70 digital camera, and a × 60 or 100/numerical aperture optics. Images were taken in either fluorescence, phase contrast or bright field mode. (2) In order to qualitatively study birefringence of the specimens, Leica DM4500 P LED polarized microscope was used as described previously.[^6] Briefly, samples were placed between two cross-polarizers and interference colour angles corresponding to differences in order parameter were determined at ±45°. (3) Leica TCS SP5 confocal microscope equipped with x 100/1.2 water objective was used to studying the nucleation and growth of the coacervates. Images were taken either in fluorescence, phase contrast or bright field modes.

1.9. Dynamic light scattering (DLS)

DLS measurements were performed using a Malvern Zetasizer Nano-ZS90 at 173 backscattering angle equipped with a 633 nm He–Ne laser and controlled temperature at 22 ±1 °C. Results are illustrated as mean with standard deviation after fifteen measurements (N=15).

1.10. Turbidity measurement
Coacervation induced by potassium phosphate were detected by changes in the turbidity of the samples at OD_{600}. Samples were prepared by intermixing a 1:1 ratio of protein to potassium phosphate at the desired final concentrations with the final volume of 200 µl. Measurements were performed using cytation™ 5 multi-mode microplate reader with controlled temperature at 22 ±1 °C. For each measurement, backgrounds were subtracted from readouts.

1.11. Thioflavin T (ThT) assay

For the ThT assay, ThT powder (Sigma-Aldrich) was dissolved in Milli-Q water to make a 1mM stock solution. Before the measurement, the ThT stock solution was centrifuged at 16,000xg for 15 min followed by filtration through a 200 nm filter to remove undissolved ThT. The ThT solution was then diluted using Milli-Q water to a working concentration of 0.01 mM and added to the protein solutions. The ThT-protein solution was then allowed to equilibrate for 15 minutes at 22 ±1 °C before the addition of potassium phosphate to its desired concentration. Fluorescence measurements were carried out using cytation™ 5 multi-mode microplate reader with an excitation wavelength of 450 nm and an emission wavelength of 492 nm at 22 ±1 °C. Measurements include: (1) ThT in combination with protein and absence of potassium phosphate, (2) potassium phosphate solutions in the absence of protein and in Milli-Q water, and (3) ThT in Milli-Q water. Backgrounds for all the measurement were subtracted from readouts.

1.12. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)

ATR-FTIR measurements were performed using a Unicam Mattson 3000 FTIR spectrometer equipped with diamond crystal plate (PIKE Technologies GladiATR). Spectrums collected using 120 scan samples were scanned for 120 time in absorbance mode within the range of 400–4000 cm⁻¹, with a resolution of 2 cm⁻¹. Samples were dried for a minimum of 12 hours before measurement (stated in the text if otherwise), to minimize the noise from water molecules.

1.13. Circular dichroism (CD)

Chirascan™ CD equipped with a temperature-controlled unit (23 ±1 °C) was used to collect spectra, using a QS quartz cuvette with 1 mm path length. For the protein solutions, data acquisition was performed across the wavelength range of 190–260 nm and 1nm bandwidth, with 1 nm steps and averaging time of 0.5 s. For each sample, measurements were repeated eight times, and all spectrums were averaged and smoothed. For the CNC-
coacervate suspensions, data acquisition was performed in the same manner; however, the 190-800 nm wavelength range was scanned.

1.14. Scanning electron microscopy (SEM)

SEM imaging was carried out using Zeiss FE-SEM field emission microscope with variable pressure, operating at 1–1.5 kV. All samples were sputtered with a 5 nm platinum layer before imaging. ImageJ Fiji (versions 1.47d) software package was used for visualization and analysis of the micrographs.^[7]

1.15. Analytical scanning transmission electron microscopy (STEM), selected area electron diffraction (SAED) and energy dispersive spectroscopy (EDS)

Imaging was carried out using JEM-2800 Transmission Electron Microscope operated at 200 kV in a TEM mode or 3kV in STEM mode. Samples were handled using 200 mesh carbon (CFT200-Cu) or Quantifoil 3.5/1 holey carbon copper grids with 3.5 μm holes which were cleaned using a Gatan Solarus 9500 plasma cleaner. Selected area electron diffraction (SAED) and energy dispersive spectroscopy (EDS) mapping was also performed with the same equipment using standard settings.

1.16. Tomography

Tomography was performed according to the earlier method.^[8] Briefly, 3 μL of the coacervate suspension was vitrified by plunging it into a mixture of ethane and propane (-180°C). Samples were kept frozen under liquid nitrogen and transferred into a FreeZone 4.5 Liter Cascade Benchtop Freeze Dry System equipped with a collector cooling chamber at -110 °C. STEM JEOL JEM-2800 microscope was used to acquire tomographic tilt series. Specimens were tilted between ± 72° angles with 8° increment steps. Correlation alignment was performed using the IMOD software package. The maximum entropy method (MEM) was used for reconstruction, using a custom made program on a Mac with a regularization parameter value of λ= 1.0e–3. Chimera software package was used for visualization and analysis of the tomograms.

1.17. Simultaneous synchrotron wide/small-angle X-ray scattering (WAXS/SAXS) and X-ray fluorescence (XRF)

WAXS/SAXS scattering and XRF measurements were performed at the μSpot beamline at BESSY II (Berliner Elektronenspeicherring-Gesellschaft für Synchrotronstrahlung, Helmholtz-Zentrum Berlin, Germany). The
energy exposure used was 15 keV (0.82656 Å) using a silicon 111 monochromator and a beam size of either 50 or 100 μm. Data was recorded using an Eiger X 9M detector with a pixel size of 75x75 μm. XRF was performed by positioning a seven-channel Silicon drift detector (80 mm² surface area) at a 60-degree angle with respect to the beam path to simultaneously acquire both scattering and fluorescence signals. All measurements were performed at 50% relative humidity and ambient temperature. Dried specimens were clamped into a custom made sample holder, which was mounted on a motorized stage, enabling positioning of the samples perpendicular to the beam path. Diffractograms were recorded either from several points along each sample (100 μm apart) or specimens were mapped. To map the specimens, a mesh of diffractograms were acquired (including the empty space in the surrounding specimen) with each spot separated 100 μm apart in x and y directions for a total of 1787 diffraction patterns. The in-situ assembly-dehydration experiment was carried out by casting 0.5 mL of pre-assembled protein/CNC suspension across a flat stainless steel mesh and allowing dehydration over a four hour time period, while collecting the WAXS/SAXS signal by guiding the x-ray beam through the center of each mesh. Intensities around the equator and meridian were integrated radially using the DPDAK program with a built-in algorithm from the pyFAI software package. For all measurements, air scattering and dark current were subtracted from the diffractogram. Azimuthal intensity profiles at the (200) and (004) reflection were extracted by sector-wise integration after masking the diffractogram to show only either the (200) or (004) reflection ring. Subsequently, from the azimuthal intensity profile, the Hermans orientation parameter was calculated according to equations 2 and 3 and the orientation index according to equation 4. Matlab and OriginLab were used for processing and presenting the data.

\[
S = \frac{3}{2} \langle \cos^2 \Phi \rangle - \frac{1}{2}
\]

\[
\langle \cos^2 \Phi \rangle = \frac{\sum_0^\pi I(\Phi) \sin \Phi \cos^2 \Phi}{\sum_0^\pi I(\Phi) \sin \Phi}
\]

\[
\Pi = \frac{180 - FWHM}{180}
\]

where FWHM (Φ) is the Full-Width-at-Half-Maximum.
1.18. Nuclear magnetic resonance (NMR) spectroscopy

HSQC $^{13}$C-$^1$H correlation and HETCOR $^1$H-$^{13}$C NMR spectra were acquired for non-coacervate and coacervated samples to identify the conformationally sensitive Alanine Cβ and Cα chemical shifts according to our earlier study.\[8\] Briefly, the HSQC signal was obtained using the Bruker AVANCE III 500 NMR equipped with a magnetic flux density of 11.7 T, 5 mm BBFO probe head and inverse geometry. D2O was substituted with 50% H2O (1:1 D$_2$O:H$_2$O) to minimize noise. Protein concentration for the measurements was 15 mg/ml unless otherwise stated in the text. Collected spectral midpoint and the width for proton was 4.7 ppm and 13 ppm respectively with an average value of 145 Hz for one-bond J-coupling between protons and carbons. A minimum of 4 scans were obtained for every 512 increments, 1.5 s successive scan and acquisition time of 200 ms in the indirectly detected dimension. Spectral width of 220 ppm, acquisition time of 360 ms, and 4096 scans with a 1.5 s repetition delay were recorded. The solid-state spectra acquired for the protein was obtained using a MAS frequency of 12.5 kHz. A linear ramp from 49.0 to 61.2 kHz radio-frequency (RF) field amplitude on $^1$H and 65.8 kHz amplitude on $^{13}$C was used for CP, 0.5 ms. Spinal 64 decouplings were applied during acquisition with a $^1$H RF field amplitude of 83.3 kHz, a pulse length of 5.8 μs, an acquisition time of 10 ms and a recycle delay of 2.5 s.

All solid-state NMR spectra for the mineralized samples were measured with an Agilent DD2 600 NMR spectrometer with a magnetic flux density of 14.1 T, equipped with a 3.2 mm T3 MAS NMR probe operating in a double resonance mode. Samples were packed in ZrO$_2$ rotors, MAS rate in experiments was set to 10 kHz. Cross polarization $^{31}$P{$^1$H} experiments were performed with a 1.4 ms contact time and 3.5 s delay between successive scans, the number of scans was 5000. The two dimensional $^{31}$P{$^1$H} frequency-shifted Lee-Goldburg heteronuclear correlation (FSLG-HetCor) spectra were acquired with 128 scans for 64 increments in indirect dimension, with other parameters being the same as for 1D cross-polarization experiment. Protons were decoupled during acquisition using SPINAL-64 proton decoupling with a field strength of 80 kHz. $^{31}$P 90 degree pulse durations and Hartmann-Hahn match for cross-polarization were calibrated using diammonium hydrogen phosphate (NH$_4$)$_2$HPO$_4$. The spectra were processed using TopSpin 4.0 software.

1.19. Nanoindentation measurements

All samples were equilibrated at 50% relative humidity for at least for 24 hours at 22 ±1 °C temperature before measurement. All mechanical measurements were performed at 50% relative humidity ±2 and 22 ±1 °C temperature. Nanoindentation was performed under controlled conditions using Anton Paar Tritec’s Ultra nano hardness tester (UNHT) with a Berkovich diamond tip. The effect of the topography was minimized by finely
polishing the sample surface. A brick-shaped sample was cut with dimensions of L=5mm, W=3, T=0.3. Indentations were performed using a maximum load of 0.3 mN with loading and unloading rates of 0.6 mN/min with a dwell time of 20 s at the peak load to account for creep behaviour. At different regions across the specimens, two adjoined lines were measured with steps of 20 μm between lines and 8 μm between each indent along the line to prevent that plastically deformed zones from previous indents do not affect the measurements. To obtain reduced modulus and hardness, the load-displacement curves were analyzed using the methods described by Oliver and Pharr.\(^{[10]}\) Accordingly, hardness was calculated based on equation 5 and modulus using equation 6 and 7.

\[
H = \frac{P_{\text{max}}}{A}
\]

Where, \(H\) is defined as hardness, \(P_{\text{max}}\) is defined as the maximum load, \(A\) is defined as indentation area.

\[
S = \frac{dP}{dh} = \frac{2}{\sqrt{\pi}} E_r \sqrt{A}
\]

Where, \(S\) defined as stiffness can be obtained from the slope of the initial portion of the unloading curve \(dP/dh\). \(A\) is defined contact area. \(S\) can be used to calculate Young’s modulus.

\[
\frac{1}{E_r} = \frac{(1-\nu_s^2)}{E_t} + \frac{(1-\nu_f^2)}{E_i}
\]

Where \(E_r\) defined as reduced modulus which accounts for elastic displacement for both specimen and the indenter. \(E_t\) is defined as the elastic modulus and \(\nu_s\) is defined as Poisson’s ratio for the specimen, while the \(E_i\) and \(\nu_i\) are the same quantities but for the indenter.

### 1.21 Tensile testing

Measurements were performed with a 5 kN tensile/compression module (Kammrath & Weiss GmbH, Germany) using either 5 or 100 N load cells with an elongation speed of 2 μm/second and a gauge length of 5 mm. For tensile tests, the film samples were cut into 2.5 mm wide and 15 mm long stripes. The specimens were glued 5 mm from their ends between two pieces of abrasive sandpaper to prevent any slippage during the measurement. The elongation and stress were calculated according to equation 8 and 9 respectively.\(^{[11]}\) The strain was calculated by dividing the absolute elongation with the original gauge length and multiplied by 100%. The ultimate strength was taken as the highest stress point before the sample underwent catastrophic failure. The modulus was defined as the average slope of the stress-strain curve in the elastic region of the stress-strain curve.
before the yield point. Toughness was defined by calculating the area under the stress-strain curve. Cross-sectional areas were determined from SEM images. All data were processed using an in-house written script in Matlab to extract the mean values and standard deviations for Young’s modulus, maximum strength, maximum strain and toughness (n = 7).

\[
\varepsilon = \frac{\Delta L}{L_0} = \frac{L - L_0}{L_0}
\]

(8)

Where, \(\varepsilon\) defined as strain, \(\Delta L\) is defined as a change in gauge length, \(L_0\) the initial gauge length, and \(L\) as final length.\[^{[11]}\]

\[
\sigma = \frac{F_n}{A}
\]

(9)

Where, \(\sigma\) is defined as stress, \(F\) is defined as tensile force and \(A\) is the nominal cross-section.\[^{[11]}\]

1.22 Fracture Mechanics Measurements

i) Initiation Fracture Toughness (\(K_{ic}\)) measurements:

Fracture toughness measurements were conducted using three-point bending tests of bulk specimens performed on an Instron 5565 A equipped with a 500 N load cell at a loading rate of 2 \(\mu\)m/second with a support span of 10 mm in which the applied loading direction was perpendicular to the micro-platelet basal surface. All the specimens were cut into a rectangular shape with a width of 2 mm and thickness of 2 mm. For a single-edge notched bend similar specimens were made; however, they were notched to 50\% of their original width using a sharp diamond cutter and further sharpened using a stainless steel razor blade to make a notch with a final radius of approximate 50 \(\mu\)m. Measurements were performed at a constant displacement rate of 1 \(\mu\)m/second. Equations 10 and 11 were used to calculate fracture toughness (\(K_{ic}\))\[^{[12]}\].

\[
K_{ic} = \frac{P_{ic}S_n}{BW^{3/2}} f(a/W)
\]

(10)

Where, \(P_{ic}\) is defined as the ultimate in a single-edged notched beam test, and \(S\) is support span, \(B\) is the thickness of the specimen, \(W\) the width of the specimens, and the \(a\) is the depth of the notch, and \(f(a/W)\) the non-dimensional geometrical factor given by:
\[ f(a/W) = \frac{3(a/W)^{1/2}[1.99 - (a/W)(1 - a/W)(2.15 - 3.93a/W + (a/W)^2)]}{2(1 + 2a/W)(1 - a/W)^{3/2}} \]

Where, \( a/W \) represents the relative crack length.

**ii) R-curve measurements**

Crack-resistance curves (R-curves) illustrate the increase in fracture resistance \( (K_{eq}) \) with the crack extension \( (\Delta a) \) and were obtained as described previously.\(^{13}\) The equivalent fracture toughness after stable crack propagation is defined as the value right before final catastrophic fracture and is calculated from equations 12-15 using both \( J_{el} \) and \( J_{pl} \) contributions, related to the \( J \)-integral calculation. In this calculation, \( J_{el} \) is defined as the elastic contribution according to linear elastic fracture mechanics, \( J_{pl} \) is the plastic contribution, and \( A_{pl} \) is the area under the load-displacement curve. \( K_{eq} \) values can be calculated using the \( J \)-values (Equation 15), where \( E' \) is equal to \( E(1-\nu^2) \), \( E \) is Young’s modulus, and \( \nu \) is the Poisson’s ratio.

\[ J = J_{el} + J_{pl} \]

\[ J_{el} = \frac{K_{lc}^2}{E'} \]

\[ J_{pl} = \frac{2A_{pl}}{B(W - a)} \]

\[ K_{eq} = (JE')^{1/2} \]

The crack extension \( (\Delta a) \) was indirectly calculated according to equation 16-18 using:\(^{14}\)
\[ a_n = a_{n-1} + \frac{W - a_{n-1}}{2} \cdot \frac{C_n - C_{n-1}}{C_n} \]

Where \( a_n \) is the crack length, \( u_n \) is the displacement, \( f_n \) is the force, and \( C_n \) is the compliance calculated at each unloading point during stable crack propagation.

\[ C_n = \frac{u_n}{f_n} \]

\[ \Delta a = a_n - a \]

1.23. Dental implant crown formation

To formulate a material that highly resembles the intricate complex multi-layer architecture of the ductile club into a bilayer dental implant crown, we combined the composition and outstanding mechanical properties of both engineered proteins in combination with CNC. We used a numerical precision computer control machine to prepare a mould in the actual shape of the canine, first and second premolars. The mould was made from stainless steel and had a superhydrophobic coating (Figure S20). To formulate the bulk material for use in the dental implant, we first increased the dry mass of CNC from 10% w/v to 80% w/v under pressure by passing the suspension through a porous Teflon tubing connected to a syringe pump at room temperature while maintaining the reinforcing protein 4 (RP4) at 10% relative to the dry mass of CNC. This enabled the gradual removal of water while maintaining the CNC-protein suspension inside the tubing. In doing so the free-flowing suspension was transformed into a relatively stiff and injectable hydrogel. Next, the hydrogel of RP4 10% / CNC 80% w/v was injected into the moulds for the crowns (Figure S20) and allowed to dry completely under axial pressure maintained with a piston at 60°C. Crowns were then partly rehydrated in a stepwise manner by incubating them at 50%, 80% and 100% relative humidity for a period of 24, 48, 72 hours respectively. After this, they were immersed in the suspension of the condense coacervated phase of MP1 for 15 min without agitation. Excess MP1 was washed away from the surface of the crowns, by immersing them into MQ water mixed with 10mM chromophore stilbene photochroslinker for 2 min after which specimens were placed back at high relative humidity to partially dry for 48 hours. Before biomineralization, specimens were cross-linked using UV irradiation and immersed in 10 ml of biomineralization solution for 16 days. Biomineralization solution was replaced every day. Samples containing stilbene chromophore (Stilbene 420, Exciton) were cross-linked using...
UVC radiation from a low-pressure UV lamp (G36T5VH, Serve Tool Inc.) for 3 h by emitting both 185 and 254 nm wavelengths at 40 W. Crowns were then placed back into the mould and dried under pressure at 60°C with a final heat treatment in a stepwise manner by increasing the temperature in increments of 10°C from 60°C up to 120°C over 3 hours after which they were incubated for an additional 2 hours while heat was maintained at 100°C. To facilitate detachment of the crowns, the mould was then immediately cooled down using liquid nitrogen. The surface of the crowns were finely polished and smoothed by removing excess materials and all visible roughness.

1.24. X-ray micro-computed tomography

We used an RX Solutions X-ray micro-CT EasyTom 160 equipped with a micro-focus tube (XRay150, RX-Solutions, France), a tungsten filament and a flat panel detector (CsI scintillator). The equipment was operated at 100 kV acceleration tube voltage, 10 W tube power and 100 μA tube current. The sample do detector distance was 659.65 mm and source objective distance was 88.28 mm. Images were collected using an isometric voxel size of 17 μm, and acquisition of $n = 1120$ filtered back projections in the continuous 360-degree rotation. Image stacks were reconstructed with a cone-beam algorithm in the X-ACT software (RX-Solutions). 3D reconstruction was performed using 3D slicer (Version 4.10.2).

1.25. Cytocompatibility assay

All the chemicals including AHDF (product number 106-05A), Fibroblast Growth Medium (116-500), Trypsin-EDTA solution (T3924), Trypsin Inhibitor (T6414), Cytotoxicity Detection Kit (11644793001) were purchased from Sigma-Aldrich unless otherwise stated in the text. Alamar Blue™ (DAL1025) was purchased from thermofisher. Statistical analyses are presented as the mean ± standard error of mean. One-way analysis of variance ANOVA with post hoc Bonferroni was used for the statistical analysis. Results were considered significant if $P < 0.05$. In vitro cytocompatibility assessment on the surface of the crown was performed by culturing AHDF on the surface of the 2D film with the same composition as the outer surface of the bioinspired crown. As a comparison, we also cultured AHDF on the widely used tissue culture plate (TCP). The amount of released LDH was measured using a thirty-minute coupled enzymatic assay based on the conversion of a 2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride into a colored (red) formazin product in which the quantity of created color correlates to the number of lysed cells. For the measurements a 1:1 ratio of substrate mixture to sample prepared (50 μl : 50μl) was used (substrate prepared according to manufacturer instruction). Samples were then covered with aluminium foil to prevent light exposure and incubated for thirty minutes after which the reaction was stopped using 1M acetic acid (50 μl) solution. Attenuance ($D$) was then read at 450 nm.
using cytation™ 5 multi-mode microplate reader. Cell proliferation, cytotoxicity and viability was analysed using Alamar Blue™. To do so, Alamar Blue™ was added to the cell culture medium at a concentration of 10% (v/v). A 0.5 ml portion of the Alamar Blue™-cell culture medium mixture was then added to each sample, followed by a 4 hour incubation step. After this, about 200 μl of the samples were collected and their attenuation at fluorescence λex=530 nm and λem=620 nm was measured using a Fluoroscan Ascent FL spectrophotometer (Thermo Labsystems). The remaining cell-Alamar Blue™ mixture was replaced with fresh cell culture medium to continue the culture of the cells.

**Figure S1.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation of CBM-ELP-CBM, CBM-eSilEl-CBM, CBM-BGW6-CBM CBM-eBGWSiEl(+)4-CBM and CBM-eBGWSiEl(+)4-eCMP1(Asp) (MW: Molecular Weight).
**Figure S2.** Coacervation of CBM-ELP-CBM, CBM-eSilEl-CBM, CBM-BGW6-CBM, CBM-eBGWSiEl\(^{(+)}\)4-CBM, and CBM-eBGWSiEl\(^{(4)}\)-eCMP1\(^{(Asp)}\). In all cases, a 200 \(\mu\)M protein solution was intermixed with 1 M salt solution and incubated for 4 hours after which coacervates in the solution were sedimented using centrifugal force for better visualization.

**Figure S3.** Circular dichroism (CD) spectra collected for 20 \(\mu\)M CBM-ELP-CBM, CBM-eSilEl-CBM, CBM-BGW6-CBM, CBM-eBGWSiEl\(^{(+)}\)4-CBM, and CBM-eBGWSiEl\(^{(4)}\)-eCMP1\(^{(Asp)}\) before and after intermixing.
with 1 M potassium phosphate. Spectra illustrate conformational conversion from α-helical identified from the negative signature bands at ~210 and ~223 nm to β-sheet identified in a shift toward a single negative band for CBM-BGW6-CBM, CBM-eSilEl-CBM and CBM-eBGWSilEl(+)4-CBM. This shift is predominantly triggered by the presence of long poly-alanine crystalizing stretches. However, we noted the conformation of the CBM-ELP-CBM remains unchanged which can be identified due to the lack of poly-alanine stretches.

**Figure S4.** Coacervation phase diagram constructed for CBM-eBGWSilEl(+)4-CBM (right) and CBM-eBGWSilEl(+)4-eCMP1(Asp) (left) as a function of protein concentration and potassium phosphate (pH 7.4) concentration according to turbidity measurements. Protein concentrations ranging from 0.05 to 120 µM were tested along with potassium phosphate salt concentrations ranging from 0.05 to 1 M. Changes in the turbidity of the samples that accompany phase-separation and coacervation were detected using absorbance at 600 nm which resulted in the phase-diagrams at 22 ± 1 °C. For both proteins, the coacervation boundary was found to be at the critical concentration of 1 µM (protein) versus 0.05 M (salt) with a concave up-decreasing shape.
Figure S5. Monitoring the kinetics of coacervation for CBM-eBGWSiEl\textsuperscript{(+)}4-CBM and CBM-eBGWSiEl\textsuperscript{(-)}4-eCMP1\textsuperscript{(Asp)} using turbidity measurements (N=3), the relative fluorescence intensity of Thioflavin T (ThT) (N=3), and dynamic light scattering (DLS) (N=3) as a function of time for 20 µM protein and 1 M salt over twelve hours. The onset of turbidity was found to be between 0.1 and 0.19 a.u. for both proteins. Independent of protein type, turbidity increased gradually for about two hours followed by a slight decrease in the turbidity (potentially due to partial sedimentation) and a plateau region. This plateau remained unchanged until the end of the measurement. We monitored conformational conversion using Thioflavin T (ThT) dye. ThT can be used as a detection marker as it very specifically binds to the β-sheet structures of proteins resulting in enhanced fluorescence intensity at 492 nm. We noted an increase in the fluorescence intensity for both protein species. Readouts contained a short lag phase (0 to 1 hour), longer growth phase (1 to 8 hours) and a stationary phase (remaining measurements). We noted an increase in the signal one hour into the incubation period, after which intensity rapidly increased demonstrating conformational conversion from coil/α-helical to more β-sheet content. Both proteins showed assemblies with a size of about 30 nm at the onset of incubation. Increasing the incubation time resulted in an exponential growth of the coacervates. Looking at the readout we noted a rapid increase of standard deviation for the datapoints after four hours. This may potentially relate to the fact that the coalescence and the growth of the assemblies become a diffusion-limited process, which results in the formation of non-uniformly sized coacervates. Furthermore, coacervates may not remain as individual assemblies as they can bind together and form large clusters. It is evident from the ThT assay that over time β-sheet content of the coacervates increases. Coacervates would then behave as hard gels and may not merge together as fluid droplets as would be the case at the beginning of the assembly process. DLS typically has low-resolution for samples with a large polydispersity index. Therefore results after 4 hours may not be reliable.

Figure S6. The upper and lower plot illustrates fluorescence recovery after photobleaching (FRAP) measurements after four hours of incubation for coacervated CBM-eBGWSiEl\textsuperscript{(+)}4-CBM and CBM-eBGWSiEl\textsuperscript{(-)}4-eCMP1\textsuperscript{(Asp)} respectively. For both 100 µM protein and 1 M salt were used. ROI = 2 µm circular area; FRAP intensities normalized to one in all cases. We noted a substantial decrease in diffusion dynamics for both
proteins. Both samples demonstrated approximately 50% recovery due to the increase of the strength of the intermolecular interactions. This illustrates prominent partial liquid/solid-like characteristics due to physical crosslinking from β-sheet and formation of the bicontinuous network.

**Figure S7.** Extracted Young’s modulus versus maximum strength for the corresponding samples shown in (Figure 2i). Mean values ± SD (n = 7) are shown.

**Figure S8.** a) Extracted Young’s modulus versus maximum strength for pristine CNC film made from 6% w/v suspension and composite film at different protein (RP4) ratios with respect to the total dry mass of the CNC. Mean values ± SD (n = 7) are shown. b) Extracted Young’s modulus versus toughness for the corresponding samples shown in (a). Mean values ± SD (n = 7) are shown.
Figure S9. Calculated Hermans orientation parameter from azimuthal broadening (FWHM) of the (200) equatorial diffraction for the corresponding measurement shown in (f). Mean values ± SD ($n=3$) are shown.
**Figure S10.** Time-resolved in situ 1D synchrotron WAXS for the drying CNC (6% w/v)-CBM-eBGWSiEl4-CBM (10%) suspension. Data cover a total period of 00:05 to 04:00 hours.

![Graph showing intensity (I) vs. q (nm⁻²) for different time points (00:05, 01:00, 02:00, 04:00).]

**Figure S11.** Time-resolved in situ 1D synchrotron SAXS for the drying CNC (6% w/v)-CBM-eBGWSiEl4-CBM (10%) suspension.

![Graph showing orientation parameter vs. time (hh:mm).]

**Figure S12.** Calculated Hermans orientation parameter from azimuthal broadening (FWHM) of the (200) equatorial diffraction patterns from time-resolved *in-situ* synchrotron WAXS for the drying of pristine CNC.
(pink rectangles) and CNC (6% w/v)-CBM-eBGWSiElI(4)-CBM (10%) suspension. Data cover a total time span of 00:05 to 04:00 hours. Mean values ± SD (n = 3) are shown.

**Figure S13.** Time-resolved *in-situ* synchrotron WAXS for drying of CBM-eBGWSiElI(4)-CBM coacervated suspension alone.
**Figure S14.** Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) of the dried CBM-eBGWSiEl(4)-CBM samples before and after coacervation (4 hours into the coacervation). Spectra illustrate conformational conversion from alpha-helical to beta-sheet with a clear shift of the amide I band from 1635 (dominantly α-helical) to 1622 (β-sheet).
Figure S15. 500 MHz 2D 1H-13C HSQC before coacervation and 1H-13C HETCOR after coacervation of CBM-eBGWSilEl4-CBM, demonstrating the chemical shift for the Alanine Cβ and Cα. The chemical shift for 13C is found to be sensitive to slight differences in the structural conformation. The alanine shows strong peaks at 52.98 and 16.28 ppm. These correspond respectively to Cα and Cβ of Ala residues in the α-helical
conformation. In contrast after coacervation, we noted a clear shift ($C_\alpha = 49.98$ ppm and $C_\beta = 21.26$ ppm) for both carbons, indicating conformational conversion to $\beta$-sheet.

**Figure S16.** Calculated Hermans orientation parameter from azimuthal broadening (FWHM) of the (200) equatorial diffraction for the composite CNC-CBM-eBGWSilEl$^{(+)}$4-CBM with different contents of CNC while maintaining the CBM-eBGWSilEl$^{(+)}$4-CBM content of all specimens at 10%.
**Figure S17.** Calculated Hermans orientation parameter from azimuthal broadening (FWHM) of the (200) equatorial diffraction with CNC samples for reference at the various concentrations corresponding to Figure S16.
Figure S18. SEM micrographs of fractured cross-sections of pristine CNC after three-point flexural bending testing at low (top) and high (bottom) magnifications.
**Figure S19.** 2D solid-state $^1$H–$^{31}$P HETCOR corresponding to Figure 4c for samples incubated from 4 to 16 days during the apatite biomineralization process.
Figure S20. Full-width half max (FWHM) for sharpest $^{31}$P signal correlated with OH$^{-1}$ at $\delta$ ($^1$H) at 0.0 ppm corresponding to ordered $\text{PO}_4^{3-}$ of the apatite crystallite for Figure 4c and S18. Lower FWHM inversely correlates with higher-order and crystallinity.
Figure S21. Calculated hydroxyapatite precipitates as a function of time corresponding to polarized light microscopy images in Figure 4a.

Figure S22. In-house built injection moulding set up for the fabrication of the dental implant’s crown cap.

Sequence information:

CBM-ELP-CBM

MGNLKVEFYNSNPSDTTNSINPQFKVTNTGSSAIDLSKLTLRYYTVGDQKDQTFWCDHAIIIGSNGSY NGITSNVKTFVKMSSSTNNADTYLEISFTGGTLEPGAHVQIQGRFAKNDWSNYTQSNDYSFKSASQFVEWDQVTAYLNGLVWGKEPLEHHHHH
CBM-eSilEl-CBM

MGNLKVEFYNNSPDTSINIPQFKVTNTGSSAIDLSKLTLRYYTVDGQKDJTWFCDHAIIIGNSGYNGITSNVKGTFVKMSSTTNADTYLEISFTGTLTEPAHVGIIQGRFAKNDWSNYTQNSNDYSFKSASQFVEWEDQVTAYLNGVLVWGKE

CBM-BGW6-CBM

MGNLKVEFYNNSPDTSINIPQFKVTNTGSSAIDLSKLTLRYYTVDGQKDJTWFCDHAIIIGNSGYNGITSNVKGTFVKMSSTTNADTYLEISFTGTLTEPAHVGIIQGRFAKNDWSNYTQNSNDYSFKSASQFVEWEDQVTAYLNGVLVWGKE

CBM-eBGWSilEl\textsuperscript{(+)}-4-CBM

MGNLKVEFYNNSPDTSINIPQFKVTNTGSSAIDLSKLTLRYYTVDGQKDJTWFCDHAIIIGNSGYNGITSNVKGTFVKMSSTTNADTYLEISFTGTLTEPAHVGIIQGRFAKNDWSNYTQNSNDYSFKSASQFVEWEDQVTAYLNGVLVWGKE
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