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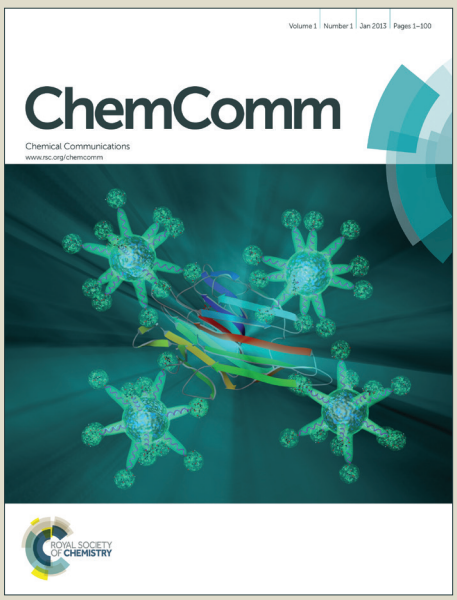

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Ceramophilic chitin and biohybrid materials enabled by a genetically engineered bifunctional protein

Cite this: DOI: 10.1039/x0xx00000x

Jani-Markus Malho,[a] Hanna Heinonen,[a] Inkeri Kontro,[b] Ngesa E. Mushi,[c] Ritva Serimaa,[b] Hans-Peter Hentze,‡[a] Markus B. Linder*[a,d] and Géza R. Szilvay*[a]

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/x0xx00000x

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A bifunctional protein composed of a highly negatively charged oyster shell protein and a chitin binding domain enabled the formation of biohybrid materials through non-covalent surface modification of chitin nanofibres. The results demonstrate that specific biomolecular interactions offer a route for the formation of biosynthetic materials.

Many materials in biological systems have remarkable mechanical properties, yet their material components are held together by weak interactions. [1] Cohesiveness between the components is an essential factor that contributes to the formation of strong materials from relatively weak components. [2] The key to the cohesive role of proteins in materials may be their multifunctionality afforded by multi-domain structures. Protein domain duplication and shuffling is an important mechanism in the evolution of new biological functions. [3] Each domain has a specialized function that can together contribute to the macroscopic material properties. Understanding the role of multifunctional proteins in the materials should bring new important insight to the architecture of biological materials and to the development of synthetic materials inspired by biology. [4]

In Nature biomineralization is an efficient way to create stiff and tough materials using genetic information. [5] A careful control of crystal polymorph, size and shape is achieved using multiple weak interactions. In nacre, for example, brittle aragonite $CaCO_3$ platelets are combined with proteins and chitin to form a mechanically superior material. [6] Therein, a number of multifunctional proteins have been identified that seem to interact with $CaCO_3$ aragonite platelets and an extracellular organic matrix, which may confer cohesion in this material. [7-9] Consequently, multifunctional proteins seem to be contributing to the mechanical properties of natural materials. [2, 10]

There is an on-going effort to mimic these structures for creating novel synthetic materials with already promising results. [11-14] Many recent studies highlight approaches to mimic Nature´s materials structurally and mechanically. [15-18] However, mimicking Nature`s design principles for guiding the construction of synthetic materials is as important as the final structures and functions. In this work we show how a genetically engineered bifunctional protein can be employed to self-assemble novel biomimetic materials *via* surface functionalization of chitin nanofibres with a mineralization domain. Importantly, our study highlights the direct relationship between specific molecular interactions and the mechanical properties of a material *via* molecular self-assembly and biological interactions.

We engineered a hybrid protein with two separate functionalities, one for binding chitin and one for interacting with cations and minerals. The hybrid protein contains a chitin-binding domain (ChBD) from a bacterial chitinase enzyme [19] and a fragment of aspein, a shell specific protein from the pearl oyster *Pinctada fucata.* [20] The aspein gene from pearl oyster was chosen because of its unusually high aspartate residue content and thus highly acidic nature, herein containing a block of 16 (28%) negatively charged residues (Fig. 1 a). A synthetic gene encoding a fusion of ChBD and aspein fragment (residues 20 – 77) was expressed in *Escherichia coli* and the resulting protein was purified as described in the ESI†.

First the functions of both protein blocks were assessed separately. A chitin binding assay showed that ChBD-aspein bound to the chitin substrate as expected with a similar association constant as reported for an isolated ChBD (Fig. 1 b). [21] For determining the effect of ChBD-aspein on $CaCO_3$ mineralization a double-jet experiment was used where a controlled and simultaneous addition of $CaCl_2$ and Na_2CO_3 solutions results in rapid nucleation of homogeneous crystals. [22] The formed crystals where washed and dried, and then imaged using SEM. In presence of ChBD-aspein the mineral particles had a uniform size of about 0.5 - 1 µm in diameter, in contrast to control samples where the particle size was about an order of magnitude larger (Fig. 1 c and d). All formed crystals where $CaCO_3$ vaterite according to powder X-ray diffraction analysis. While crystal polymorph was not affected in these conditions (absence of Mg^{2+}-ions), the protein had a clear influence on the morphology of the formed crystals.

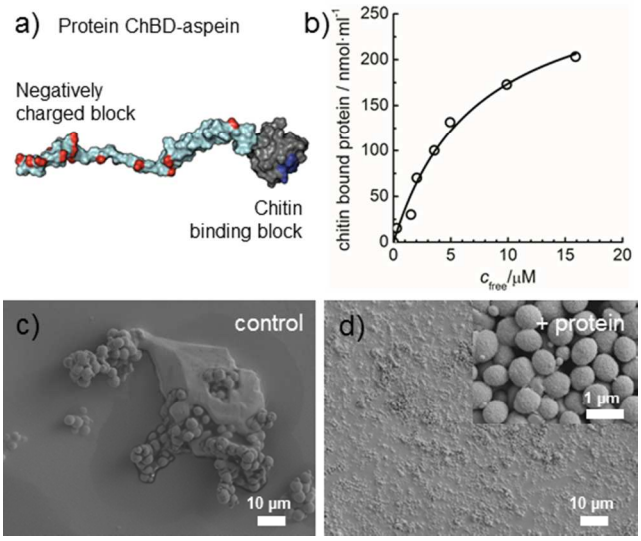

Fig. 1 Interaction of the bifunctional protein ChBD-aspein with chitin and influence on $CaCO_3$ mineralization. a) Molecular model of ChBD-aspein generated using the NMR structure of ChBD and a model structure of the aspein fragment. [23, 24] This model should be considered as an aid in visualizing the relative dimensions of the protein and not as a detailed structural view. b) Chitin binding isotherm of ChBD-aspein protein. c, d) $CaCO_3$ mineralization in the presence (d) and absence of ChBD-aspein (c) observed using SEM.

ChBD-aspein binding to chitin introduces a net charge of -15 in an area of about 3 nm^2 to the polysaccharide surface (at pH 8). This very high local negative charge on the chitin surface should attract positive ions and render chitin "ceramophilic". $CaCO_3$ mineralization was performed in the presence of ChBD-aspein decorated chitin beads or unmodified beads. Optical microscopy showed that in solution the formed crystals were preferentially bound to the ChBD-aspein functionalized beads and not to the unmodified beads (see the ESI Fig. S3). Taken together the results show that ChBD-aspein binds to chitin and affects $CaCO_3$ crystal morphology.

Chitin functionalization requires typically covalent chemical modifications that can change the original structure and properties of the fibres. We set out to explore how genetically engineered proteins can be used to non-covalently functionalize chitin nanofibres. ChBD-aspein functionalized and negatively charged chitin should readily interact with cations and especially with Ca^{2+} *via* aspartate residues. We used lobster shells as the chitin source and produced chitin nanofibres by passing the material several times through a microfluidizer (see the ESI). The chitin dispersion was stable, yet some aggregation was observed as native chitin is hydrophobic by nature (Fig. S4 a in the ESI).

Films were created from chitin nanofibre suspensions by vacuum filtration with and without $CaCl_2$, where the amount of added ChBD-aspein was 50 wt-% in relation to the chitin nanofibres. The presence of bound ChBD-aspein improved the stability of the nanofibre dispersion, likely due to repulsive forces afforded by the high local negative charge on the nanofibre surface. All samples produced a translucent solid film with a thickness of about 3 – 5 μm.

Tensile testing (see the ESI) showed that the mechanical properties of unmodified chitin nanofibre films were comparable with previously reported chitin and chitin based nanocomposites. [25] Films prepared from nanofibrillated chitin suspensions containing both ChBD-aspein and Ca^{2+} ions showed 80% increased stiffness (7.1 GPa modulus) compared to the unmodified chitin film (4 GPa) (Figure 2). Moreover, the ultimate tensile strength of these films was 120 MPa which is 70% higher than that of the unmodified chitin films. Interestingly, films prepared identically but in the absence of Ca^{2+} ions showed no enhancement in strength and only a minor increase in stiffness as compared to the control.

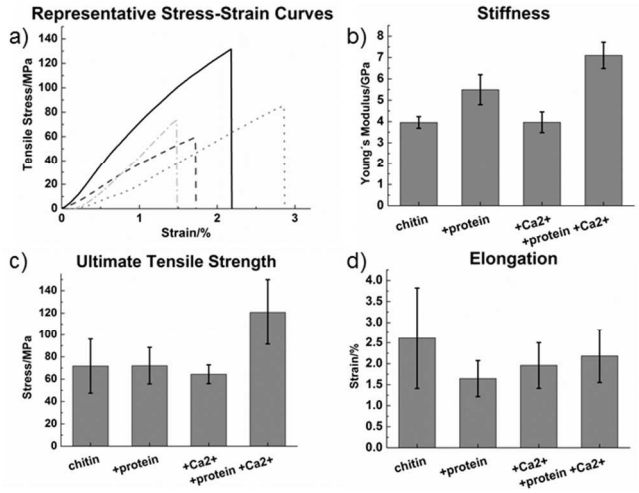

Fig. 2 Mechanical properties of bioinspired materials formed from nanofibrillated chitin and ChBD-aspein protein in the presence of $CaCl_2$. Representative stress-strain curves (a) are shown for unmodified chitin nanofibre (dotted grey line), chitin and ChBD-aspein (dash dotted grey line), chitin and Ca^{2+} (dashed grey line), as well as chitin and ChBD-aspein with Ca^{2+} (solid black line) films. Average values with standard deviations of the material stiffness (b), ultimate tensile strength (c) and strain-to-failure (d) are shown.

The presence of Ca^{2+} ions in the films was confirmed by SEM with energy dispersive X-ray spectroscopy (EDX) imaging. The EDX spectrum from a cross-section of a chitin film prepared with ChBD-aspein and Ca^{2+} shows a clear peak for calcium (Fig. S4 b in the ESI). Thus, the results indicate that the observed improved mechanical properties induced by Ca^{2+} ions arise from ionic interactions between the multiple binding sites introduced by ChBD-aspein and divalent ions. Similarly, randomly distributed ionic bonds have previously been suggested to play a role in providing sacrificial bonds in biological and biomimetic materials. [12, 26]

Encouraged by the finding that ChBD-aspein could functionalize chitin nanofibres and form modified materials through multivalent ionic interactions we attempted to form $CaCO_3$ minerals within the chitin/ChBD-aspein matrix by biomimetic mineralization. Using the double-jet setup, $CaCl_2$ and Na_2CO_3 solutions were introduced to a suspension of nanofibrillated chitin functionalized with ChBD-aspein protein. Freestanding films were prepared from the dispersions and analysed for the presence and polymorph of $CaCO_3$ minerals by wide angle X-ray scattering (WAXS) and mechanical properties by a tensile tester (Fig 3).

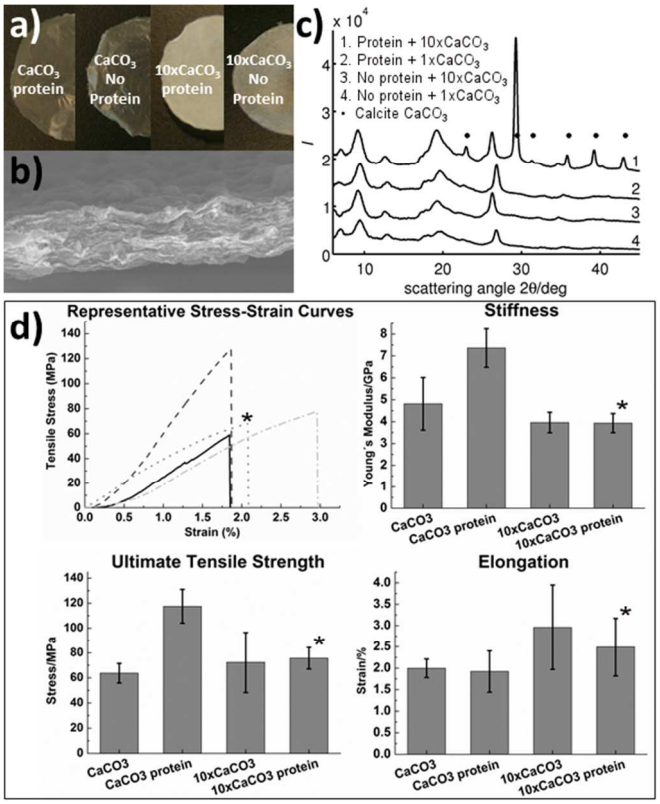

Fig. 3 Biomimetic $CaCO_3$ mineralization within the chitin nanofibre scaffold. a) Mineralized films formed with or without ChBD-aspein protein prepared from suspensions containing 0.6 (1x) and 6.0 mM (10x) $CaCl_2$ and Na_2CO_3. b) A cross-sectional SEM image of the ChBD-aspein functionalized chitin film prepared with 10x $CaCO_3$. c) Scattering intensities of the films obtained using WAXS. Characteristic peaks for calcite $CaCO_3$ are marked with black dots. d) Mechanical properties of the chitin nanofibre based films. Representative stress-strain curves are for samples prepared with 1x$CaCO_3$ (solid line), 1x$CaCO_3$ with protein (dashed line), 10x$CaCO_3$ (dash-dotted line), and 10x$CaCO_3$ with protein (dotted line). Average values with standard deviations for stiffness, ultimate tensile strength and strain-to-failure are also shown. Films shown to contain mineral crystals are marked with an asterisk.

WAXS peaks from crystalline chitin were observed in all samples as expected. When about an equimolar amount of Ca^{2+} (and CO_3^{2-}) in relation to carboxyl groups in the ChBD-aspein functionalized chitin nanofibres was introduced no $CaCO_3$ crystals were observed (Fig. 3 c). This indicates that at these low salt concentrations the carbonate ions were outcompeted by the protein carboxyl groups and Ca^{2+} ions preferentially bound to the latter, thus preventing mineralization. As observed earlier in the films prepared without CO_3^{2-} ions (see Fig. 2), it is likely that the bound Ca^{2+} ions form multiple ionic interactions within the matrix. This was observed in the enhanced mechanical properties (Fig 3 d). However, using a 10-fold excess of Ca^{2+} and CO_3^{2-} ions resulted in retaining of $CaCO_3$ crystals within the matrix when ChBD-aspein was present as shown by the characteristic scattering peaks. The crystal polymorph was identified as calcite based on the peak pattern and a crystal size of 50.2±1.2 nm in the (104) direction was obtained. The presence of calcium within the film cross-section was verified using EDX spectroscopy (Fig. 3 b and ESI Fig. S5-6). To verify that the incorporation of $CaCO_3$ within the film was enabled by the ChBD-aspein a film without the protein was prepared, and, as expected, no detectable $CaCO_3$ peaks were observed in WAXS.

The film that was shown to retain $CaCO_3$ minerals (ChBD-aspein and 10x $CaCO_3$) surprisingly maintained its stiffness and strength as compared to plain chitin films (Fig. 3 d). Incorporating relatively large crystals in the chitin network potentially could have reduced the strength by disturbing the well percolated fibre network. [27] However, this was not observed in our system, indicating the formation of a well-integrated hybrid material. The results show that biomimetic mineralization *via* the ChBD-aspein protein enabled the retention of $CaCO_3$ mineral particles within the chitin nanofibre network. The role of ChBD-aspein may be in interfacing the minerals with the chitin network and providing cohesion between the components. Mineral particle shape and size are likely to have a major influence on the mechanical properties; however, these parameters were not controlled in this study.

In this work we have shown how a genetically engineered protein was used for the construction of two biomimetic hybrid materials. Considerable improvements in mechanical properties of the chitin network were achieved by introducing surface charges and counter-ions and thereby creating cohesion in the material through multivalent ionic interactions. Furthermore, the high density of charged groups introduced by the engineered protein enabled the formation and retention of biomimetic $CaCO_3$ crystals within the chitin matrix. A schematic view of the functionalized "ceramophilic" chitin is shown in Fig. 4. We believe that a control over crystal size and shape, and self-assembly at longer length scales will yield improved mechanical performance in the future.

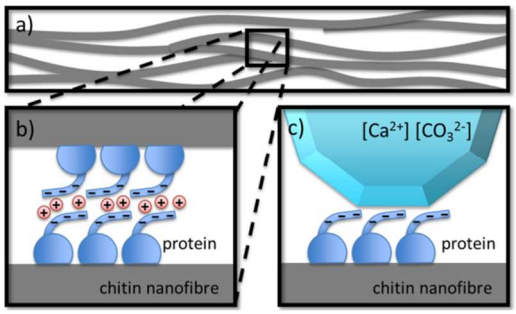

Fig. 4 Schematic presentation of the formed chitin based nanocomposites. a, b) The bifunctional ChBD-aspein protein confers negative charge to the chitin nanofibres and thus improves the dispersion stability and attracts Ca^{2+}-ions. The cations provide multiple ionic interactions across the chitin network and thus enhance the mechanical properties through improved and increased interactions in the chitin network. c) ChBD-aspein protein on chitin provides nucleation sites for $CaCO_3$ crystallization and enables the retention of formed crystals within the chitin nanofibre matrix.

The work herein demonstrates that simple but specific functionalities found in biology combined in multifunctional proteins can be utilized in the development of novel biohybrid materials for e.g. biomedical applications. We anticipate that the highly specific functions found in biology will be applied to self-assembly concepts in supramolecular chemistry yielding future materials that reach and exceed the properties of natural materials.

We would like to acknowledge VTT, the Academy of Finland, Emil Aaltonen Foundation and Bioregs Graduate School for financial

support. This work made use of the Aalto University Nanomicroscopy Center (Aalto-NMC) premises.

Notes and references

[a] VTT Technical Research Centre of Finland, PO Box 1000, 02044 VTT, Finland.
[b] University of Helsinki, Department of Physics, PO Box 64, 00014 HU, Finland.
[c] Royal Institute of Technology, Fibre and Polymer Technology, SE-100 44 Stockholm, Sweden.
[d] Aalto University, Department of Biotechnology and Chemical Technology, PO Box 16100, 00076 Aalto, Finland.
‡ Present address: DuPont Nutrition & Health, Edwin Rahrs Vej 38, DK-8220 Brabrand, Denmark.
† Electronic Supplementary Information (ESI) available. See DOI: 10.1039/c000000x/

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