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Nanomaterials

Genetic Engineering of Biomimetic Nanocomposites: Diblock Proteins, Graphene, and Nanofibrillated Cellulose[**]

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Nature has materials with extraordinary stiffness, strength, and toughness that is based on aligned, tailored self-assemblies.[1] They have inspired biomimetic nanocomposites with drastically better properties[2] than synthetic composites.[3] Herein we show a new approach to making biomimetic nanocomposites based on the exfoliation of graphite into a matrix of genetically engineered proteins and native nanofibrillated cellulose. The protein was genetically engineered to incorporate a hydrophobin[4] block, which binds to graphene,[5] and a cellulose-binding block,[6] which binds to nanofibrillated cellulose,[7] thereby bringing about both the self-assembly and adhesion between the nanoscale components. The aligned co-assembly leads to remarkably good mechanical properties (modulus: 20.2 GPa, strength: 278 MPa, strain-to-failure: 3.1 %, and work-of-fracture 57.9 kJ m^{-2}). The bifunctional protein was crucial for the excellent mechanical properties. This concept shows how high-performance biomimetic composites can be built through the binding and self-assembly of advanced biomolecules that have been genetically tailored.

Biology shows numerous composite materials wherein aligned hard and soft self-assembled components are bound together to result in excellent mechanical properties such as the combination of toughness, strength, and stiffness. Such materials are, for example, nacre, plant tissue, bone, silk, and tendon.[1,8] Factors contributing to their advantageous properties include the chemical nature of the hard-reinforcing and soft-dissipating components, their molecular interactions, their mechanical interlocking, dimensions, and alignment, which contributes to the mechanics of crack propagation. The soft matrix is especially interesting as it acts as glue that keeps the hard components together and allows dissipation of fracture energy.[9] Still, very little is known about, for example, how the matrix proteins of nacre function.[10]

A rational route towards a controlled interconnectivity between the self-assembled domains in biomimetic composites is suggested by the design principles of block copolymers, which are used in materials science, for example, to interface two different polymers in mixtures[11] or to stabilize colloidal systems, even for responses[12] or functions.[13] In this work we show the feasibility of genetically engineered proteins having two well-defined binding blocks, denoted as diblock proteins, that bind and assemble the structural components for biomimetic composites.

Previously we have shown that the adhesive surfactant-like proteins, hydrophobins,[4] allow exfoliation of graphite to give single- or few-layer flakes of graphene in aqueous solutions.[5] Here, the same route to disperse single- or few-layer flakes of graphene using proteins in a cellulose matrix was employed to form biomimetic nanocomposite materials. The dispersions of the single- or few-layer flakes of graphene are referred to herein simply as graphene dispersions, although there may be a range of flake thicknesses present. A genetically modified hydrophobin was used to combine graphene[14] and native nanofibrillated cellulose (NFC), also called nanocellulose or microfibrillated cellulose. The structure of the resulting composite resembles that of nacre where self-assembled, aligned platelet-like aragonite reinforcements are embedded in a protein matrix containing nanofibrillar chitin.[2,15] By using engineered molecules that contain unusual combinations of binding abilities, it is possible to build composites from components that do not occur in natural materials. This technique allowed us to combine flakes of graphene, one of the strongest materials presently known,[14c] and nanofibrillated cellulose having a modulus approaching the one of steel[7,16] in a nanocomposite material.

The protein was genetically engineered to connect graphene and NFC, so that it self-assembles at the interfaces, thus leading to cohesion and alignment (Figure 1 a). Binding to graphene was achieved by a hydrophobin, more specifically the class II hydrophobin HFBI, which self-assembles on various interfaces[4] and surfaces,[17] including graphene.[5] Binding to cellulose was achieved by using a protein denoted as a cellulose-binding domain (CBD) found in cellulose-

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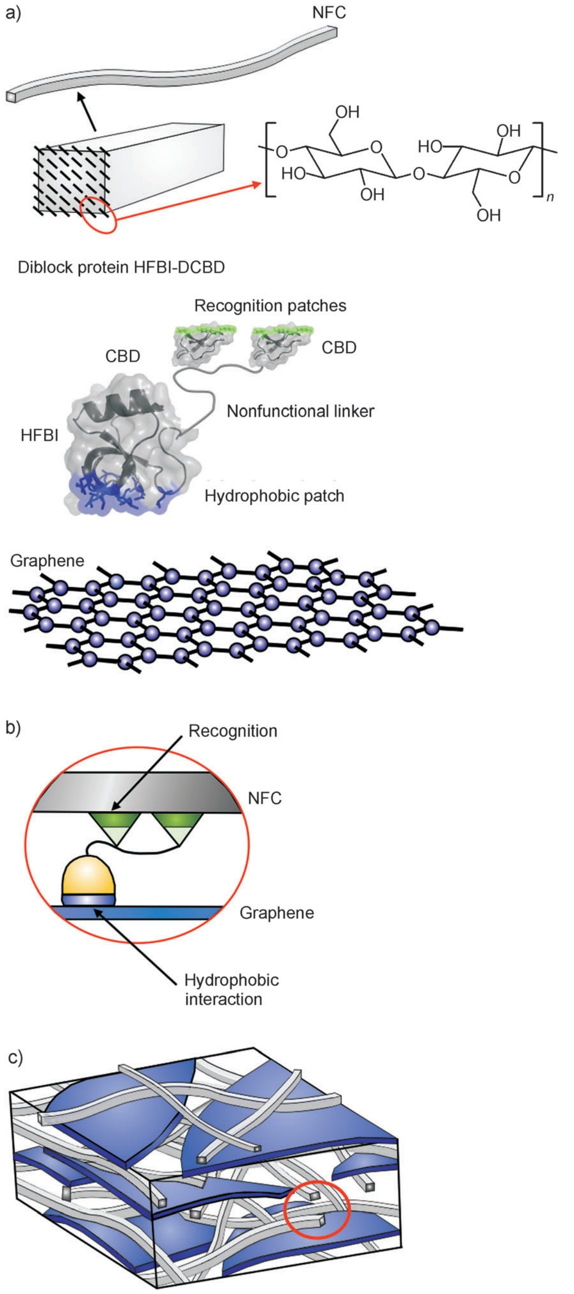

Figure 1. A schematic presentation of the structure of the composite. a) At the molecular level there are two functional blocks of the fusion protein HFBI-DCBD and its target surfaces, that is, graphene and nanofibrillated cellulose (NFC). The amphiphilic hydrophobin (HFBI) attaches to graphene and the cellulose-binding domains (CBDs) to NFC. b) Diblock binding protein. The fusion protein is able to assemble at the interface between cellulose and graphene. For enhanced and balanced binding, two cellulose binding domains are located in tandem positions. c) Graphene/NFC/diblock binding protein assembly. At the microscopic level the composite has a layered structure wherein graphene flakes are interlocked by the NFC fibrils.

degrading enzymes. Therefore we named the fusion protein HFBI-DCBD, where DCBD stands for double cellulose-binding domain as two CBDs were used in tandem to increase the binding efficiency; the CBDs are small in comparison to the hydrophobin HFBI.[18] Because the protein contains two functional blocks, one for binding to graphene and one to cellulose, it is an example of a diblock protein. The functional blocks were tethered by amino acid linkers that had no specific functions or affinity towards the target materials.

Exfoliation of graphite by the HFBI-DCBD fusion protein (concentration 2 $\mathrm{g\,L^{-1}}$) into water solutions with and without NFC (concentration 2 $\mathrm{g\,L^{-1}}$) was studied (Figure 2a).

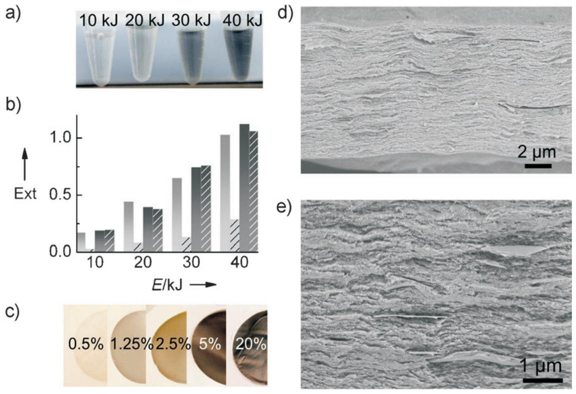

Figure 2. a) Kish graphite aqueous dispersions in a mixture of NFC and HFBI-DCBD (both 2 $\mathrm{g\,L^{-1}}$) at different sonication energies. The same output power (60% of the nominal 750 W) was used in all the experiments. b) Optical extinction (ext) measured for samples at 10 times dilution at 660 nm as a function of the sonication energy immediately after sonication (solid bars) and after three days (highlighted bars). Suspensions containing only protein and graphite are presented in light gray whereas suspensions containing NFC as well are presented in dark gray color. c) Photographs of NFC/HFBI-DCBD films containing a varying amount of graphene. Graphene content is given as weight percentage compared to nanocellulose. d) A SEM image of a cross-section of NFC/HFBI-DCBD film with 20 wt% of graphene relative to NFC. e) A detailed image of the same film.

The value of the optical extinction at 660 nm was used as a qualitative measure of exfoliation and it showed a linear dependency on the sonication energy as expected (Figure 2b).[19] Although the fusion proteins were partly bound to the NFC matrix,[20] the efficiency of graphene exfoliation with or without NFC was similar. However, a major difference was observed in the sedimentation of graphene. Three days after the exfoliation, major sedimentation of graphene was observed in the absence of NFC, whereas no sedimentation was observed for the samples containing NFC. This was probably a result of the steric stabilization of the graphene flakes provided by the NFC and mediated by HFBI-DCBD. Importantly, this indicates that NFC gels promote colloidal stabilization of the graphene flakes. A TEM image of a few-layer-thick flake of graphene embedded in NFC is shown in Figure SI-1 in the Supporting Information.

Composite films were prepared by vacuum filtration of such aqueous dispersions of NFC, HFBI-DCBD, and exfoliated graphene flakes (Figure 2c). The nominal NFC and HFBI-DCBD amounts were equal before filtration and the graphene content, given as a weight percentage (wt%) compared to NFC, was varied. Besides the amount of graphene, the contribution of bonding between the graphene flakes and NFC was also studied by preparing films containing mixtures of the cellulose-binding HFBI-DCBD fusion protein and the wild-type monofunctional HFBI protein, which only contains the hydrophobin part. A notable decrease in the stability of the graphene suspensions containing less than 70 % of HFBI-DCBD was observed and also resulted in a much less homogeneous appearance of the final composite material (see Figure SI-2 in the Supporting Information).

The exfoliated graphite flakes were identified as few-layer flakes of graphene by Raman mapping (see Figure SI-3 in the Supporting Information). The scanning electron microscopy (SEM) image in Figure 2 d shows that the film was very dense and had a lamellar alignment of the graphene flakes and NFC. A higher magnification in Figure 2 e suggests a uniform mixing of cellulose fibrils and graphene flakes although the thickness of graphene and thin graphite flakes vary. Consequently, protein coating enabled good dispersion of graphene into the cellulosic matrix, which resulted in a homogeneous structure at microscale.

The mechanical properties of the composite films were studied by tensile tests. The Young's modulus, the ultimate tensile strength, and work-of-fracture were extracted from the stress-strain curves. A summary of the results and typical stress-strain curves with different graphene contents are presented in Figure 3. All mechanical properties were significantly enhanced when graphene was added. Interestingly, the mechanical properties did not follow the common rule of mixtures for composite materials, but demonstrated a synergetic performance at an optimum composition. The best values for Young's modulus (20.2 GPa), ultimate tensile strength (278 MPa) and toughness (57.9 kJ m^{-2}) were obtained for 1.25 wt % of graphene relative to NFC.

The optimal performance at such a composition might be explained by saturation of NFC by the protein. This type of NFC can bind only 20 μmol of HFBI-DCBD per gram of NFC,[20] which corresponds to 0.04 μmol and approximately 0.96 m^2 in terms of the area of hydrophobic patches under these conditions. This value corresponds roughly to the area of 1.25 % of graphene and might explain why higher amounts of graphene cannot bind to NFC and thus do not enhance the composite properties.

The importance of the linkage between graphene and NFC was studied by replacing different fractions of HFBI-DCBD by a wild-type HFBI protein which is not able to bind to cellulose (Figure 4). The composite containing only HFBI clearly exhibits weaker mechanical properties with a Young's modulus of 12.2 GPa, tensile strength of 73 MPa, and strain-to-failure of 0.7 %. Even if the modulus and strength remain relatively high, the work-of-fracture approaches zero, which clearly shows the importance of the adhesion between the graphene flakes and NFC.

A further comparison of our composite with the relevant benchmark materials, such as NFC nanopapers,[21] graphene (oxide) nanopapers,[22] and graphene (oxide) nanocomposites,[14b,19,23] demonstrates the dramatic effect of the combination of fusion protein and a small amount of graphene on the mechanical properties of the NFC composites. The addition of only 1.25 wt % graphene leads to a roughly 50 % higher stiffness, yield stress, and stress-at-break compared to unmodified NFC nanopapers, and also gives rise to a significantly enhanced work-of-fracture. Given only the small quantities of graphene, the materials only show half of

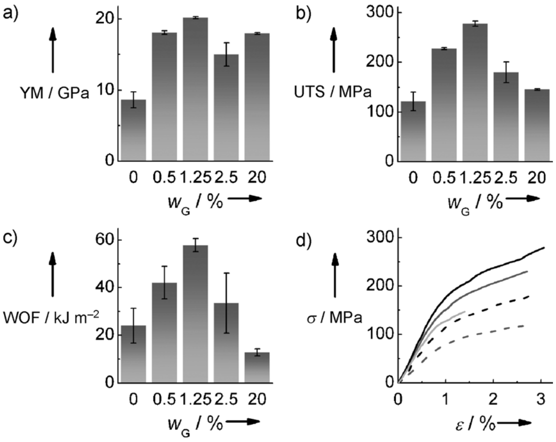

Figure 3. Mechanical properties of composites containing different amounts of graphene (wt% versus the mass of NFC). The Young's modulus (YM; a), ultimate tensile strength (UTS; b), and work-of-fracture (WOF; c) of the composite reached a maximum level at a graphene content of 1.25 wt%. Work-of-fracture was calculated from the area under the stress-strain curves; strain transformed into meters. d) Stress-strain curves from samples containing 0 wt% (dashed gray line), 0.5 wt% (solid gray line), 1.25 wt% (solid black line), 2.5 wt% (dashed black line), and 20 wt% (solid light gray line) of graphene versus NFC. Modulus and strength of the film containing only NFC and HFBI-DCBD protein showed slightly smaller values than what is reported in literature for NFC nanopapers.[21] Error bars are based on standard deviation of the measurements.

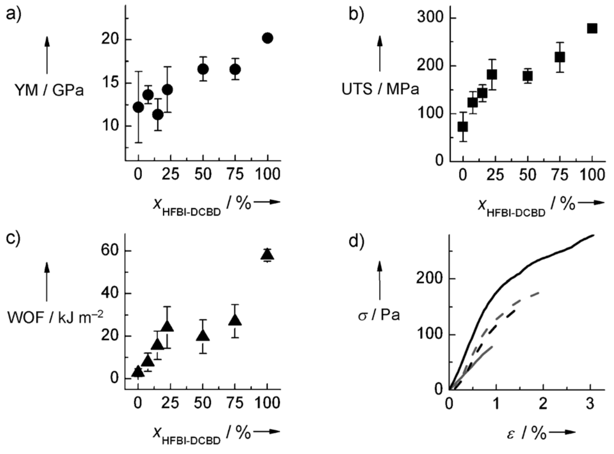

Figure 4. The effect of binding between graphene and NFC on the mechanical properties. The amount of graphene is kept constant (1.25 wt% versus NFC) and a mixture of bifunctional HFBI-DCBD and monofunctional HFBI is used to modulate the binding ($X_{\mathrm{HFBI-DCBD}}$ is a molar fraction of HFBI-DCBD in the mixture HFBI-DCBD/HFBI). The Young's modulus (a) and ultimate tensile strength (b) become considerably improved by adding HFBI-DCBD whereas the effect is particularly drastic for work-of-fracture (c). d) Example stress-strain curves with molar fractions 0 mol% (solid gray line), 7.5 mol% (dashed black line), 22.5 mol% (dashed gray line) and 100 mol% (solid black line) of HFBI-DCBD in the HFBI-DCBD/HFBI mixture.

the stiffness of pure graphene nanopapers, however comparable ultimate tensile strength and much higher maximum strain are found owing to the high-strength NFC network. This feasible combination of a fibrillar NFC scaffold with an intrinsic toughening mechanism and stiff graphene flakes that are "glued" together by our diblock proteins also enables mechanical performance that is among the best of those reported for graphene and graphene oxide composites thus far.[19,22–23] This data suggests that specific binding between the CBD domains and the cellulose plays the decisive role in build-up of the mechanical properties.

We have shown how a rationally designed diblock copolymer such as a genetically engineered bifunctional protein can be used to interconnect the components in a composite material. This approach depends on the efficient interactions between the proteins and the different components, cellulose and graphene in this case. Interestingly it has been recently found that the organic matrix of nacre contains proteins that are analogous to the one used here and have a structure that putatively enables specific bridging of the components of the material.[10] The Pif-97 protein found in the nacre of the pearl oyster *Pinctada fucata* is also a type of diblock copolymer having one aragonite-binding domain and one chitin-binding domain. Thus it has the ability to bind the rigid aragonite platelets to the soft dissipative chitin-containing protein matrix. The role as a cross-linker has not been proved for Pif-97, but it has been clearly shown that it has a pivotal role in the formation of nacre.

In recent years, there have been different approaches to making biomimetic nanocomposites. One particularly simple approach has been to self-assemble synthetic polymeric or colloid-like objects, which leads to some success. At the other extreme, designed peptide synthesis allows mimicking of biological nanocomposites in more detail, but the rational design can become challenging. Here we suggest a fundamentally new approach where we select high-performance components, relevant for the functionalities pursued, and we genetically engineer biomolecular components that allow tuning of the interconnectivity and properties. Therein the possibilities to precisely engineer the molecular structures open endless opportunities to design and fine-tune materials properties. With the rapid development of different techniques enabling directed evolution in the laboratory it will be possible to engineer completely new materials with truly advanced properties on the basis of molecular and distinct control of the interactions. We expect that in this way the special properties of graphene and NFC can ultimately be exploited for high-tech materials having extraordinary mechanical and electrical properties.

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