Bacterially Produced, Nacre-Inspired Composite Materials

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The impressive mechanical properties of natural composites, such as nacre, arise from their multiscale hierarchical structures, which span from nano- to macroscale and lead to effective energy dissipation. While some synthetic bioinspired materials have achieved the toughness of natural nacre, current production methods are complex and typically involve toxic chemicals, extreme temperatures, and/or high pressures. Here, the exclusive use of bacteria to produce nacre-inspired layered calcium carbonate-polyglutamate composite materials that reach and exceed the toughness of natural nacre, while additionally exhibiting high extensibility and maintaining high stiffness, is introduced. The extensive diversity of bacterial metabolic abilities and the possibility of genetic engineering allows for the creation of a library of bacterially produced, cost-effective, and eco-friendly composite materials.

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In this work, we used two strains of bacteria to assemble a bioinspired, layered, nacre-like composite material via alternating deposition of calcium carbonate and γ-polyglutamate (PGA) layers. Calcium carbonate was generated by microbially induced calcium carbonate precipitation with the bacterium *Sporosarcina pasteurii* in a crystallization medium containing urea. *S. pasteurii* expresses urease, an enzyme that cleaves urea into ammonia and carbonate ions. The resultant increase in pH shifts the equilibrium of carbonate, causing precipitation of calcium carbonate. *Bacillus licheniformis* was used to produce the anionic polymer PGA to act as an organic matrix between the calcium carbonate layers, as well as providing equally distributed negative charges as nucleation centers for CaCO3 crystallization.

The deposition process (Figure 1 and Figure S1, Supporting Information) began with the formation of a layer of calcium carbonate on poly(methyl methacrylate) (PMMA) slides by horizontal suspension of the slides within a culture of *S. pasteurii*. This step was followed by placing the calcium carbonate-covered PMMA slides into a solution of PGA collected from *B. licheniformis* cultures. The process was iteratively repeated to produce layered composites, here called “bacterial composite,” with a thickness of ≈200 µm. A bacterially produced CaCO3 material without PGA (“bacterial CaCO3”) and a purely inorganic calcium carbonate material precipitated via a chemical slow diffusion method (“chemical CaCO3”) were also deposited on PMMA slides and used as controls.

Scanning electron microscopy (SEM) analysis of freshly fractured sections of bacterial composite revealed structural similarity to *Mytilus edulis* nacre, a natural nacre chosen as an additional control. *M. edulis* and bacterial composite both showed layered structures with a layer thickness of 1.4 ± 0.3 µm (Figure 2a,b) and 11.2 ± 6.3 µm (Figure 2d,e), respectively. The variation of the layer thicknesses in the bacterial composite might have been caused by heterogeneous distribution of PGA deposited onto the surface of the material, or by variation in the ambient humidity or in the concentration of ions or small molecules between different rounds of bacteria-mediated CaCO3 crystallization. The layered calcium carbonate platelets in bacterial composite were formed by dense needle-like structures (Figure 2f) and were oriented parallel to each other, as in natural nacre (Figure 2b,e). This morphology suggests that PGA can promote layered crystal growth, as reported elsewhere. In contrast, the bacterial CaCO3 specimens displayed a prevalence of randomly distributed plates, with less than 10% of the cross-sectional area containing layered zones (Figure 2g,h). The layers in bacterial CaCO3 samples were limited to spherical crystal structures (spherulites) that occasionally appeared in the bacterial composite samples as well (Figure 2d). In contrast, the chemically produced calcium carbonate material showed no clear formation of small particles but rather large crystals with no layered structure (Figure 2j–l).

On the nanoscale, the two bacterially produced materials showed a nanospheric granular texture similar to *M. edulis* nacre (which contained nanoasperities of ≈20–40 nm in diameter, Figure 2c, comparable with other natural nacres). Nanoasperities in the bacterial composite showed a similar diameter of ≈10–70 nm (Figure 2f), while in the bacterial CaCO3 they were somewhat larger (~60–90 nm, Figure 2i). Chemically produced calcium carbonate showed neither granular texture nor nanoasperities (Figure 2l), and the average crystallite size was significantly larger (Figure S2, Supporting Information). The nanogranular morphology found only in the biomimeralized samples (nacre and bacterially produced materials) may be the result of the various (organic) components present during crystallization, such as PGA or urea in the bacterial growth medium, which might influence crystal formation and the polymorph (crystal structure) (Figures S3 and S4, Supporting Information).

The mechanical properties of the bacterially produced materials were determined at different length scales: nano- or microscopically with nanoindentation, microscopically with microindentation, and macroscopically with three-point bending tests. Using mechanical tests at these three hierarchical levels allowed a determination of how the properties changed throughout the different length scales (Figure S5, Supporting Information). Nanoindentation showed a high stiffness.
(indentation modulus) in all samples: 77.7 ± 29.1 GPa in *M. edulis*, 49.3 ± 8.2 GPa in bacterial composite, 47.5 ± 6.1 GPa in bacterial CaCO3, and 59.3 ± 8.1 GPa in chemical CaCO3 (n ≥ 3, at least 60 indents per sample type). No significant difference in stiffness between the two bacterial material types was measured (p = 0.83), while the differences with respect to *M. edulis* nacre and the chemically precipitated CaCO3 were significant (p < 0.01, Figure S6a,b, Supporting Information).

Similar trends were observed in microindentation, but the average indentation moduli were lower, which is likely related to the higher hierarchical level of the materials tested (20.2 ± 10.5 GPa in *M. edulis*, 17.6 ± 2.3 GPa in bacterial composite, 16.4 ± 2.7 GPa in bacterial CaCO3, and 13.5 ± 3.6 GPa in chemical CaCO3; n ≥ 3, at least 60 indents per sample type; Figure S6c,d, Supporting Information).

At the macroscale, characteristic parameters such as toughness, flexural stiffness, and extensibility (strain at failure) were determined in three-point bending experiments[21] (Figure 3 and Figure S7, Supporting Information). Remarkably, we could not identify any macroscopic cracks in the bacterial composite at high strains, at strain regimes prior to where the crystallization substrate (PMMA) begins to dominate the mechanical response (Figure 3a). This behavior contrasted with that of the other two sample types not containing PGA (bacterial CaCO3 and chemical CaCO3), where large cracks were detected (Figure 3a). The toughness (work of fracture) of bacterial composite was 1.7 ± 1.0 kJ m⁻² (n = 6), within the range of toughness reported for natural nacre (0.3–13.0 kJ m⁻²)[5,9] (Figure 3c). Since we could not detect macrocracks in these samples, we used video data and comparisons to the three-point bending curves of pure PMMA substrates to gauge the point of failure. Therefore, our bacterial composite may have even higher toughness than we were able to measure. The measured toughness of the bacterial composite constituted an approximately fourfold increase over the bacterial CaCO3 (0.4 ± 0.1 kJ m⁻², n = 4) and an almost sixfold increase compared to the chemically produced samples (0.3 ± 0.1 kJ m⁻², n = 3, Figure 3b). We have not found significant differences in macroscopic flexural stiffness of the bacterially produced materials (7.9 ± 5.5 and 7.5 ± 3.7 GPa for bacterial composite, n = 6, and bacterial CaCO3, n = 4, respectively) and the chemically produced samples (2.3 ± 1.4 GPa, n = 3, Figure S7d, Supporting Information), while the flexural strength was significantly higher in bacterial composite (Figure S7e, Supporting Information). The extensibility of the bacterial composite (0.31 ± 0.05) was almost twice that of bacterial CaCO3 and chemical CaCO3 (0.16 ± 0.04 and 0.18 ± 0.09, respectively, p < 0.05). The distinct cracking behaviors of the samples with and without PGA lead us to hypothesize that the bacterial composite material may employ several toughening mechanisms in common with mollusk-produced nacre, including crack deflection upon entering the layer interfaces,
increased crack path length and energy absorption,[22] or nanoasperities.[5] Moreover, the organics might act as a viscoelastic adhesive, and the irregularity of the layers may enable layer interlocking.[5,6]

The bacterial composite developed here demonstrates improved toughness and extensibility, without sacrificing stiffness (Figure 3c), a combination of properties that is difficult to achieve in man-made materials.[23] Our method created materials that are as tough or tougher than other artificial nacre-mimetic materials (in terms of \( K_i \)) recolculated from the measured toughness of bacterial composite, which was ≈3.6 MPa m^{1/2} compared to up to 1.9 MPa m^{1/2} in artificial nacre, see the Supporting Information[11,24] and also some natural nacres (e.g., 2.4 MPa m^{1/2} in Cristaria plicata shells).[11]

Additionally, the nanoindentation stiffness of our bacterial composite (≈45 GPa) is above the range achieved by other man-made nacre-mimetic materials (38–43 GPa)[7,24] without losing the extensibility (0.31) that is comparable to the other successful nacre-mimetic approaches (0.23–0.38).[11,24] These improved properties may result from the hierarchical design that our composite material and other nacre-mimicking materials[11,24] share with natural nacre. PGA likely plays an important role in the emergence of the improved mechanical properties in the bacterial composite. The equally spaced charges provided by PGA allow CaCO_3 crystallization to occur in layers;[16] we also observed layer formation when applying PGA between rounds of chemical CaCO_3 deposition (Figure S8, Supporting Information). Besides influencing crystallization, PGA might act as a viscoelastic glue between the crystals, in a manner comparable to the organic matrix in nacre.[25]

One key advantage of our method of bioinspired materials production is that it is performed exclusively with bacteria: under ambient conditions, using only ecologically friendly and renewable components, and without producing toxic waste. The alternative production processes involve high temperatures (e.g., 80 °C[24]) and pressures (e.g., 100–200 MPa[11,24]) or the use of toxic organic solvents.[7,24,26] Our bacterially based PGA production is significantly cost-effective compared to industrial PGA production (€1 g^{-1} vs €800–2000 g^{-1}).[27,28] These costs could be further decreased in the future through utilization of bacteria strains that do not require supplemental glutamic acid in order to produce PGA, or by streamlining the PGA purification procedure. Bacteria are readily available for genetic engineering, show an enormous diversity of metabolic activities, and are already used extensively in biotechnology and synthetic biology for the production of chemicals.[23] Moreover, complex or irregular (3D-printed) structures,
made from, for example, PLA, can be easily covered with the bacterially deposited materials (Figure S9, Supporting Information), which constitutes a rare feature of the bacterial composite compared to the other tough nacre-mimicking materials available.\(^{[27,29,34]}\)

Our method demonstrates a first step toward the bacterial production of completely new composite materials that can be tailored toward a wide range of applications. Removal of the substrate, for example, by dissolving the PMMA layer using organic solvents, could further expand the range of applications for our material. Furthermore, our approach has the potential to be extended to multimaterial coatings, incorporating layers of different, tunable inorganic materials. Natural nacre is highly biocompatible, as are the components of our bacterial composite.\(^{[30]}\) The biofriendliness of our material will enable its use in medicine\(^{[29]}\) as, for example, tough artificial bones, or in the food industry, as a sustainable packaging material for food. Our composite material can be deposited on-site since its production does not require the use of complex instruments, and its chemical resistance is predicted to be comparable to that of other calcium carbonate-based coatings, which offer excellent protection against chemical degradation and weathering. Our bacterial composites are therefore promising for applications, for example, in civil engineering for crack prevention or remediation in bioconcrete,\(^{[30,31]}\) in the automotive and aerospace industries\(^{[32–36]}\) for the production of lightweight structural components, as protective coatings for dust or erosion control,\(^{[37–40]}\) or for conservation of ornamental stone or cultural heritage items.\(^{[41–43]}\)

**Experimental Section**

Experimental details are shown in the Supporting Information.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Keywords**

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