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Bacterially Produced, Nacre-Inspired Composite Materials

Ewa M. Spiesz, Dominik T. Schmieden, Antonio M. Grande, Kuang Liang, Jakob Schwiedrzik, Filipe Natalio, Johann Michler, Santiago J. Garcia, Marie-Eve Aubin-Tam, and Anne S. Meyer**

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.

The development of simple, environmentally friendly methods for the production of advanced structural materials is becoming increasingly important. The use of bacteria as cell factories is a well-established and cost-effective biotechnological process for industrial-scale production of compounds such as polymers (e.g., poly(lactic-*co*-glycolic acid) (PLGA),[1] polyhydroxyalkanoate (PHA)[2]), cellulose,[3] and inorganic materials (e.g., calcium carbonate[4]). Such bacterially produced materials are typically far simpler than the complex hierarchical materials made by living organisms in nature, which in turn results in poorer mechanical properties.

Natural materials such as tooth enamel, nacre, or bone attain their superior mechanical properties from combining organic and inorganic components into hierarchical composite structures spanning across different length scales.[5,6] For instance, nacre, the tough, iridescent layer constituting the inner surface of mollusk shells, consists of a tessellated structure[6,7] of layered calcium carbonate platelets interconnected by an organic matrix to create a hierarchical composite structure.[7,8] Nacre is comprised of ≈95 wt% calcium carbonate in its aragonite polymorph, while the rest of the material is a complex organic matrix containing β-chitin, lustrin, and silk-like proteins. Despite consisting mainly of ceramic calcium carbonate, nacre behaves very distinctly from the brittle monolithic calcium carbonate. While the Young's modulus (i.e., the stiffness) of 70 GPa is comparable to pure calcium carbonate,[7] its toughness (0.3–13.0 kJ m^{-2})[5,9] is estimated to be up to 1000 times higher than the toughness of pure aragonite crystal. The high toughness of nacre derives from its diverse mechanisms for effective energy dissipation, including crack deflection, organic components that act as a viscoelastic matrix, and nanoasperities (nanospheric texture) that resist inelastic shearing.[10,11] These features enable the composite material of nacre to withstand dramatically higher loads than the equivalent bulk calcium carbonate material, which in turn enables nacre's function as a mechanical shield against predators.[10]

As a response to the selective pressures in their ecological niches, mollusks have evolved the ability to produce nacre from easily attainable, renewable components under ambient conditions, and with a relatively low expenditure of energy. The complex, hierarchical composite structure including organic and inorganic phases is highly advantageous in terms of material design, but to our best knowledge, has not yet been

Dr. E. M. Spiesz, Dr. D. T. Schmieden, K. Liang, Dr. M.-E. Aubin-Tam
Department of Bionanoscience
Delft University of Technology
Van der Maasweg 9, 2629 HZ Delft, The Netherlands
E-mail: m.e.aubin-tam@tudelft.nl

Dr. A. M. Grande
Department of Aerospace Science and Technology
Politecnico di Milano
Via Giuseppe La Masa, 34, 20156 Milan, Italy

Dr. J. Schwiedrzik, Prof. J. Michler
Laboratory for Mechanics of Materials and Nanostructures
EMPA Swiss Federal Laboratories for Materials Science and Technology
Überland Str. 129, 8600 Dübendorf, Switzerland

Dr. F. Natalio
Weizmann Institute of Science
234 Herzl St., Rehovot 7610001, Israel

Dr. S. J. Garcia
Faculty of Aerospace Engineering
Delft University of Technology
Kluyverweg 1, 2629 HS Delft, The Netherlands

Prof. A. S. Meyer
Department of Biology
University of Rochester
Hutchison Road, Rochester, NY 14620, USA
E-mail: anne.meyer@rochester.edu

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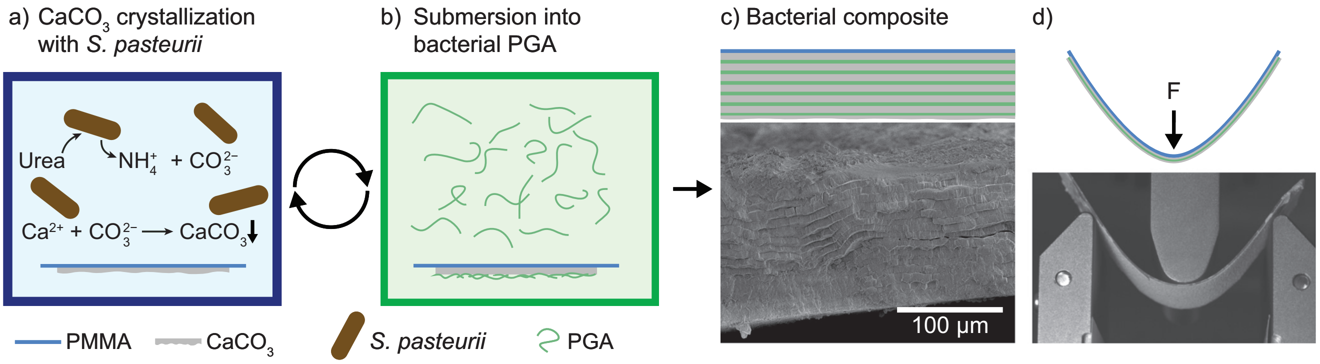

Figure 1. Production of bacterial composite. a) PMMA slide was submerged in a growth medium containing *Sporosarcina pasteurii* to form calcium carbonate in the presence of urea and calcium ions. b) The slide was then immersed in bacterially produced γ-polyglutamate (PGA) solution, which resulted in PGA binding to the calcium carbonate. c) Both steps were repeated 23 times to form the ≈200 µm thick bacterial composite deposited onto the slide. d) High extensibility and toughness were revealed by three-point bending tests.

successfully utilized in biologically based production of structural composite materials. Biological production methods that could not only successfully reproduce nacre's superior mechanical properties, but which could also mimic the environmentally friendly production process, would be a substantial asset for fields including medicine, space exploration, or civil and aerospace engineering.

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*[4] in a crystallization medium containing urea. *S. pasteurii* expresses urease, an enzyme that cleaves urea into ammonia and carbonate ions.[4] The resultant increase in pH shifts the equilibrium of carbonate, causing precipitation of calcium carbonate.[10] *Bacillus licheniformis*[12] 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 CaCO$_3$ crystallization.[13]

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 CaCO$_3$ material without PGA ("bacterial CaCO$_3$") and a purely inorganic calcium carbonate material precipitated via a chemical slow diffusion method[14] ("chemical CaCO$_3$") 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 2**a,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 CaCO$_3$ crystallization.[15] 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.[16] In contrast, the bacterial CaCO$_3$ 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 CaCO$_3$ samples were limited to spherical crystal structures (spherulites[14]) 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[17]). Nanoasperities in the bacterial composite showed a similar diameter of ≈10–70 nm (Figure 2f), while in the bacterial CaCO$_3$ 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 biomineralized 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).[18,19]

The mechanical properties of the bacterially produced materials were determined at different length scales:[20] nanoscopically 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

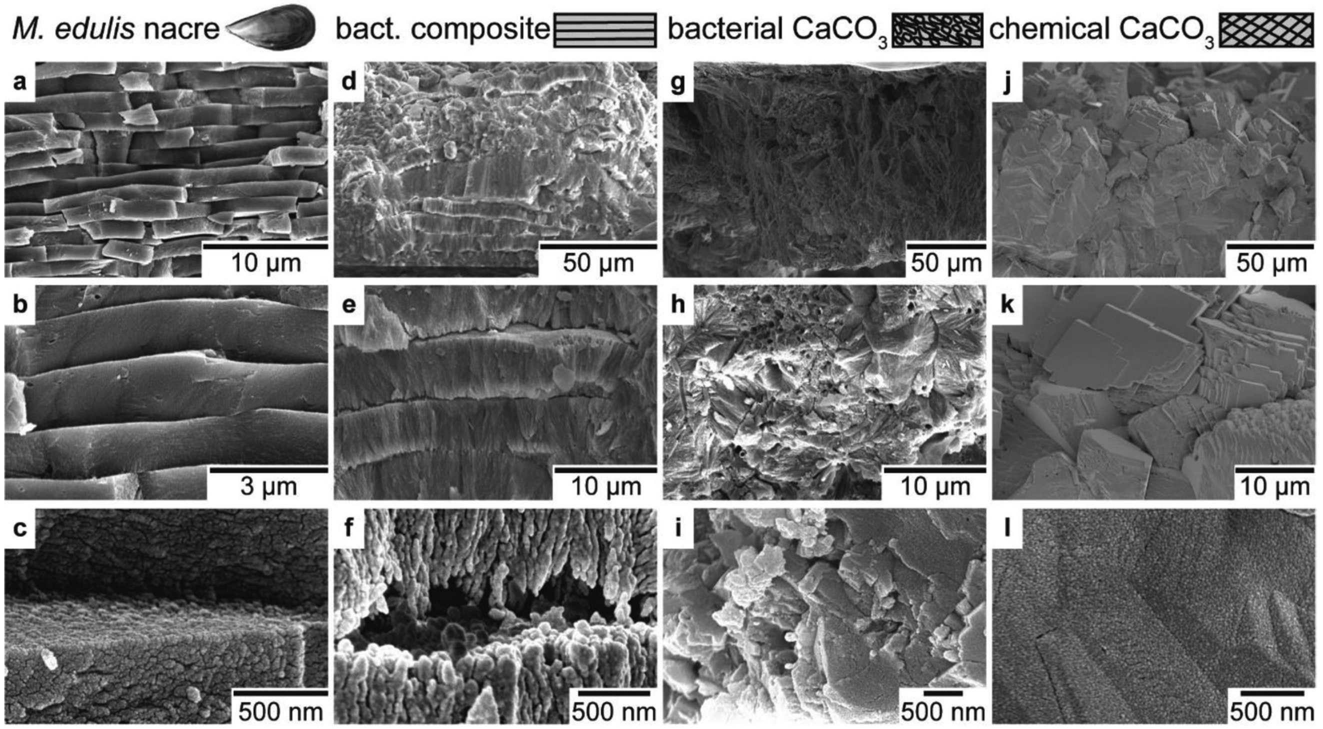

Figure 2. Bacterially produced composite contained calcium carbonate layers similar to natural nacre. Samples were fractured, and cross sections were imaged by SEM. a) *Mytilus edulis* nacre showed a characteristic layered structure with b) layer irregularity enabling interlocking and c) nanoasperities (nanospheric texture) that resist inelastic shearing, both acting as toughening mechanisms. d) Bacterial composite, produced through alternate deposition of calcium carbonate by *S. pasteurii* and submersion in PGA produced with *B. licheniformis*, also displayed e) irregular layers and f) nanoasperities (nanospheric texture), which might contribute to increased toughness. g–i) Bacterial calcium carbonate material produced by repeated deposition with *S. pasteurii* without PGA showed few-layered structures. j) Chemically produced calcium carbonate material generated by slow diffusion of ammonia and CO_2 into a $CaCl_2$ solution showed k) no layers and l) no nanoasperities.

(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 $CaCO_3$, and 59.3 ± 8.1 GPa in chemical $CaCO_3$ ($n \geq 3$, 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 $CaCO_3$ 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 $CaCO_3$, and 13.5 ± 3.6 GPa in chemical $CaCO_3$, $n \geq 3$, 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 $CaCO_3$ and chemical $CaCO_3$), 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 $CaCO_3$ (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 $CaCO_3$, $n = 4$, 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 $CaCO_3$ and chemical $CaCO_3$ (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,

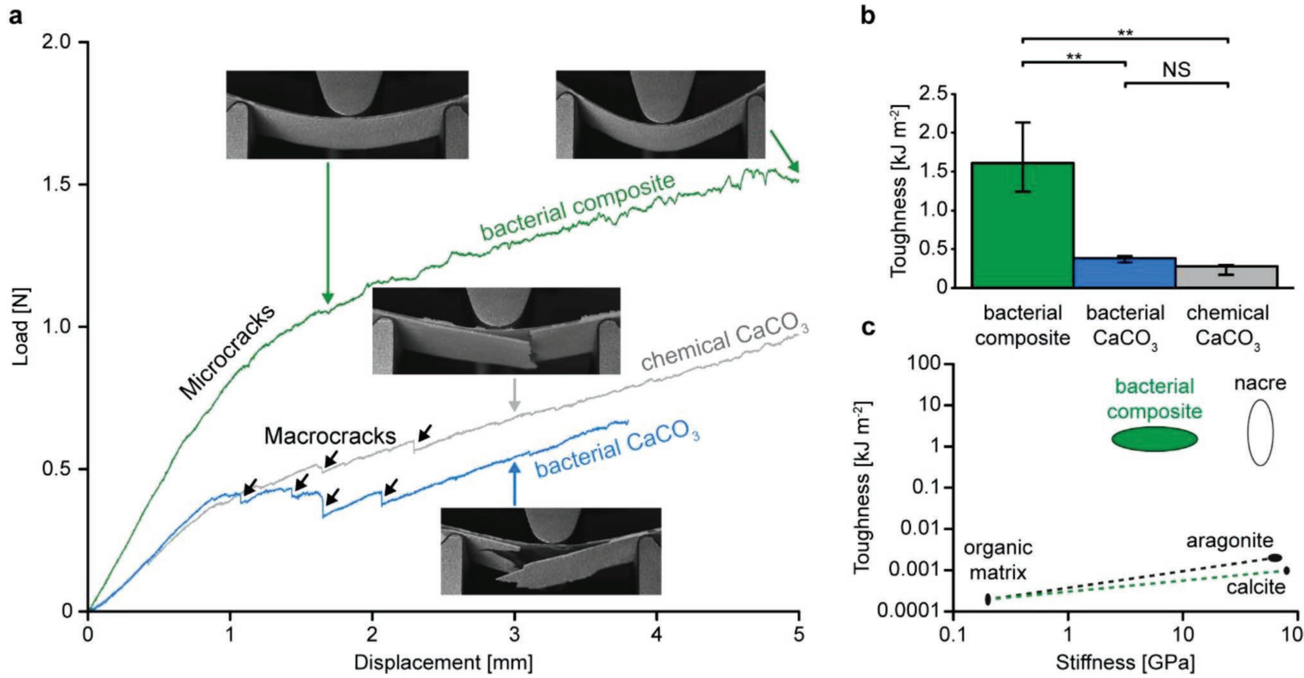

Figure 3. Bacterial composite shows a substantially higher toughness and distinct cracking behavior from both chemically and bacterially produced $CaCO_3$. a) Representative three-point bending force–displacement curves and images of the samples at a given displacement, highlighting the lack of macrocrack formation during bacterial composite (green line) deformation, even at high displacement. In contrast, distinct macrocracks could be observed in the responses of bacterially and chemically produced $CaCO_3$ (blue and gray lines, respectively, macrocracks indicated by black arrows). Images of high displacements for chemical and bacterial $CaCO_3$ demonstrate the complete damage of the coating material and its detachment from the substrate. The three-point bending curves of all samples are depicted in Figure S7 (Supporting Information). b) Toughness measured in three-point bending experiments. Data represent medians with quartiles 1 and 3 ($n \geq 3$, individual results presented in Figure S7a, Supporting Information). Significant differences marked as * for $p < 0.05$, ** for $p < 0.01$, and NS for not significant. c) The rule of mixtures (dashed lines) is a simplified model for estimating mechanical properties of composite materials based on the properties of components and their volume fractions.[5] Both natural nacre and bacterial composite produced in this work violate this rule, indicating that more complex toughening mechanisms are involved.

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_{Ic} recalculated 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.[7,11,24]

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.[28] 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 biocement,[30,31] in CO_2 sequestration,[32,33] in the automotive and aerospace industries[34–36] 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

bacterially induced materials, biological materials, biomaterials, biomimetic materials, hierarchical materials, nanocomposites

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