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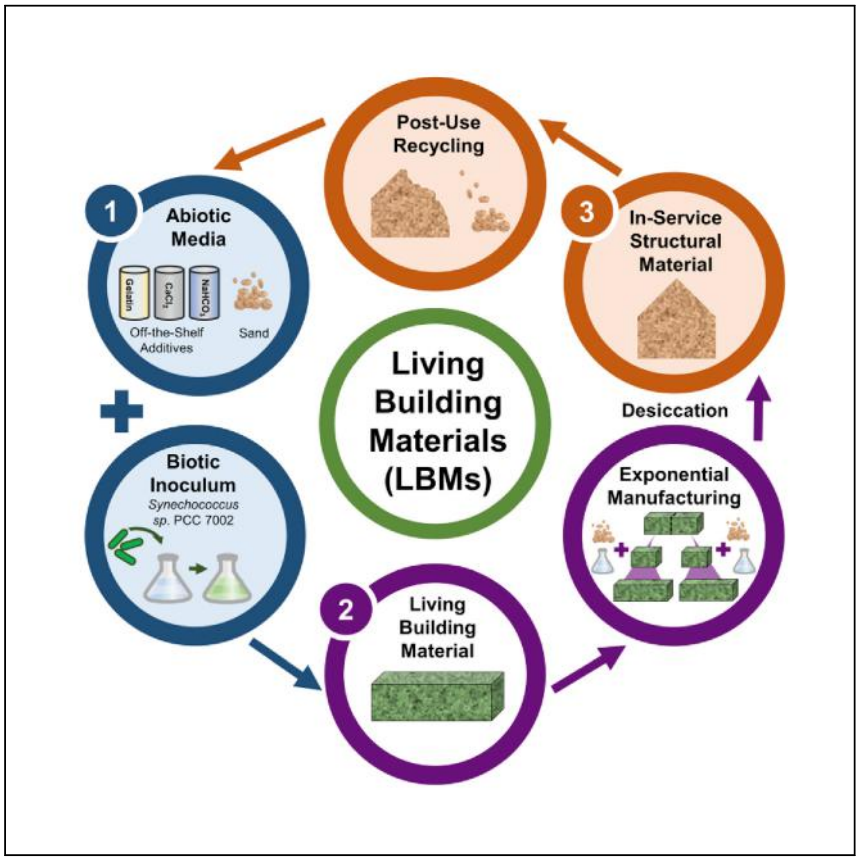
Biomineralization and Successive Regeneration of Engineered Living Building Materials



Living building materials (LBMs) were engineered using photosynthetic cyanobacteria and an inert sand-gelatin scaffold. Microorganisms biomineralized LBMs with calcium carbonate, which imparted higher fracture toughness compared with no-cell controls. The microorganisms maintained relatively high viability in LBMs as long as sufficient humidity conditions were provided. The microorganisms were capable of on-demand exponential regeneration in response to temperature and humidity switches. Looking forward, LBMs represent a new class of structural materials that can be engineered to exhibit multiple biological functionalities.

Benchmark
First qualification/assessment of material properties and/or performance

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HIGHLIGHTS

Living building materials (LBMs) were grown and regrown using physical switches

Cyanobacteria biomineralized hydrogel-sand scaffolds

Biomineralization increased the fracture toughness of LBMs

Three child generations of LBMs were grown from one parent generation

Microbial viability in the living building materials was maintained through 30 days

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Biomineralization and Successive Regeneration of Engineered Living Building Materials

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SUMMARY

Living building materials (LBMs) were engineered that are capable of both biological and structural functions. LBMs were created by inoculating an inert structural sand-hydrogel scaffold with *Synechococcus* sp. PCC 7002, a photosynthetic cyanobacterium. The scaffold provided structural support for *Synechococcus*, which toughened the hydrogel matrix via calcium carbonate biomineralization. Temperature and humidity switches were utilized to regulate the metabolic activity of the microorganisms and achieve three successive regenerations of viable LBMs from one parent generation. Microbial viability in LBMs maintained in at least 50% relative humidity for 30 days was 9%–14%, which far exceeded literature values of microorganisms encapsulated in cementitious materials for similar timeframes (0.1%–0.4%). While structural function was maximized at ultradesiccated conditions, prolonged dehydration compromised microbial viability. Despite this tradeoff in biological-structural function, LBMs represent a platform technology that leverages biology to impart novel sensing, responsive, and regenerative multifunctionality to structural materials for the built environment.

INTRODUCTION

Today, microbially induced calcium carbonate ($CaCO_3$) precipitation (MICP) is utilized for soil stabilization,[1] *in situ* concrete crack repair,[2–4] fracture sealing of oil and gas wells,[5] bioremediation of metals,[6,7] and mitigating leakage from geologically sequestered carbon dioxide (CO_2).[8] During MICP, the metabolic activity of microorganisms increases the saturation state local to the bacterial cell and promotes $CaCO_3$ precipitation.[1,2] While ureolytic microorganisms have been the focus of most MICP applications,[1,2,9] several alternative metabolic pathways also achieve $CaCO_3$ precipitation, such as carbonic anhydrase[10,11] and the carbon-concentrating mechanism of cyanobacteria.[12] Reparative (i.e., self-healing) applications of MICP require prolonged microorganism viability. However, ureolytic bacteria have only limited viability in the harsh, high-pH environment of cementitious materials.[4,13–15] While viability is improved somewhat by utilizing spore-forming bacteria strains or by encapsulating microorganisms in a protective bead or matrix,[4,16] long-term survival of the initial inoculum is still limited in these enhanced systems.[14,15,17,18]

If long-term viability were improved, microorganisms could be utilized to create or "grow" living building materials (LBMs) with structural and sustained biological functions. LBMs necessitate two principal components: (1) an inert scaffold that provides structural support for (2) a living component that, together with the structural

Progress and Potential

Engineered living building materials (LBMs) use biology to confer multiple functionalities to materials for the built environment. Microorganisms can be leveraged for multiple purposes in the design of LBMs, including increasing the rate of manufacturing, imparting mechanical benefit, and sustaining biological function. In this work, we used photosynthetic microorganisms to biomineralize inert sand-gelatin scaffolds to create LBMs. These materials are capable of exponential regeneration of the living component in response to physical switches. Thus, from one starting generation of material, multiple regenerations are produced on demand. In this study, microorganism-precipitated calcium carbonate conferred high fracture toughness to the LBMs. More broadly, LBMs represent a platform technology whereby biology can be leveraged to potentially deliver multiple functionalities to infrastructure materials by design.

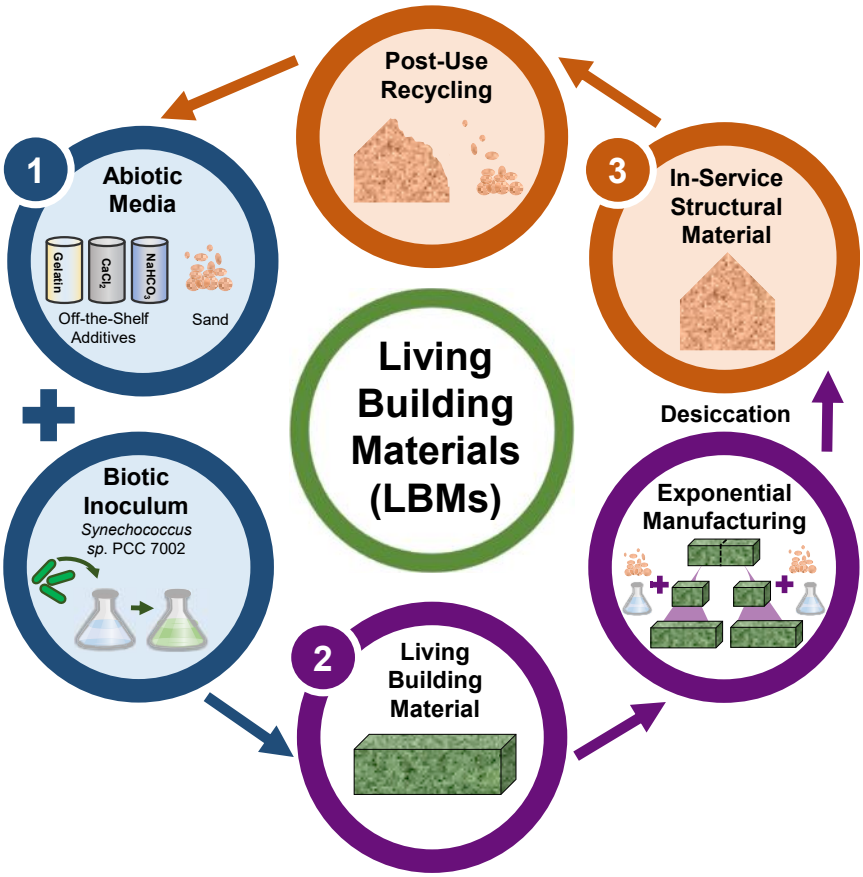

Figure 1. The Life Cycle of Living Building Materials

(1) LBMs are created by mixing *Synechococcus* sp. PCC 7002 cells with calcium-containing nutritional media, gelatin, and sand. (2) LBMs can be exponentially regenerated from an original LBM through use of temperature and humidity switches. (3) LBMs gain structural integrity through desiccation. After service as a load-bearing structural material, LBMs could be deconstructed and recycled as an aggregate source for new LBMs.

scaffold, endows the LBM with structural and biological function. The living component must be robust to a range of environmental conditions and respond to physical switches (e.g., temperature, pH, light, moisture, pressure) with changes in metabolic activity. Microorganisms capable of MICP, for example, could be used to grow load-bearing building materials with self-sustaining functions.[19] Control over microbial metabolism through environmental switches would enable on-demand growth, biomineralization, dormancy, and subsequent regeneration of LBMs. These environmental switches could enable regeneration of LBMs from one parent inoculum, which would enable new possibilities for infrastructure material manufacturing, use, and post-use remanufacturing (Figure 1).

In this work, we engineered LBMs capable of successive regeneration in response to environmental switches. LBMs were created with a sand-hydrogel structural scaffold inoculated with *Synechococcus* sp. PCC 7002 (*Synechococcus*)—a robust photosynthetic cyanobacterium capable of MICP (Figure 2). First, we inoculated sand with dissolved gelatin, media, and *Synechococcus*. Gelatin was chosen because its melting point (37°C) is compatible with bacterial viability and also because gelatin scaffolds gain strength through physical crosslinking during dehydration.[20] The LBM was then cooled to form a three-dimensional hydrogel network[21] reinforced with biogenic

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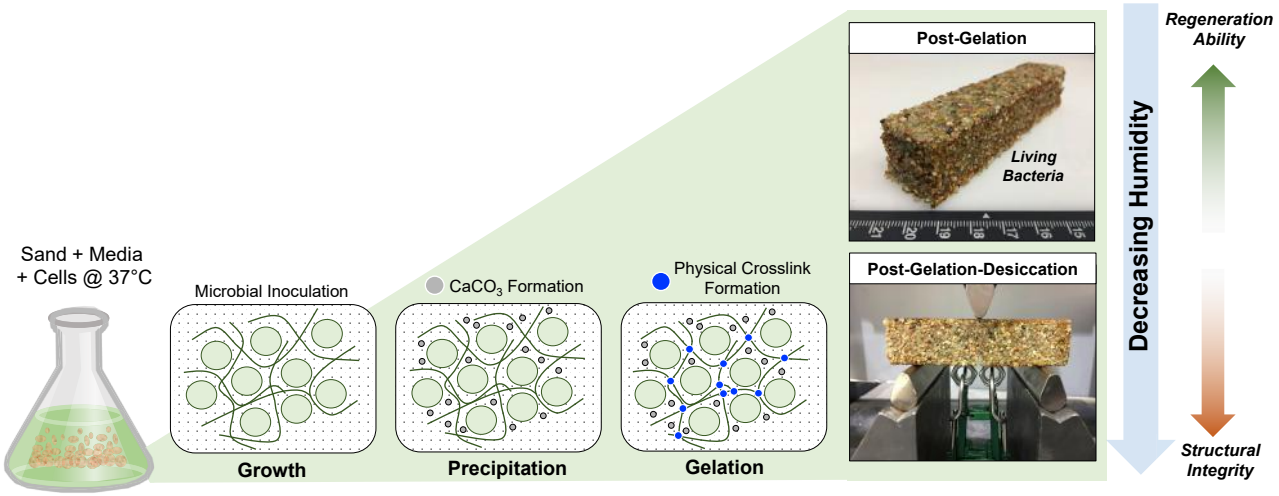

Figure 2. Formation of the LBM Mineralized Scaffold

The physically crosslinked hydrogel together with bacterial calcite precipitation supports the LBM. With decreased humidity, the scaffold gains structural integrity (i.e., improved mechanical properties).

$CaCO_3$. *Synechococcus* utilizes the enzyme Rubisco to convert CO_2 to sugars during photosynthesis. In low-CO_2 media, O_2 competitively binds to the Rubisco active site and diminishes CO_2 carboxylation efficiency. *Synechococcus* surmounts this limitation by concentrating HCO_3^- from media to CO_2 within the cell and exporting OH^- outside of the cell,[12] thereby increasing local pH and promoting $CaCO_3$ precipitation. Cyanobacteria are a diverse phylum of microorganisms well known to survive extreme environmental conditions, including high and low temperature, salinity, and humidity.[12] These characteristics make *Synechococcus* particularly suitable for LBMs, since in-service environmental conditions of building materials (i.e., fluctuating moisture and temperature) can impart physical stress to microorganisms. Using *Synechococcus*, LBMs were generated and successively regenerated from one parent inoculum using temperature and humidity switches. The mechanical properties, mineral characteristics, cell viability, and regeneration ability of LBMs are reported here.

RESULTS

Viability and Regenerative Potential

We first assessed whether cyanobacteria would maintain long-term viability in the parent LBMs at ambient temperatures (20°C), refrigerated temperatures (4°C), or both (Figure 3). At ambient temperature and relative humidity (RH), samples were viable at the time of demolding (9.5 h) but were not viable at 7 days. Because *Synechococcus* sp. PCC 7002 was expected to have slower metabolism at lower temperatures, viability was again assessed for refrigerated samples. When maintained at either 50% or 100% RH, ~69% of the initial inoculum survived up to 14 days. When assessed at 30 days, ~9% and 14% of the initial inoculum survived in the LBM stored at 50% RH and 100% RH, respectively. As expected, LBMs stored at 4°C with ambient RH (24%) exhibited reduced viability compared with those stored at higher humidity. At this lower humidity, ~ 37% of the initial inoculum survived at 14 days while viability was not detectable at 30 days. This loss of viability closely aligned with the time to reach equilibrium mass via controlled dehydration (Figure S1).

Next, we evaluated whether parent LBMs could be utilized to regenerate successive generations of viable LBMs. For the parent generation, viability was measured at

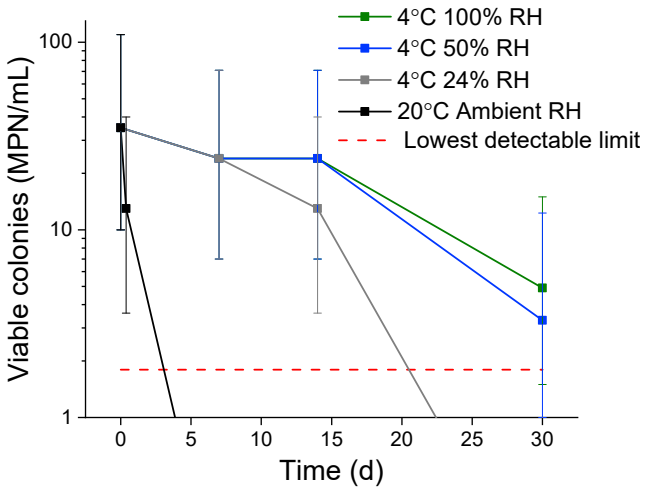

Figure 3. Viability of *Synechococcus* sp. PCC 7002 over 30 Days in LBMs

Low temperature (4°C) and high relative humidity (RH) maintains microorganism viability. Most probable number (MPN) viability was maintained through 30 days (d) for 50% and 100% RH conditions.

0 days and 7 days. For each generation (first, second, third), new abiotic media (i.e., sand and calcium-containing nutritional media) were added to form two new LBMs from one parent LBM. Thus, in three generations separated by 7 days each, eight specimens were formed from one initial parent LBM. Within each generation, viability decreased from day 0 to day 7. However, the addition of new medium at each regeneration and low-temperature storage sustained LBM viability through three generations for 50% RH and 100% RH (Figure 4). As expected, greater viability was maintained for 100% RH compared with 50% RH. Viability measured at day 7 of the third generation was 20% and 40% of the viability of the initial inoculum (i.e., parent generation, day 0) for 50% and 100% RH, respectively.

Microstructural Characterization

Because $CaCO_3$ can spontaneously precipitate, we sought to understand whether the composition and morphology of mineral formation was influenced by the presence of the gelatin matrix and cyanobacteria. Two controls were used to assess whether mineral precipitated in the presence of cyanobacteria differed from mineralization spontaneously occurring in the absence of biotic influence. The "abiotic control" was created with the same medium as the cyanobacterial LBMs, but without cells. As with the initial LBM, the abiotic control was set to pH 7.6. The "abiotic high-pH control" was an abiotic control set to pH 10 to incite maximum precipitation of $CaCO_3$. Mineral deposited from cyanobacteria and controls were compared for samples prepared without and with gelatin.

From X-ray diffraction (XRD), LBMs and the abiotic control each produced a mixture of calcite and gypsum minerals, which were detected along with halite introduced by the media. By contrast, some calcite and halite (but not gypsum) were precipitated in the abiotic high-pH control (Figure S2). From scanning electron microscopy (SEM), larger minerals were precipitated by the abiotic control compared with the LBMs and high-pH control (Figure 5). Energy-dispersive spectroscopy (SEM-EDS) showed that most of the sampled minerals in the abiotic controls were calcium sulfate (likely gypsum) (Figure S3). Most of the minerals sampled by SEM-EDS for the abiotic high-pH control as well as the LBMs were $CaCO_3$.

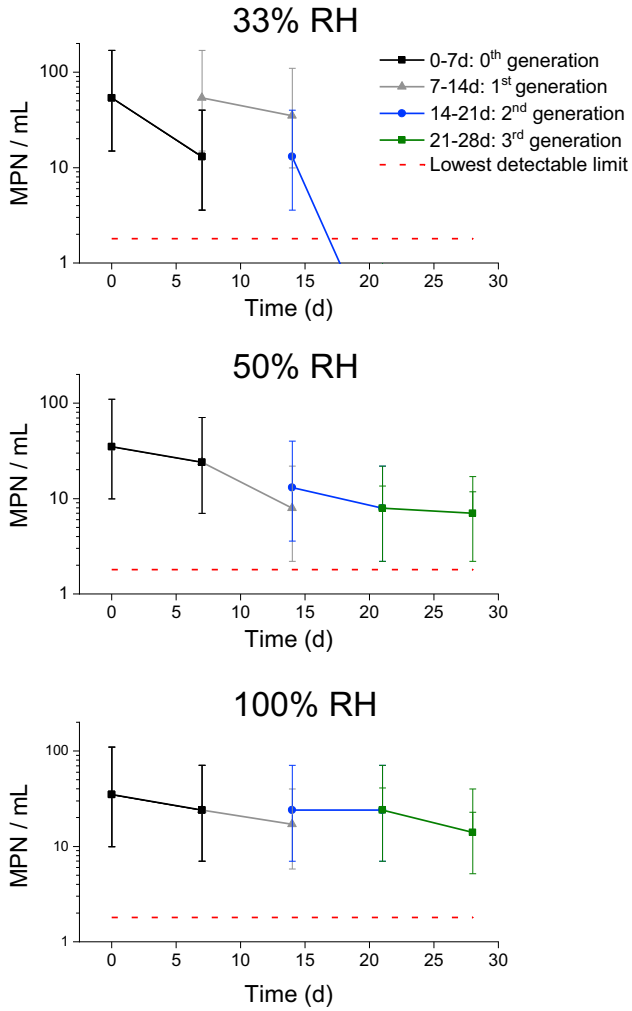

Figure 4. Regeneration of *Synechococcus* sp. PCC 7002 in LBMs

To form the next generation of LBMs, the previous generation was subjected to a high-temperature switch and refreshed with abiotic medium (high-humidity switch). The new LBM was then gelled (low-temperature switch). Viability for each generation was measured at 0 days and again at 7 days of storage at 4°C. Greater RH (50%–100%) allows at least three viable regeneration events. Data indicate the mean and 95% confidence intervals.

SEM-EDS revealed that the regenerated LBMs were able to biomineralize gelatin, similar to the parent generation. SEM-EDS confirmed the presence of $CaCO_3$ in the parent, first, second, and third generations (Figure 6). The abundance of $CaCO_3$ appeared to increase over subsequent generations.

Mechanical Properties

As observed in nature, biomineral deposits within polymer matrices can yield composites with high toughness.[22–26] Thus, the fracture energy of LBMs was assessed and the results compared with the fracture energies of the abiotic and abiotic high-pH controls cured at room temperature. Rectangular prism LBMs and controls were desiccated to equilibrium mass at ambient temperature before testing (Figure S4). While these desiccated samples do not retain microorganism viability, dehydration best enabled direct assessment of the mechanical benefit imparted by microbial biomineralization.

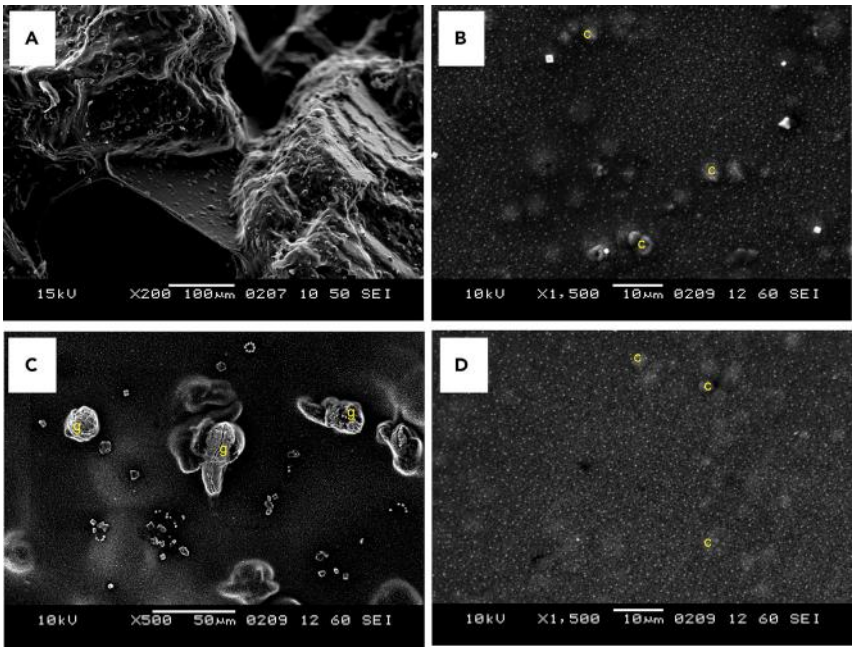

Figure 5. Mineralization of the Gelatin Scaffold for LBMs and Controls

(A) For LBMs as well as controls, gelatin bridges sand particles and provides a substrate for mineralization.

(B) *Synechococcus* sp. PCC 7002 induces precipitation of $CaCO_3$, identified with SEM-EDS, in ALS-gelatin medium.

(C) The abiotic control (pH 7.6) forms large gypsum particles as well as minor deposits of $CaCO_3$.

(D) The abiotic high-pH (pH 10) control forms $CaCO_3$, although these precipitates are smaller than when bacteria are present. Representative SEM-EDS spectra are provided in Supplemental Information.

Post hoc testing revealed that fracture energy was significantly higher for the LBMs than for the abiotic controls (+15.6%, $p < 0.05$) and abiotic high-pH controls (+17.0%, $p < 0.05$) (Figure 7). From ANOVA, specimen type (i.e., LBM, abiotic control, abiotic high-pH control) and gelatin batch both had significant main effects on fracture energy. There was no interaction between these factors.

Compressive strength, assessed for mortar cubes, was not influenced by gelatin batch but was significantly affected by specimen type (Table 1). Specifically, the abiotic high-pH control had significantly lower compressive strength than either the abiotic control (−29.3%, $p < 0.05$) or the LBMs (−28.4%, $p < 0.05$). Notably, both the LBMs and abiotic controls had strength similar to the minimum acceptable strength for ordinary Portland cement-based mortars (~3.5 MPa).[27]

DISCUSSION

We engineered LBMs with the capacity for regeneration and biomineralization (Figure 1). Specifically, at least three successive generations of LBMs with viable *Synechococcus* were regenerated from one parent generation (Figure 4). The regenerative ability of LBMs demonstrates a potential for exponential "growth" in material manufacturing. For each subsequent generation, one LBM from the previous generation was supplemented with new abiotic medium and sand to form two new LBMs. Thus, in three generations, one LBM formed eight new specimens from one parent microbial inoculum.

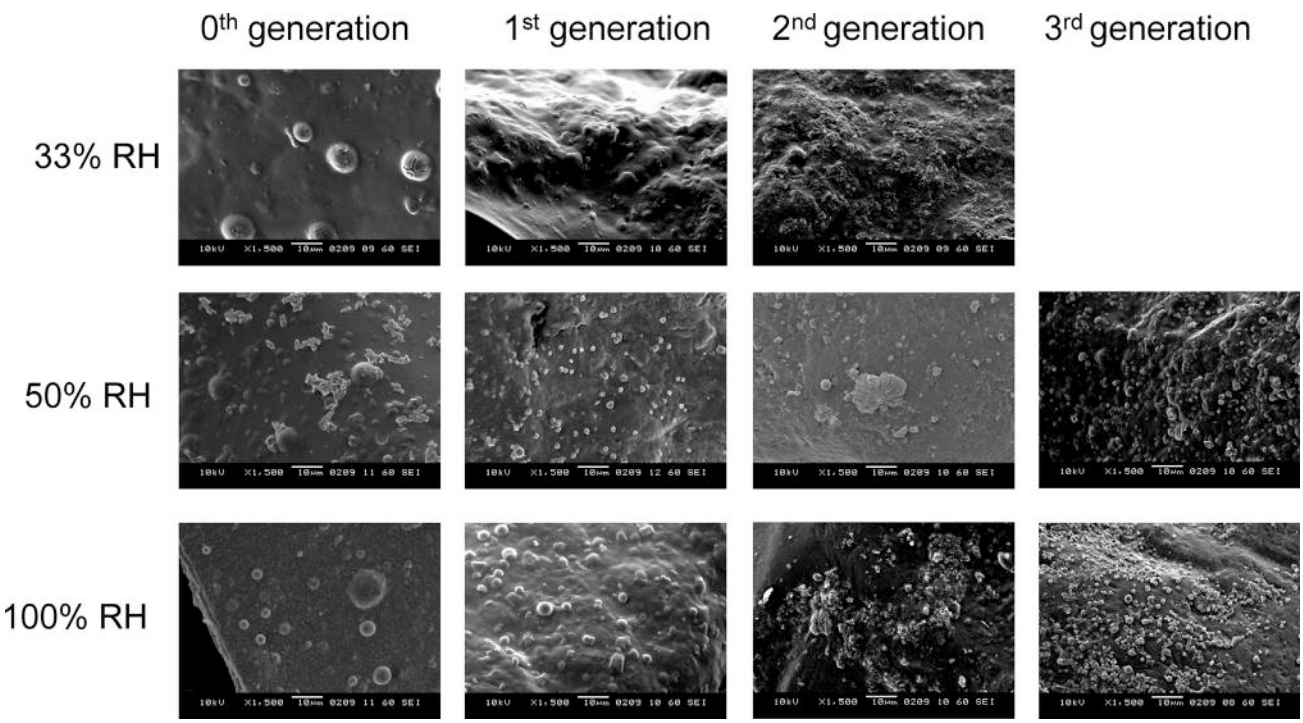

Figure 6. Mineralization of Three Regenerations of LBMs

Mineralization was assessed with SEM-EDS for all viable generations of LBMs at each RH. $CaCO_3$ mineralization qualitatively increased in abundance with each subsequent generation. All images are shown at 1,500×. Representative SEM-EDS spectra are provided in Supplemental Information.

The viability of *Synechococcus* in the sand-hydrogel composite was, in general, much higher than other reported biomineralizing microorganisms in cementitious materials. Greater viability in our system may be attributed to the lack of harsh conditions that exist inside cement paste, including ultrahigh pH (>12), high ionic strength, elevated temperatures that occur during exothermic cement hydration, and nutrient depletion.[28–31] Achal et al.[14] reported that only 0.1% of vegetative (i.e., metabolically active) *Bacillus megaterium* cells remained viable in an aged cementitious mortar; similarly, Bundur et al.[13] reported that 0.4% of vegetative *Sporosarcina pasteurii* cells remained viable in a cement paste at 28 days. Jonkers and coworkers[15,18] examined the viability of endospores (i.e., metabolically inactive) in bacterial mortars and reported that only 2% of the initial inoculum were detected in a mortar mixed with either *Bacillus cohnii* or *Bacillus halodurans* at 10 days, while 7% of the initial inoculum was detected in a mortar mixed with *Bacillus pseudofirmus* at 10 days.

The viability and regenerative potential of *Synechococcus* were enabled by the use of temperature and humidity (i.e., rehydration) switches. The first high-temperature switch corresponded to the incubation and growth temperature (37°C), which was sufficient to dissolve the gelatin matrix and encourage bacterial metabolic activity and mineral precipitation. The low-temperature switch corresponded to the storage temperature (4°C). At this temperature, the gelatin matrix effectively encapsulated the cyanobacteria and medium to form a solid LBM. The cyanobacteria remained viable at the storage temperature as long as humidity was sufficient to prevent excessive cell desiccation (50%–100% RH). Importantly, 50% RH was similarly effective in maintaining viability as 100% RH. This finding is of significance because many climates worldwide have at least 50% RH. Thus, during LBM regeneration, the

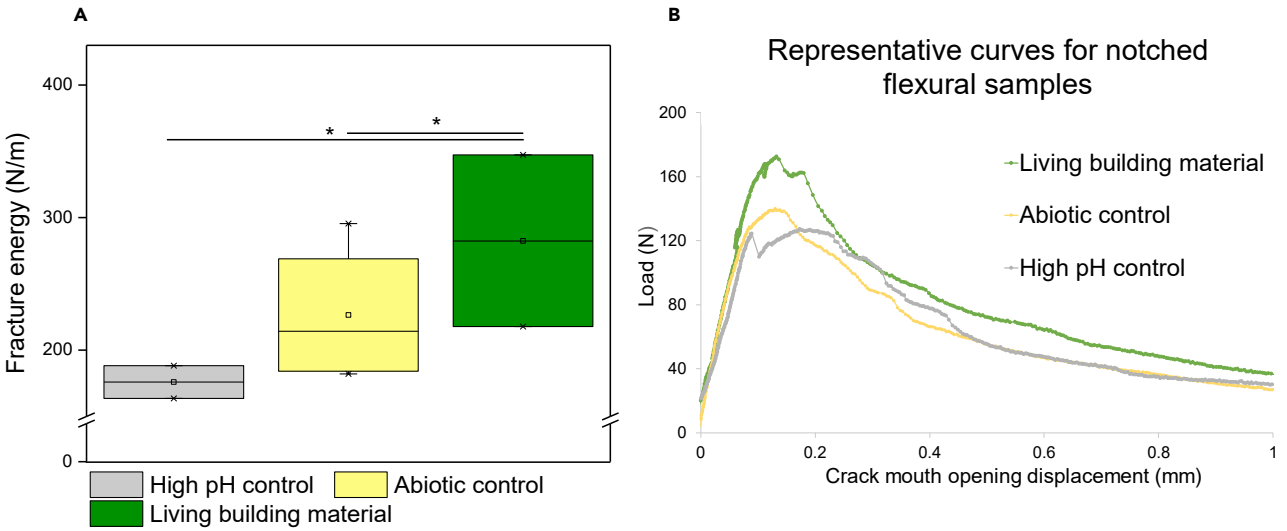

Figure 7. Fracture Energy for LBMs versus Controls

(A) Fracture energy was significantly higher for LBMs compared with either abiotic (pH 7.6) or abiotic high-pH (pH 10) controls. *$p < 0.05$ for Tukey post hoc comparisons.

(B) Representative curves from flexural testing for fracture energy calculation.

addition of new liquid abiotic media and higher temperatures were the high-humidity and high-temperature switches that rekindled metabolic activity.

From an engineering perspective, the responsiveness of LBMs to these temperature and humidity switches is opportune. Material systems that protect ureolytic microorganisms with encapsulating gels or other solid media generally require physical damage to the encapsulant in order to trigger additional biomineralization.[4,32] While this approach may be appropriate for *in situ* crack sealing, it is challenging to elicit a uniform metabolic response from embedded microorganisms. By contrast, environmental switches, such as those employed herein, can be applied uniformly to precisely control microbial activity.

$CaCO_3$ biomineralization increased with each regeneration event. Because biomineralization from *Synechococcus* is a consequence of metabolism, viable microorganisms were expected to precipitate additional $CaCO_3$ with each regeneration event. While SEM revealed a qualitative increase in mineralization with each regeneration, it is not known whether mineralization efficiency changed with regeneration. Likewise, the effect of additional biomineral content on LBM mechanics is not known and is a limitation of the present work. These questions would benefit from further study.

The increased fracture energy of LBMs compared with controls likely relates to the characteristics of the biogenic mineral. LBMs, as well as both control materials, experienced a profound toughening effect from the mineralized gelatin matrix compared with cementitious mortars with similar aggregate size.[33] Because the abiotic and abiotic high-pH controls had similar fracture energy despite precipitating predominantly gypsum and calcite, respectively, the specific phase of precipitated mineral may be less influential to material toughness than other factors. In particular, cyanobacteria themselves or their mineral precipitates may increase composite toughness. Zhang and coworkers reported that the inclusion of ureolytic bacteria conferred a toughness benefit to fiber-reinforced cement.[32] Fracture toughness

Table 1. Compression Tests on LBMs and Controls

Group	Living Building Material	Abiotic Control	Abiotic High-pH Control
Modulus (MPa)	293.9 ± 11.0	323.1 ± 11.5	219.0 ± 6.4[a]
Strain at peak stress	0.0169 ± 0.0017	0.0167 ± 0.0011	0.0015 ± 0.0006
Maximum stress (MPa)	3.60 ± 0.327[b]	3.45 ± 0.124[b]	2.38 ± 0.0141

Data are presented as mean ± standard error of the mean.
[a]Significant difference compared with abiotic control.
[b]Significant difference compared with abiotic high-pH control.

was similar for an engineered strain with higher urease activity and $CaCO_3$ precipitation compared with a wild-type control, suggesting that bacteria themselves increase crack-tip resistance. Alternatively, $CaCO_3$ precipitated in the presence of cyanobacteria may also have distinct mechanical behavior compared with abiotic calcite. $CaCO_3$ minerals from brachiopods, for example, exhibit high nanoindentation hardness compared with abiotic $CaCO_3$, likely due to nanoscale polysaccharide inclusions.[24] Calcite precipitated by ureolytic microorganisms also has high nanoindentation hardness,[34] as does nacre.[35] The cell membrane of *Synechococcus*, as with many other microorganisms, is substantially composed of polysaccharides and amino acids. $CaCO_3$ can nucleate directly on this membrane, and biological macromolecules (e.g., polysaccharides, lipids, proteins) can incorporate into the crystal,[36] perhaps conferring a toughening effect. Although outside of the scope of the present work, identifying specific toughening mechanisms is an important topic for further investigation.

Compressive strength of the LBMs and control specimens in this study were similar to the minimum compressive strengths of cementitious mortars.[27] As expected, compressive strength was not affected by cyanobacteria-mediated mineralization. Since compressive strength is predominantly influenced by the strength, size, and distribution of aggregate, strength would be minimally affected by the much smaller bacterial-precipitated $CaCO_3$ crystals. The strength and toughness of LBMs was assessed at 7 days but are not expected to change with greater curing time. While cementitious materials gain strength with hydration and it is commonplace to assess mechanical properties at 28 days, LBMs instead dehydrate to impart strength to the gelatin scaffold. Because equilibrium mass for LBMs is obtained at ambient conditions in approximately 4 days, further changes to strength and fracture energy with curing time beyond 7 days are not anticipated. Nonetheless, if comparisons are made between the mechanical properties of LBMs and cementitious materials, both types of materials should be sufficiently cured.

Our results illustrate a tradeoff between biological viability and mechanical performance for this class of LBMs. Given that gelatin gains strength with dehydration,[20] peak mechanical performance of LBMs is obtained at maximum dehydration. By contrast, viability of cyanobacteria requires sufficient humidity, and minimum mechanical performance would be obtained at maximum viability conditions (i.e., 100% RH and 4°C). While viability was compromised for LBMs that were desiccated enough for peak mechanical performance, nonviable desiccated structures could be recycled as the abiotic component for new structures made from LBMs. The tradeoff between viability and mechanical performance could be mitigated by exploring molecular additives or other strategies to improve extreme desiccation tolerance of microorganisms (e.g., trehalose).[37–39]

Engineered LBMs with a capacity to regenerate in response to controllable environmental switches represent a new frontier for exponential material manufacturing and

end-of-life reuse. The high fracture energy of LBMs suggests that these materials may be particularly well suited for applications in which resistance to crack propagation is valued. Although this technology is in its nascence, potential applications of LBMs range from temporary civil and military structures to paving, façades, and other light-duty load-bearing materials. LBMs are not intended to broadly replace cementitious materials, but instead represent a new class of materials in which structural function is complemented by biological functionalities.

Optimizing the biological and structural characteristics of LBMs (e.g., temperature, humidity, aggregate gradation, hydrogel chemistry, and inclusion of biological molecules) may extend the utility of LBMs to a myriad of advanced applications. More broadly, other microorganisms and physical switches could be deployed within the LBM framework for the design and fabrication of multifunctional building materials capable of sensing, actuation, and chemical response. For example, microorganisms could potentially sense—and respond to—toxic chemicals or reveal structural damage with fluorescence.[40–42]

Conclusions

We engineered LBMs capable of exponential regeneration through the use of physical environmental switches and microbial-induced $CaCO_3$ precipitation. The LBMs were composed of an inert structural scaffold of sand and hydrogel that structurally supported living microorganisms. The living component of the LBM, the cyanobacterium *Synechococcus* sp. PCC 7002, mineralized and toughened the gelatin. When desiccated, LBMs exhibited improved fracture energy compared with abiotic controls. Subsequent generations of LBMs were regenerated from one parent LBM. Taken together, the results presented in this work demonstrate that new classes of LBMs could be engineered to impart multiple biological functionalities to structural materials for applications within the built environment.

EXPERIMENTAL PROCEDURES

Materials: Microorganisms, Media, Hydrogel, and Sand

The cyanobacterium *Synechococcus* sp. PCC 7002 was selected for this study. A preculture protocol was established to ensure consistent cell viability and $CaCO_3$ precipitation. *Synechococcus* cells were maintained in standard A+ medium[43] and supplemented with 1 mM sodium thiosulfate and 1.5% agar. These cells were used to inoculate 50 mL of A+ medium in 150-mL Erlenmeyer flasks, which were incubated on a shaker with 200 rpm at 37°C and illumination of approximately 180 μmol (photons) m^{-2} s^{-1} provided by Cool White fluorescent lamps. To isolate cells, we centrifuged cultures at 4,300 × *g* for 20 min at room temperature (22°C) and washed them once with modified low-salt content A+ (ALS) medium.

To make the LBM, we incubated *Synechococcus* in 50 mL of ALS medium (NaCl was removed from traditional recipe) mixed with dissolved gelatin (ALS-gel) (Table S1). The ALS-gel medium was developed by modifying A+ such that it contained sufficient nutrients to maintain cell viability while reducing the tendency toward halite precipitation. The ALS-gel was prepared by slowly mixing gelatin powder into ALS warmed to 50°C to facilitate solubility in a mixing ratio of 100 g gelatin per liter of ALS (Knox, 10% [w/v]). After full gelatin solubility and reduction of temperature to approximately 40°C, 0.1 M $NaHCO_3$ was added to the solution. The pH was adjusted to 7.6, followed by slow addition of 0.1 M $CaCl_2 \cdot 2H_2O$. Cells were added when the medium had cooled to 37°C at a starting density of OD_{730} 0.3. The liquid medium with cells was incubated consistent with preculture conditions for 10 h before mixing with aggregates to cast the LBM.

A local river sand (Boulder, CO, USA) was used as the fine aggregate for the LBMs and control specimens. The sands were sieved to particle sizes ranging from 1.18 mm to 2.36 mm, treated with 4% HCl for 24 h, and rinsed with distilled water until the pH was raised to 7. Sand was then dried in an oven at 80°C for at least 48 h before mixing.

Three types of specimens were examined in this study. The LBM was prepared from ALS-gel, sand, and *Synechococcus*. The abiotic control was a prepared without microorganisms. The abiotic high-pH control was similarly prepared, but the pH was adjusted to 10 with NaOH.

Assessment of Microorganism Viability in LBMs

LBMs and controls were prepared at a binder-to-sand ratio of 0.3 (v/w). Ingredients were mixed semi-continuously by hand for 1 h before casting in 50 × 50 × 50-mm^3 cube molds at 4°C. Cube specimens were demolded after 8 h and stored at 4°C in either 24%, 50%, or 100% RH chambers. Additional samples were assessed for ambient (i.e., room temperature and humidity) conditions.

The most probable number (MPN) method was utilized to quantify *Synechococcus* viability in LBM cubes at 0, 7, 14, and 30 days. To obtain suitable aliquots for the MPN assay, we placed whole LBM cubes individually in 500-mL beakers and incubated them at 37°C and 200 rpm for 1 h until they resembled a liquid-sand suspension. Test tubes with A+ medium were inoculated from the beakers and serially diluted from 10^{-1} to 10^{-4}. Replicates (n = 5) were prepared at each dilution rate. The test tubes were incubated at 37°C, 200 rpm, and 180 μE for 7 days, at which time positive (i.e., green) tubes were counted. These data were then used to obtain MPN values (MPN/mL) and 95% confidence intervals from standard tables.[44] The MPN assay was also performed on control cubes at 30 days to eliminate the possibility of "false-positive" tubes that might occur due to contamination.

Assessment of Regenerative Ability of Microorganisms in LBMs

LBMs and controls were prepared as described for viability assessments. For assessment of whether LBMs could exhibit regenerative behavior when subjected to temperature and humidity switches, three (first, second, and third) generations of specimens were created from the parent generation of LBMs. For each generation, viability was measured using the MPN procedure at 0 and 7 days. The following procedure was employed to regenerate samples.

1. High-temperature switch: LBM cubes designated for regeneration were placed individually into 500-mL beakers and incubated on a shaker table at 37°C and 200 rpm for 1 h. During this time, the contents of the beakers were melted until they resembled a medium viscosity liquid-sand suspension.
2. Liquid abiotic addition (high-humidity switch): 54 mL of new ALS-gel medium was added to each beaker.
3. Biotic propagation: the beakers were incubated at 37°C, 200 rpm, and 180 μ μmol (photons) m^{-2} s^{-1} for 6 h. The doubling time of *Synechococcus* sp. PCC 7002 has been reported in the range of 3–4 h depending upon the growth conditions.[37] Thus, 6 h was considered sufficient for propagation of the biotic component.
4. SS Solid abiotic addition: 180 g of sand was added to each beaker.
5. Low-temperature switch: Regenerated LBMs were cast in 50-mm^3 cube molds at 4°C. Cubes were demolded after 8 h and stored at 4°C at either 50% or 100% RH.

Assessment of Mechanical Properties of LBMs

Preparation of cyanobacteria and control medium for mechanical testing was the same as described in "Materials: Microorganisms, Media, Hydrogel, and Sand," except that samples for mechanical testing were prepared with a liquid/solid ratio of 0.13 and cured at 22°C. Cubes (50 × 50 × 50 mm^3) were used for uniaxial compression testing, and 90 × 25 × 25-mm^3 prisms for notched fracture testing. Compression and notched fracture tests were performed after 7 days of sample curing at ambient temperature (22°C), which achieved mass equilibrium (Figure S4).

The uniaxial compressive failure test followed the ASTM C109 (i.e., "Standard Test Method for Compressive Strength of Hydraulic Cement Mortars [Using 2-in. or [50-mm] Cube Specimens]"). An Instron 5869 universal testing machine was used with actuator displacement rate of 0.2 mm/s, determined by trial tests so that the load ramp fell into the range stipulated in ASTM C109 (900–1,800 N/s). Loading was stopped at 5 mm of displacement.

A three-point flexural test was used to measure fracture energy for center-notched prisms. The sample was placed with the notched face downward on a 70-mm span. Two legs of an Epsilon 3542 extensometer were fastened to the bottom of the prism at both sides of the notch to allow determination of the crack mouth opening displacement (CMOD). The quasi-static actuator displacement rate of 0.01 mm/s was applied until the displacement reached 2 mm. The fracture energy was determined by dividing the load under the load-CMOD curve by the original ligament area (25 × 20 mm^2).

Mineralogical Assessment of Precipitates

Additional samples were prepared for assessment of mineral phase and morphology for LBMs and controls. These samples were prepared as described in "Materials: Microorganisms, Media, Hydrogel, and Sand," except that sand aggregate was not added. Films were dried for at least 7 days before assessment. Dried films were ground with mortar and pestle and smear-mounted on no-background silicon disks for fingerprint XRD. A Siemens D500 X-ray diffractometer analyzed samples from 5° to 65° 2θ using CuKα X-ray radiation with a step size of 0.02° and a dwell time of 2 s per step. Mineral phases were identified using Jade software (MDI, version 9) and the International Center for Diffraction Data (ICDD) 2003 database.

Microstructures of LBM, abiotic controls, and abiotic high-pH control films were evaluated using a JEOL JSM-6480 scanning electron microscope (accelerating voltage of 10 kV, working distance 9–11 mm, aperture 30 μm). Samples were first sputter coated with a 15-nm coating of gold. SEM-EDS was employed to assess the chemistry of precipitates in the gelatin. In addition, mineralization was evaluated for the parent- to third-generation samples from the regeneration experiment.

Statistical Methods

Mechanical testing outcomes (compressive strength, fracture energy) were compared between LBMs and abiotic controls using ANOVA. Because it is well known that the mechanical properties of gelatin are influenced by small variations in experimental conditions (i.e., cooking temperature), gelatin batch was designated as a blocking factor in ANOVA analyses. Post hoc testing was performed using a Tukey procedure to adjust critical alpha for family-wise error. The definition of significance was set a priori to $p < 0.05$. For all models, residuals were checked for normality and homoscedasticity. All analyses were performed using Minitab (v18).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.matt.2019.11.016.

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AUTHOR CONTRIBUTIONS

Study design: C.M.H., S.L.W., J.A., J.Q., M.H.H., S.M.C., J.C.C., and W.V.S. Data collection: C.M.H., S.L.W., J.A., and J.Q. Data analysis: C.M.H., S.L.W., and J.Q. Data interpretation: C.M.H., S.L.W., J.Q., and W.V.S. Drafting manuscript: C.M.H., S.L.W., and W.V.S. Approving final version of manuscript: all authors. C.M.H. takes responsibility for the integrity of the data analysis.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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