Genetically Programmed Engineered Living Materials as High-Performance Bioplastics

Gokce Altin-Yavuzarslan[1,2,3], Kinsey Drake[2], Shuo-Fu Yuan[4], Sierra M. Brooks[5], Eng Kwa[2], Hal S. Alper[4,5], Alshakim Nelson[1,2]

[1] Molecular Engineering and Sciences Institute, University of Washington, Seattle, WA 98195, USA

[2] Department of Chemistry, University of Washington, Box 351700, Seattle, WA 98195, USA

[3] Department of Chemistry and Chemical Biology, Northeastern University, Boston, MA 02115, USA

[4] Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, TX 78712, USA

[5] McKetta Department of Chemical Engineering, The University of Texas at Austin, Austin, TX 78712, USA

*Corresponding Authors:

Prof. Alshakim Nelson (Lead Contact)

Department of Chemistry, University of Washington, Box 351700, Seattle, WA, USA

E-mail: alshakim@uw.edu

Prof. Hal Alper

Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, TX, USA

E-mail: halper@che.utexas.edu

SUMMARY

Engineered Living Materials (ELMs) are a class of materials comprising living cells and a polymer (or biopolymer) network that together, afford a function, performance, or property that could not be achieved by the individual components. There are limited means to fabricate ELM bioplastics with arbitrary 3D shapes and requisite physical properties for mechanical performance. Herein, we use programmed bioproduction to tune the stiffness and degradation of ELMs with protein-based matrices. Using genetically engineered *Saccharomyces cerevisiae* strains, it is possible to induce production of betaxanthins and proteinase A in response to copper ions and galactose, respectively. The betaxanthins served to enhance the modulus of the bioplastics and reduce the degradative activity of native proteases, whereas proteinase A was produced for the rapid on-demand degradation of the ELM. This bio-manufacturing approach provides the means to fabricate ELMs with arbitrary 3D shapes and bio-augmented mechanical properties that also address demands for sustainability.

Key words: Engineered living materials, 3D printing, Bio-augmentation, Biomanufacturing, Genetic programming, Biodegradable, Bioplastic

Introduction

The synergy of synthetic biology and materials science yields innovative strategies for fostering environmental sustainability and improved functionality. Among these approaches, engineered living materials (ELMs) stand out as a promising platform. ELMs are a distinctive

class of smart materials that result from the integration of synthetic or non-synthetic living cells into a polymer network[1]. Recent studies highlight ELMs as alternative bioproduction platforms for chemical[2] and energy[3–5] production. Besides bioproduction capabilities, ELMs can also be designed for self-healing[6,7], shape-morphing[8], or bio-augmented stiffness[9,10]. Despite the future potential of ELMs in areas like smart packaging, textiles, soft robotics, and biosensor devices, a persistent challenge lies in the design and manufacturing of ELM bioplastics that can address the future needs for sustainability and chemical circularity[11].

Synthetic polymers and biopolymers are susceptible to acid- and base-catalyzed hydrolysis, particularly when the polymers possess ester or amide bonds[12,13], and non-enzymatic hydrolysis can be a slow process to achieve complete degradation[13]. Synthetic strategies have also been used to develop polymer networks that can be easily degraded via thiol-ester exchange or contain nano-dispersed embedded enzymes that catalyze degradation during the recycling process[14,15]. Biopolymer-based plastics (i.e., formed from proteins, cellulose, etc.) are more easily degraded than their petroleum-based counterparts, but still require external inputs such as heat or enzymes to degrade[16–18]. We previously reported two different biodegradable protein-based resin formulations, namely methacrylated bovine serum albumin (MABSA)[19] and poly(ethylene glycol)diacrylate modified bovine serum albumin (BSA-PEGDA)[20,21], for use as resins for vat photopolymerization. 3D printed samples from these formulations exhibited plastic behavior when dried, making them promising candidates for bioplastic applications.

ELMs that utilize a native or easily bio-mimicked biological extracellular matrices such as polysaccharides and proteins can utilize natural enzymes for the degradation of the matrices at the end of its life cycle of utility. While biodegradability is a benefit of biopolymer-based

ELMs, temporal control over when the biodegradation takes place presents a barrier for the implementation of such materials, especially in the built environment. During the lifetime of an ELM material, the native proteases and esterases of the encapsulated microorganisms can degrade the extracellular matrix, except in instances of heated and cell-deactivated ELMs, which affects the performance of these materials while in use[9,22]. Ideally, these enzymes should not affect the degradation of the ELM until the end of its life cycle of utility.

ELM hydrogels can utilize metabolic products from engineered microorganisms to bio-augment the mechanical properties of the hydrogel matrix[9]. For instance, L-DOPA production within BSA-PEGDA ELMs improved the stiffness of the hydrogel matrix, while betaxanthins production significantly reduced the rate of enzymatic degradation. Betalains are water-soluble, nitrogen-containing pigments responsible for the red-violet (betacyanins) and yellow (betaxanthins) colors in some fruits and vegetables[23]. Recently, we demonstrated that betaxanthins can bind to bovine serum albumin, which helps restrict the degradation of bovine serum albumin hydrogels[9]. Additionally, we reported input-responsive ELMs using genetically modified *S. cerevisiae* with multiple gene circuits wherein the cells produced betaxanthins or proteinase A in response to copper ions or galactose, respectively[2]. This approach showcased a method for temporal control over dynamic gene expression, as well as the long-term genetic expression capabilities of these systems in continuous cultures.

Herein, we demonstrate the additive manufacturing of ELMs that utilize inducible gene production to program the ELM's stiffness and temporal control of degradation (Figure 1). The 3D printed ELMs comprised BSA-PEGDA polymer matrices that encapsulated *S. cerevisiae* cells genetically engineered for the inducible production of betaxanthins or proteinase A. The betaxanthins served to enhance the modulus of the bioplastic materials and to reduce the degradative activity by native proteolytic enzymes of *S. cerevisiae*, while proteinase A was

produced for the rapid on-demand degradation of the ELM bioplastic. Thus, the manufacturing and end-of-use degradation of these mechanically functional bioplastic ELMs are genetically programmed. To highlight the practical utility of these ELM compositions, a functional wall hook was fabricated and kept in operation for 60 days, after which time the preserved microorganisms were induced to produce proteinase A for the degradation of the material. Thus, this approach demonstrates an advanced biomanufacturing scheme that utilizes additive manufacturing and genetic control to produce high-performance bioplastics.

Result and Discussion

Effects of inducible bioproduction on the polymer network

An *S. cerevisiae* strain was engineered with a multi-input genetic program (Figures 2A-B) for the dynamic expression of betaxanthins (Figures 2A, S1A) and/or Proteinase A (Figures 2B, S1B) in the presence of Cu^{2+} and/or galactose, respectively. BSA-PEGDA resins comprised the genetically engineered *S. cerevisiae* and were 3D printed on a commercial vat photopolymerization 3D printer. ELM constructs with a cylindrical shape (x =10.5 mm, y = 10.5 mm, z = 7 mm) were fabricated and their capacity for inducible bioproduction was evaluated over 15 days by culturing them with different inducers. In the presence of the Cu^{2+} inducer, ELM constructs consistently produced betaxanthins (Figures 2A, S2E), while a continuous production of Proteinase A was sustained by culturing the construct in media containing galactose inducer (Figures 2B,). Upon introduction of both Cu^{2+} and galactose inducers into the culture media, the ELMs exhibited simultaneous production of both betaxanthins and proteinase A (Figures 2A and 2B). However, the quantity of product detected was lower when both betaxanthins and proteinase A were produced concurrently, in comparison to the product obtained under single inducer conditions. Notably, a limited production was observed when ELMs were cultured in basal media devoid of inducers.

We investigated the effect of the residence time of the microbes in the printing resin on cell proliferation and bioproduction within the ELMs. Higher initial viability after photocuring should promote greater proliferation of cells from the hydrogel into the media. BSA-PEGDA resins with non-inducing media and freshly grown spk05 cells were kept at room temperature for 0, 1, and 2 h before photo-curing samples to simulate different lengths of printing times (2h is the time of the longest print). Samples were then placed in the different inducing media for 24 h and cultured. In Fig. S3A, we show that the cell proliferation resulting in outgrowth from the ELMs increased slightly with cell residence time in the resin, likely due to the increased time for cell proliferation. Samples incubated in non-inducing media exhibited higher cell outgrowth. We then normalized the bioproduction to the cell growth of each inducer set and found that the bioproduction of betaxanthins relative to cell growth of the Cu^{2+}-induced samples decreased slightly with resin holding time, suggesting that there may be a decrease in viability over extended print cycles (Fig S3B). The galactose-induced samples had consistent normalized proteinase A production that did not decrease as a result of the cell residence time in the resin (Fig S3C).

Rheometric characterization techniques are commonly used for synthetic polymeric materials to understand network properties, degradation, and viscoelastic characteristics[14,24], which is reflected in the storage and loss moduli of the material[25]. Here, we used the direct relationship between the plateau storage modulus (G′) of a polymer network and its crosslinking density (Equation S1)[24] to investigate the effects of inducible bioproduction on the ELM matrices (Figure 2C). We prepared samples by photocuring disks (x = 20 mm, y = 20 mm, z = 1 mm) at 405 nm to match the dimensions of the parallel plate geometry. Additionally, the dry mass loss over time for each of these samples was also measured (Figure 2F). We quantified these changes over the course of 15 days in response to induced expression of betaxanthins or proteinase A. To understand the extent of degradation due to hydrolysis of

peptide and ester bonds in the BSA-PEGDA network, control samples without cells – the acellular BSA-PEGDA matrix – were subjected to the same culturing conditions as the inducible ELMs (Figure 2E,H). Additionally, to understand the programmability of the inducible ELMs due to induced bioproduction, we also prepared ELMs with wild type (WT) *S. cerevisiae* under the same culturing conditions (Figure 2D and 2G). To more accurately demonstrate the relative changes in G' of each sample set across 15 days, we normalized the measured G' to the initial value (G'/G'_0) to provide a clearer comparison [26]. Critically, we did not observe significant differences among the initial storage moduli across all samples ($p=0.931$, $p = 0.498$, $p = 0.988$ for inducible ELMs, WT ELMs, and BSA-PEGDA polymer, respectively, (Table S1).

Our rheological investigations show that Cu^{2+}-induced production of betaxanthins significantly retarded the microbial degradation of BSA-PEGDA ELMs, which is consistent with our previous report[9] using *S. cerevisiae* for the constitutive production of betaxanthins. The continuous Cu^{2+}-induced production of betaxanthins over the 15-day period had a negligible effect on the storage modulus of the ELM matrix compared to the acellular BSA-PEGDA matrix (Figure 2C and 2E), as the G' values for both matrices were similar throughout the experiment. Thus, there was no significant difference between the BSA-PEGDA sample incubated in Cu^{2+} media and the Cu^{2+}-induced ELM on day 15 ($p=0.4479$). In addition, there was no significant difference among the G' of the Cu^{2+}-induced ELM sample ($p=0.8834$) from days 1 to 15. We also observed minimal changes to the dry mass over this period of time, which is consistent with the rheological results (Figure 2F). These results show that betaxanthins do not affect the mechanical properties of these water-swollen ELM networks, and that the mechanical properties do not change despite the constitutive production of proteolytic enzymes by the cells[27]. Betaxanthins have previously reporting that they retard microbial degradation of BSA-PEGDA ELMs by interacting with the BSA and altering its secondary structure[9]. In

contrast, the control experiments on non-induced ELMs shows a decrease in the G′ values over time (Figure 2C) with a concomitant decrease in the dry mass over the same period (Figure 2F). Thus, in the absence of betaxanthins, the endogenously expressed proteolytic enzymes can degrade the network slowly over time. Notably, the WT ELMs exhibited decreases in storage modulus and dry mass regardless of the inducing media they were cultured in (Figure 2E,G).

To facilitate the rapid degradation of the BSA-PEGDA ELM, galactose can be used to induce the production of proteinase A by the engineered microbes. Rheometric characterization showed a significant difference in the G′ of galactose-induced BSA-PEGDA ELMs versus the acellular BSA-PEGDA matrix (Figure 2C,E) over time. The acellular BSA-PEGDA matrix in media containing galactose had a G′ of 27.8 ± 8.6 kPa on day 1 that decreased to 11.6 ± 4.4 kPa (*p=0.0567*) after 15 days. For the galactose-inducible ELMs, there was a more significant decrease in the G′ from 37.5 kPa ± 9.6 kPa on day 1 to 7.5 kPA ± 0.3 kPa on day 15 (*p=0.0276*). Overall, the fastest rate of decrease was observed for the galactose-induced ELMs (0.0458 kPa/day), which was over 6 times the magnitude of the degradation rate for Cu^{2+}-induced ELMs. These results are supported by the extensive dry mass loss for galactose-induced ELMs that decreased from 135.9 ± 6.7 mg to 89.0 ± 5.4 mg after 15 days (Figure 2F) which show that genetic programming provides a viable strategy for on-demand degradation of BSA-PEGDA ELM matrices induced by the presence of galactose. WT ELMs did not show substantially different slopes of degradation over the 15 day period, highlighting the benefits of genetic induction rather than constitutive metabolic activity in ELMs (Table S2). Moreover, these changes can be monitored rheometrically over time.

Many proteases are known to have a higher activity in lower pH environments[28], therefore, we investigated the cell viability and degradation rate of galactose-induced ELMs in more acidic media (pH 4). Live/dead stains were used to evaluate the cell viability at pH 7 and pH 4 (Figures 3A and 3B, respectively). While the cell viability decreased from 99% at pH 7 to 79% at pH 4(Figure 3C), we observed a net increase in the proteinase A activity between these conditions (Figure 3D). Concurrently, a notable decrease in both G' and dry mass was observed in samples cultured for 15 days in galactose-containing media at pH 4. Specifically, the G' of galactose-induced ELM cultured at pH 4 (3.9 ± 1.3 kPa) was lower than at pH 7 (13.1 ± 4 kPa) after 15 days ($p = 0.0162$). The extent of mass change of galactose-induced ELMs is also notably higher under more acidic conditions (Figures 2F, 3D). To ensure that the pH of the media did not have an effect on the BSA-PEGDA network, we incubated the acellular BSA-PEGDA samples in galactose-containing media at pH 4 (Figure S5). After 15 days of incubation, the G' slightly decreased from 34.0 ± 21.3 kPa to 30.9 ± 3.8 kPa ($p = 0.5993$). Moreover, CD spectroscopy data showed that the pH reduction did not cause any structural changes to the BSA (Figures 5C, S67A).

One advantageous feature of cell-laden ELMs is their *in situ* production of proteolytic enzymes that are more effective than the exogenous addition of enzymes for biodegradation. To demonstrate this, we compared the continuous production of proteinase A (a pepsin-like aspartic proteinase[29]) within the galactose-induced ELMs to an acellular BSA-PEGDA sample incubated with pepsin (500 U/mL). Interestingly, the galactose-treated acellular BSA-PEGDA matrices at pH 4 and 7 exhibited minimal mass reduction. In contrast, the galactose-induced ELMs with *in situ* production of the protease showed a mass loss of 51.3 ± 2.6 % and 34.5 ± 4.0 % at pH 4 and 7, respectively.

Additive manufacturing of inducible ELMs

We next investigated the additive manufacturing of BSA-PEGDA ELMs and inducible bioproduction within the 3D printed ELM hydrogels. Vat photopolymerization with a stereolithographic apparatus (SLA) 3D printer was used to fabricate the lattice constructs shown in Figure 4A. These constructs were submersed in media that contained Cu^{2+} for betaxanthins production, galactose for proteinase A production, or both inducers for simultaneous production of betaxanthins and galactose. After 30 days of culturing, the Cu^{2+}-induced ELM decreased in size, but in general, maintained its initial lattice shape. In contrast the lattice construct induced by galactose broke apart into smaller fragments due to degradation of the network by proteinase A. Simultaneous induction with both inducers demonstrated a better shape integrity compared to the galactose-induced ELM but exhibited slightly less stability than the Cu^{2+}-induced ELM. To investigate the changes in material properties via inducible metabolite production, we assessed the compressive modulus of induced ELM samples following a 30-day culture with the different inducers. The ELM samples were 3D printed in a cylindrical disk shape (x = 10.5 mm, y = 10.5 mm, z = 7 mm) and cultured under four conditions: culture media without any inducing agent, culture media with galactose, culture media with Cu^{2+}, and culture media with both galactose and Cu^{2+}. The compressive moduli for these ELM hydrogels were 357 ± 30 kPa, 248 ± 20 kPa, 415 ± 18 kPa, and 328 ± 10 kPa, respectively (Figure 4B, $p < 0.05$). The increased stiffness of the Cu^{2+} induced sample and softening of the galactose-induced sample reflects the mass changes expected in the presence of betaxanthins or proteinase A (Figure S7C). CD spectroscopic analysis suggests that betaxanthins can interact with proteins such as BSA and proteinase A to disrupt their secondary and tertiary conformations (Figures 5F, S6), which is consistent with a previous report in the literature[9]. These interactions can have consequences for the mechanical properties and degradation rates of the BSA-PEGDA matrix.

Sequential induction of genetic expression within the ELM matrix

In a scheme for bio-augmented manufacturing of ELMs as high-performance materials, the as-printed constructs must first undergo the bio-enhancing step prior to the end-of-use biodegradation. We investigated the simultaneous and sequential induction of the ELMs as shown in Figures 4 and 5, respectively.

First, we monitored the G' and dry mass of the ELMs over 3 different phases: Cu^{2+} induction (phase 1) of betaxanthins, no induction (phase 2), and galactose induction of proteinase A (phase 3). Phase 1 was a period of sustained betaxanthins production over 15 days. As expected, the G' and dry mass exhibited minimal changes over this phase. Phase 2 was a transition period with inducer free media to remove residual Cu^{2+} and cease betaxanthins production (7 days). During this time, the G' and dry mass was largely unchanged. In contrast, the control sample with no inducers present showed a significant decrease in the G' after day 10 of culture (Figure 5B), which is attributed to the native proteolytic enzymes produced by *S. cerevisiae*. The production of betaxanthins during Phase 1 in the ELMs prevents this degradation from occurring (Figure S8A). Phase 3 was the degradation phase, wherein galactose-containing media at pH 4 was used to degrade the ELM matrix (15 days). During this period of time, the G' decreased due to the proteolytic degradation of the BSA-PEGDA matrix by proteinase A (Figure S8A). The dry mass change of ELM samples over the three phases showed similar trends with changes to G' (Table S3).

The rate of degradation of the induced ELM samples was higher than the control sample that was not subjected to Cu^{2+} or galactose. This difference could be due to the difference in pH between the galactose-induced sample during phase 3 (pH 4) and the non-induced sample at the same time period (pH 7). As described earlier, a higher proteolytic activity was observed at pH 4 (Figure 3D). Furthermore, we observed pH-dependent interactions between

betaxanthins and the BSA-PEGDA matrix. Betaxanthins stability is affected by pH[30], and we observed pH-dependent structural changes of both BSA and proteinase A in the presence of betaxanthins. At neutral pH, circular dichroism (CD) spectroscopy experiments showed a red-shift in BSA in the presence of betaxanthins compared to pH 4, suggesting changes in the alpha helix structure of BSA (Figures S6A, 5C). Similarly, when performing UV-Vis (Figures S9A,C) and fluorescence experiments (Figure S10A) of BSA in the presence of betaxanthins, we observed an apparent decrease in BSA-betaxanthins interactions under acidic conditions. Our CD experiments of proteinase A in the presence of betaxanthins also showed structural changes to the enzyme which may suggest an inhibitory effect of betaxanthins towards proteinase A and other secreted enzymes (Figures 5F, S6B).

Additive manufacturing and bio-augmentation of ELMs as high-performance bioplastics

We next demonstrated the manufacturing, bio-augmentation, and bio-degradation life-cycle for ELM bioplastics that serve as functional components. To showcase the versatility of the additive manufacturing process, we fabricated several items that included spoons, and interlocking puzzle pieces (Figures S11). All of these ELMs were then subjected to Cu^{2+}-induced betaxanthins production and then dried to remove the water and afford the ELM bioplastic. These objects underwent isotropic de-swelling during the 3-day drying process, losing approximately 62-70% of their mass due to water loss (Table S4). This is consistent with previously prepared BSA-PEGDA bioplastic samples[21]. We observed that the presence of Cu^{2+} in the culture media caused concentration-dependent softening of the BSA-PEGDA material (Figure S12C), likely due to the bridging effect of divalent cations increasing BSA aggregation[31]. As such, we found that the optimal culturing conditions for stiffening the ELMs was induction with 0.05 mM Cu^{2+}.

The compressive modulus of the Cu^{2+} induced bioplastic ELMs after 5 days of culturing was 343 ± 11 MPa, exceeding that of the non-induced ELM (296 ± 17 MPa) and the galactose-induced (pH 4) ELM (218 ± 22 MPa, $p = 0.0017$) (Figure 6A). This value exceeds previous work with acellular BSA-PEGDA bioplastics with compressive moduli of 168.0 ± 5.2 MPa[21] and 162 MPa[20]. We also 3D printed dogbone specimens and performed uniaxial tensile experiments after 5 days of culturing in Cu^{2+}, galactose (pH 4), and non-inducing media (Figure 6B, S12), and found significant differences among the samples (p = 0.0416). Notably, the and found that the young's modulus of the Cu^{2+} induced samples (262 ± 58 MPa) was much higher than that of the galactose induced samples (146 ± 23 MPa). Thus, the improvement in the mechanical stiffness of the ELM bioplastics in the presence of betaxanthins produced *in situ* demonstrates the successful bio-augmentation of these materials. The ELM bioplastic objects were stable over indefinite periods of use. For example, we demonstrated the versatility and usability of the inducible ELM system by 3D printing wall hooks that could be further assembled via a dovetail joint. After printing, the hooks were easily assembled and left to dry in the bioplastic state for 7 days (Figure 7A). One of the many advantages of 3D printing is the versatility—we printed the hooks in two sizes (14 mm and 20 mm once dried) without any changes to our existing printer hardware. We cultured the hooks in Cu^{2+} containing media to improve their bioplastic stiffness via betaxanthins production as demonstrated in our compression and tensile experiments (Figure 6). After drying into the stiffened bioplastic form, the hooks could be attached to walls and support objects such as sweaters, scissors, wrenches, and keychains up to 40 times their weight (Figure 7B).

ELMs can preserve encapsulated microorganisms under dehydrated conditions for extended periods of time, and these cells can be reactivated upon rehydration with media[32,33]. We investigated the preservation and reactivation of ELM bioplastics after 60 days of use under ambient conditions in the bioplastic state. We observed that the engineered *S. cerevisiae* in the

3D printed hooks were reactivated upon introduction to culture media, and the bioproduction of the samples remained responsive to the inducer type in the culture media (Figure S14).

At the end of their usage period as a bioplastic, the samples were first incubated with non-inducing media for 7 days before being transferred to galactose-containing media at pH 4 to induce Proteinase A expression to accelerate degradation (Figure 7C). After 5 days, the hooks lost their structural integrity, and after 19 days of galactose induction at pH 4, the hooks lost 72% of their mass (Figure S15A). Similar results were observed for 3D printed interlocking puzzle pieces and spoons (Figures S11, S15). As a control, we also prepared identical 3D bioplastics without cells and observed minimal mass change over the same culturing conditions and time period (S16).

Conclusion

In this study, we developed the bio-augmented additive manufacturing of ELMs as high-performance bioplastics with end-of-use biodegradation. The materials were genetically programmed to undergo mechanical stiffening with the Cu^{2+}-induced production of betaxanthins and proteolytic degradation with galactose-induced production of proteinase A. Betaxanthins enhanced the modulus of the bioplastic materials and reduced the degradative activity of native proteolytic enzymes of *S. cerevisiae*, while proteinase A enabled the rapid on-demand degradation of the ELM bioplastic. The ELMs were 3D printed using a vat photopolymerization process, which afforded different objects that included spoons, wall hooks, and puzzle pieces. 3D printed ELMs not only provide a new approach toward bio-manufacturing, but they also represent a new strategy toward the hybrid manufacturing wherein the encapsulated cells can augment the native properties of the material. Additional genetic programming enables the incorporation of other material functions that can include biodegradation as we have shown. Moreover, the cellular dynamics are preserved for extended

periods of time in the bioplastic state (over 60 days), after which time the genes can still be induced using small molecules. These smart materials have the potential to significantly contribute to more environmentally conscious and sustainable solutions for the plastics economy.

Experimental Procedures

Materials

Bovine serum albumin was purchased from Nova Biologics. Polyethylene glycol diacrylate (M_w 700 g/mol), sodium persulfate and $Cu_2SO_4.5H_2O$ were purchased from Sigma-Aldrich. Tris(2,2′-bipyridyl) ruthenium (II) chloride hexahydrate ($Ru(bpy)_3Cl_2$), yeast extract, peptone, glucose, galactose and geneticin were obtained from Fisher Scientific.

Strains, plasmids, media

The *S. cerevisiae* strains, plasmids, and primers were all previously published[2].

3D printing of Inducible ELM Constructs

Inducible ELM constructs comprising *S. cerevisiae* spk05 cells encapsulated within a BSA-PEGDA matrix were fabricated by vat photopolymerization 3D printing using a Form 2 printer from Formlabs. The BSA-PEGDA resin was prepared according to a protocol described in a previous study[9]. Briefly, 20 g of BSA-PEGDA conjugate solution, 10 wt% PEGDA was dissolved in 11.2 g culture media. Then 30 wt% BSA in powder form was added to the PEGDA solution in small portions and vortexed for 1 min to react PEGDA with BSA. The BSA-PEGDA conjugate solution was stored overnight at 4 ℃ while the conjugation reaction continued. The following day, freshly grown *S. cerevisiae* spk05 cells, which were incubated overnight at 30 ℃ with orbital shaking at 220 RPM in 4 mL YPD media (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) with 50 g/mL geneticin (G418), were added to BSA-PEGDA conjugate solution. The cell density was measured at OD_{600} and 1 mL of the cell suspension

that contained $3x10^7$ cell/mL was added to 20 g of resin. Then, the photo initiator system (0.075 wt% $Ru(bpy)_3Cl_2$ and 0.24 wt % sodium persulfate) was introduced to the formulation. For the 3D printing, the Form 2 printer was operated in Open Mode using a layer height of 100 μm and photocured using a 405 nm violet laser (250 mW) with 140 μm laser spot size at 21 °C. Autodesk Fusion 360 was used to create the 3D models, or they were downloaded from Thingiverse.

Induction systems and bioproduction

3D printed ELM constructs were prepared by *S. cerevisiae* spk05 cells in the non-induced state. Different inducers were used to start different products as follows. *Betaxanthins production:* ELM constructs cultured with YPD media contains 0.5 mM $Cu_2SO_4.5H_2O$ inducer. *Proteinase production:* ELM constructs cultured with 2 wt% galactose containing culture media (10 g/L yeast extract, 20 g/L peptone and 50 mg/L geneticin*). Control group*: ELM constructs cultured with YPD, no inducer. To determine bioproduction, samples were collected from culture media different time points as follow; day 2, day 4, day 7, day 10 and day 15, centrifuged at 4400 rpm for 10 min, the supernatants were collected and stored at -20 ℃ until analysis was performed. Betaxanthins bioproduction was determined by fluorescence intensity measurement at excitation/emission: 485 nm/520 nm using a Fluoroskan Ascent FL Fluorescence Microplate Reader from Thermo Labsystems. Amplite® Universal Fluorimetric Protease Activity Assay Kit (AAT Bioquest) was used to quantify the amount of functional proteinase A. The assay was performed according to the procedure provided by the manufacturer. To monitor continuous bioproduction, at each time point, ELM constructs were first washed with appropriate culture media, then placed in new culture tubes, and cultured in fresh media.

Pepsin degradation experiments

Control degradation experiments were conducted on acellular BSA-PEGDA samples in 2 wt% galactose containing culture media (10 g/L yeast extract, 20 g/L peptone and 50 mg/L geneticin*)* media with 1 mg/mL of pepsin from porcine pancrease (500 U/mg). Samples were incubated under identical conditions to induced ELM samples (30°C with orbital shaking at 220 rpm).

Fluorescence microscopy imaging

Cell viability was calculated from fluorescence microscopy images of stained cells after 48 h of culturing. Biotium Live/Dead Yeast staining kit was used for cell staining according to the procedure provided by the manufacturer.

Monitoring the effect of different induction cycles on mechanical properties

In order to examine how induction cycles affect mechanical properties a load frame was used to measure compressive moduli, and a rheometer was used to measure the storage and loss moduli.

Measurement of loss and storage moduli: ELM constructs were prepared by casting the resin in cylindrical disks (x = 20 mm, y = 20 mm, z = 1 mm) under 405 nm for 12 min. Samples were removed from their mold and placed in appropriate cell culture media and incubated at 30 °C with orbital shaking at 220 rpm. After a specified number of days, constructs were removed from culture media and rinsed twice with distilled water and swelled to equilibrium overnight in distilled water. A TA Instruments Rheometer was used to perform the testing. Before testing, samples were trimmed to match the rheometer's 20 mm parallel plate geometry (x = 20 mm, y = 20 mm) and weighed to obtain their wet mass. Samples were loaded onto a Peltier plate set at 21°C with the aforementioned geometry and compressed at 2N axial force until stable storage and loss moduli values were obtained (0.5% tolerance, 5 consecutive points). Samples were measured in triplicate at 0.2% strain, 1 Hz for 180 seconds to obtain

storage and loss moduli values. To obtain the dry mass, samples were left to dry overnight at ambient conditions.

Measurement of compressive modulus: 3D printed ELM constructs were fabricated as a cylindrical disk (x = 10.5 mm, y = 10.5 mm, z = 7 mm) shape. Then, they were dried at room temperature for a week. Dried ELM constructs were incubated in different culture medias which are media containing $Cu_2SO_4 \cdot 5H_2O$ inducer, media containing galactose inducer, media contained both $Cu_2SO_4 \cdot 5H_2O$ inducer and galactose inducer. As a control group ELM construct culture in inducer free media. A Newton Test Machine electromechanical test frame with 1 kN load cell and a crosshead rate of 1.3 mm/min was used to perform compression experiments on the hydrogel samples. For the samples in the bioplastic state, samples were rinsed twice with diH2O then dried at ambient conditions for 3 days. An Instron Test Machine with a 50 kN load cell and a crosshead rate of 1 mm/min was used to assess the compressive modulus. The compressive modulus was calculated based on the slope of the stress (kPa)-strain (mm/mm) curve for strain values of 0.05 (mm/mm) - 0.1 (mm/mm).

Measurement of bioplastic tensile properties: 3D printed ELM constructs were 3D printed as tensile dogbones following ISO 727-2 (ISO 527-2/5B/6). After culturing for 5 d, specimens were rinsed twice with diH_2O, vacuum dried for 90 min, then equilibrated at room temperature overnight prior to the mechanical test. Tensile tests were performed with a Newton Test Machine electromechanical test frame with 1 kN load cell at a speed of 5 mm/min until mechanical failure of the samples. The Young's modulus was calculated based on the slope of the stress (kPa)-strain (mm/mm) curve for strain values in the linear region.

Statistical analysis

One-way ANOVA, Welch Test (p value of < 0.05) were used to calculate statistical differences by Igor Pro (Version 8.04) software. For multi-group comparisons, Tukey HSD, (p value of < 0.05) were used to calculated statistical differences by JMP Pro (Version 16.0)

software. The means and standard deviations (± S.D.) of replicates were calculated in JMP Pro (Versions 16.0) and Excel.

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Alshakim Nelson (alshakim@uw.edu).

Materials Availability

All unique/stable reagents generated in this study are available from the lead contact with a completed materials transfer agreement.

Data statement

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Acknowledgments

This work was supported by the United States National Science Foundation (NSF) Emerging Frontiers in Research and Innovation award 2223537. Part of this work was conducted at the Washington Nanofabrication Facility / Molecular Analysis Facility, a National Nanotechnology Coordinated Infrastructure (NNCI) site at the University of Washington with partial support from the National Science Foundation via awards NNCI-1542101 and NNCI-2025489.

Author Contributions

G.A.Y and K.D contributed equally to this project, and they are first authors of this paper. G.A.Y, K.D, A.N and H. S. A conceived the project and designed the experiments. S.M.B and S.F.Y designed metabolically engineered yeast. E.K synthesized protein-based polymer and

performed cell imaging. G.A.Y wrote the manuscript with contributions from all authors. All authors have given approval to the final version of the manuscript.

Declaration of interests

None of the authors have any conflicts of interest with the work presented.

References

1. Rodrigo-Navarro, A., Sankaran, S., Dalby, M.J., del Campo, A., and Salmeron-Sanchez, M. (2021). Engineered living biomaterials. Nat Rev Mater *6*, 1175–1190. https://doi.org/10.1038/s41578-021-00350-8.
2. Sugianto, W., Altin-Yavuzarslan, G., Tickman, B.I., Kiattisewee, C., Yuan, S.-F., Brooks, S.M., Wong, J., Alper, H.S., Nelson, A., and Carothers, J.M. (2023). Gene expression dynamics in input-responsive engineered living materials programmed for bioproduction. Materials Today Bio, 100677. https://doi.org/10.1016/j.mtbio.2023.100677.
3. Freyman, M.C., Kou, T., Wang, S., and Li, Y. (2020). 3D printing of living bacteria electrode. Nano Res. *13*, 1318–1323. https://doi.org/10.1007/s12274-019-2534-1.
4. Su, Y., McCuskey, S.R., Leifert, D., Moreland, A.S., Zhou, L., Llanes, L.C., Vazquez, R.J., Sepunaru, L., and Bazan, G.C. (2021). A Living Biotic–Abiotic Composite that can Switch Function Between Current Generation and Electrochemical Energy Storage. Advanced Functional Materials *31*, 2007351. https://doi.org/10.1002/adfm.202007351.
5. Choi, J., Min, J., Kim, D., Kim, J., Kim, J., Yoon, H., Lee, J., Jeong, Y., Kim, C.-Y., and Ko, S.H. (2023). Hierarchical 3D Percolation Network of Ag–Au Core–Shell Nanowire-Hydrogel Composite for Efficient Biohybride Electrodes. ACS Nano *17*, 17966–17978. https://doi.org/10.1021/acsnano.3c04292.
6. Duraj-Thatte, A.M., Courchesne, N.-M.D., Praveschotinunt, P., Rutledge, J., Lee, Y., Karp, J.M., and Joshi, N.S. (2019). Genetically Programmable Self-Regenerating Bacterial Hydrogels. Advanced Materials *31*, 1901826. https://doi.org/10.1002/adma.201901826.
7. McBee, R.M., Lucht, M., Mukhitov, N., Richardson, M., Srinivasan, T., Meng, D., Chen, H., Kaufman, A., Reitman, M., Munck, C., et al. (2022). Engineering living and regenerative fungal–bacterial biocomposite structures. Nat. Mater. *21*, 471–478. https://doi.org/10.1038/s41563-021-01123-y.
8. Rivera-Tarazona, L.K., Shukla, T., Singh, K.A., Gaharwar, A.K., Campbell, Z.T., and Ware, T.H. (2022). 4D Printing of Engineered Living Materials. Advanced Functional Materials *32*, 2106843. https://doi.org/10.1002/adfm.202106843.
9. Altin-Yavuzarslan, G., Brooks, S.M., Yuan, S.-F., Park, J.O., Alper, H.S., and Nelson, A. (2023). Additive Manufacturing of Engineered Living Materials with Bio-Augmented Mechanical Properties and Resistance to Degradation. Advanced Functional Materials *33*, 2300332. https://doi.org/10.1002/adfm.202300332.
10. Paunović, N., Meyer, D., Krivitsky, A., Studart, A.R., Bao, Y., and Leroux, J.-C. (2023). 4D printing of biodegradable elastomers with tailorable thermal response at physiological temperature. Journal of Controlled Release *361*, 417–426. https://doi.org/10.1016/j.jconrel.2023.07.053.
11. An, B., Wang, Y., Huang, Y., Wang, X., Liu, Y., Xun, D., Church, G.M., Dai, Z., Yi, X., Tang, T.-C., et al. (2023). Engineered Living Materials For Sustainability. Chem. Rev. *123*, 2349–2419. https://doi.org/10.1021/acs.chemrev.2c00512.
12. Li, X., Tsutsui, Y., Matsunaga, T., Shibayama, M., Chung, U., and Sakai, T. (2011). Precise Control and Prediction of Hydrogel Degradation Behavior. Macromolecules *44*, 3567–3571. https://doi.org/10.1021/ma2004234.
13. Meyvis, T.K.L., De Smedt, S.C., Demeester, J., and Hennink, W.E. (2000). Influence of the Degradation Mechanism of Hydrogels on Their Elastic and Swelling Properties during Degradation. Macromolecules *33*, 4717–4725. https://doi.org/10.1021/ma992131u.

14. Carberry, B.J., Hernandez, J.J., Dobson, A., Bowman, C.N., and Anseth, K.S. (2022). Kinetic Analysis of Degradation in Thioester Cross-linked Hydrogels as a Function of Thiol Concentration, pKa, and Presentation. Macromolecules *55*, 2123–2129. 10.1021/acs.macromol.1c02396.
15. DelRe, C., Jiang, Y., Kang, P., Kwon, J., Hall, A., Jayapurna, I., Ruan, Z., Ma, L., Zolkin, K., Li, T., et al. (2021). Near-complete depolymerization of polyesters with nano-dispersed enzymes. Nature *592*, 558–563. 10.1038/s41586-021-03408-3.
16. Iyer, H., Grandgeorge, P., Jimenez, A.M., Campbell, I.R., Parker, M., Holden, M., Venkatesh, M., Nelsen, M., Nguyen, B., and Roumeli, E. (2023). Fabricating Strong and Stiff Bioplastics from Whole Spirulina Cells. Advanced Functional Materials *33*, 2302067. 10.1002/adfm.202302067.
17. Erdal, N.B., and Hakkarainen, M. (2022). Degradation of Cellulose Derivatives in Laboratory, Man-Made, and Natural Environments. Biomacromolecules *23*, 2713–2729. 10.1021/acs.biomac.2c00336.
18. Su, J., Zhao, K., Ren, Y., Zhao, L., Wei, B., Liu, B., Zhang, Y., Wang, F., Li, J., Liu, Y., et al. (2022). Biosynthetic Structural Proteins with Super Plasticity, Extraordinary Mechanical Performance, Biodegradability, Biocompatibility and Information Storage Ability. Angewandte Chemie International Edition *61*, e202117538. 10.1002/anie.202117538.
19. Smith, P.T., Altin, G., Millik, S.C., Narupai, B., Sietz, C., Park, J.O., and Nelson, A. (2022). Methacrylated Bovine Serum Albumin and Tannic Acid Composite Materials for Three-Dimensional Printing Tough and Mechanically Functional Parts. ACS Appl. Mater. Interfaces *14*, 21418–21425. 10.1021/acsami.2c01446.
20. Sanchez-Rexach, E., Smith, P.T., Gomez-Lopez, A., Fernandez, M., Cortajarena, A.L., Sardon, H., and Nelson, A. (2021). 3D-Printed Bioplastics with Shape-Memory Behavior Based on Native Bovine Serum Albumin. ACS Appl. Mater. Interfaces *13*, 19193–19199. 10.1021/acsami.0c22377.
21. Yu, S., Sadaba, N., Sanchez-Rexach, E., Hilburg, S.L., Pozzo, L.D., Altin-Yavuzarslan, G., Liz-Marzán, L.M., Jimenez de Aberasturi, D., Sardon, H., and Nelson, A. (2024). 4D Printed Protein-AuNR Nanocomposites with Photothermal Shape Recovery. Advanced Functional Materials *34*, 2311209. 10.1002/adfm.202311209.
22. Jeon, H.J., and Kim, M.N. (2016). Comparison of the functional characterization between alkane monooxygenases for low-molecular-weight polyethylene biodegradation. International Biodeterioration & Biodegradation *114*, 202–208. 10.1016/j.ibiod.2016.06.012.
23. Zhao, H.-S., Ma, Z., and Jing, P. (2020). Interaction of soy protein isolate fibrils with betalain from red beetroots: Morphology, spectroscopic characteristics and thermal stability. Food Research International *135*, 109289. 10.1016/j.foodres.2020.109289.
24. Caprioli, M., Roppolo, I., Chiappone, A., Larush, L., Pirri, C.F., and Magdassi, S. (2021). 3D-printed self-healing hydrogels via Digital Light Processing. Nat Commun *12*, 2462. 10.1038/s41467-021-22802-z.
25. Danielsen, S.P.O., Beech, H.K., Wang, S., El-Zaatari, B.M., Wang, X., Sapir, L., Ouchi, T., Wang, Z., Johnson, P.N., Hu, Y., et al. (2021). Molecular Characterization of Polymer Networks. Chem. Rev. *121*, 5042–5092. 10.1021/acs.chemrev.0c01304.
26. Kloxin, A.M., Kasko, A.M., Salinas, C.N., and Anseth, K.S. (2009). Photodegradable Hydrogels for Dynamic Tuning of Physical and Chemical Properties. Science *324*, 59–63. 10.1126/science.1169494.

27. Seredyński, R., Wolna, D., Kędzior, M., and Gutowicz, J. (2017). Different patterns of extracellular proteolytic activity in W303a and BY4742 *Saccharomyces cerevisiae* strains. Journal of Basic Microbiology *57*, 34–40. 10.1002/jobm.201600228.
28. Hofer, F., Kraml, J., Kahler, U., Kamenik, A.S., and Liedl, K.R. (2020). Catalytic Site pKa Values of Aspartic, Cysteine, and Serine Proteases: Constant pH MD Simulations. J. Chem. Inf. Model. *60*, 3030–3042. 10.1021/acs.jcim.0c00190.
29. Parr, C.L., Keates, R.A.B., Bryksa, B.C., Ogawa, M., and Yada, R.Y. (2007). The structure and function of *Saccharomyces cerevisiae* proteinase A. Yeast *24*, 467–480. 10.1002/yea.1485.
30. Cai, Y., Sun, M., Schliemann, W., and Corke, H. (2001). Chemical Stability and Colorant Properties of Betaxanthin Pigments from *Celosia argentea*. J. Agric. Food Chem. *49*, 4429–4435. 10.1021/jf0104735.
31. Navarra, G., Giacomazza, D., Leone, M., Librizzi, F., Militello, V., and San Biagio, P.L. (2009). Thermal aggregation and ion-induced cold-gelation of bovine serum albumin. Eur Biophys J *38*, 437–446. 10.1007/s00249-008-0389-6.
32. Yuan, S.-F., Brooks, S.M., Nguyen, A.W., Lin, W.-L., Johnston, T.G., Maynard, J.A., Nelson, A., and Alper, H.S. (2021). Bioproduced Proteins On Demand (Bio-POD) in hydrogels using *Pichia pastoris*. Bioactive Materials *6*, 2390–2399. 10.1016/j.bioactmat.2021.01.019.
33. Johnston, T.G., Yuan, S.-F., Wagner, J.M., Yi, X., Saha, A., Smith, P., Nelson, A., and Alper, H.S. (2020). Compartmentalized microbes and co-cultures in hydrogels for on-demand bioproduction and preservation. Nat Commun *11*, 563. 10.1038/s41467-020-14371-4.

Figure and Scheme Titles and Legends

Figure 1. Genetically programmed, bioaugmented manufacturing using inducible ELMs. An aqueous BSA-PEGDA resin is formulated with genetically engineered *S. cerevisiae* that includes genetic circuits to produce betaxanthins or proteinase A induced by Cu^{2+} or galactose, respectively. The resins were 3D printed using a light-based process (vat photopolymerization) to fabricate 3D ELM constructs. The 3D printed ELMs differentiate into a stiffer material in the presence of Cu^{2+} to afford mechanically functional bioplastic constructs. At the end of its life cycle of use, the ELM bioplastic degrades in the presence of galactose inducer for the genetically programmed biodegradation.

Figure 2. Inducible bioproduction and effects on the mechanical properties of ELMs. A) Betaxanthins bioproduction with Cu^{2+} inducer over 15 days. B) Proteinase A bioproduction with galactose inducer over 15 days. C-E) Change in normalized storage modulus (G'/G'_0) over time for (C) inducible ELM samples, (D) WT samples, and (E) acellular BSA-PEGDA matrices. Each graph includes a sample with Cu^{2+}, galactose, or no inducer. F-H) Change in dry mass over time for (F) inducible ELM samples (G) WT samples, and (H) acellular BSA-PEGDA matrices. Each graph includes a sample with Cu^{2+}, galactose, or no inducer. Cu^{2+} treated samples are 0.5 mM Cu^{2+} in YPD media, galactose treated samples are 2% galactose in YP media. Data presented as mean ± SD; n=3 or more replicates.

Figure 3. The performance of galactose induced ELM at pH 4. A-C) Cytotoxicity evaluation of inducible *S. cerevisiae* (spK05) in different pH conditions. A) *S. cerevisiae* (spK05) cells incubated at pH 7. B) *S. cerevisiae* (spK05) cells incubated at pH 4. Live cells are represented in green and dead cells are represented in orange - yellow. C) Live and dead cell ratio of *S. cerevisiae* (spK05) cells at pH 7 and pH 4. Total counted cells 1168 and 431 for pH 7 and pH 4, respectively. D) Proteinase A activity in pH 7 and pH 4 environments. E) Change in normalized storage modulus (G'/G'_0) over time for galactose induced ELM at pH 4 and acellular BSA-PEGDA in pepsin solution. F) Dry mass change of galactose induced ELM at pH 4 over 15 days. BSA-PEGDA polymer exogenously treated with pepsin was used as a control group. Data presented as mean ± SD; n=3 or more replicates.

Figure 4. Changes in material properties by long-term inducible bioproduction of ELMs (30 days). A) Optical images of 3D printed ELM in lattice forms which were induced for bioproduction of different compounds for 30 days. B) Uniaxial compression experiments were performed to obtain the compressive moduli of ELMs in hydrogel state which were cultured for betaxanthins production (Cu^{2+} induced ELM), for proteinase A production (galactose induced ELM), for both betaxanthins and proteinase A production (Cu^{2+} and galactose induced ELM). Non-induced ELM cultured in inducer free media as control group (* = $p < 0.05$). All scale bars represent 2 mm. Data presented as mean ± SD; n=3 or more replicates.

Figure 5. Effects of long-term switchable inducible bioproduction on ELM polymer network. Inducible ELM samples cultured in the following phases: Betaxanthins bioproduction with Cu (II) inducer media over 15 days, inducer free media for 7 days, proteinase A bioproduction with galactose inducer media at pH 4 for 15 days. A) Change of normalized storage modulus (G'/G'_0) over inducible bioproduction. B) Change of normalized storage modulus (G'/G'_0)

storage modulus (G') in inducer free culturing. C) CD spectroscopy of BSA and betaxanthins and BSA-Betaxanthins complex at pH 4. D) Change of dry mass over inducible bioproduction. E) Change of dry mass in inducer free culturing. F) CD spectroscopy of proteinase A and betaxanthins and proteinase A-Betaxanthins complex at pH 4. Data presented as mean ± SD; n=3 or more replicates.

Figure 6. Effects of inducible bioproduction on mechanical stiffness of bioplastic ELMs. A) Uniaxial compression experiments and B) tensile experiments were performed to obtain the compressive and Young's moduli of non-induced, Cu^{2+} induced, and galactose induced ELMs in bioplastic state after 5 days of culturing. (* $p < 0.05$, ** $p < 0.01$) Data presented as mean ± SD; n=3 or more replicates.

Figure 7. 3D printed inducible ELMs structures are in bioplastic form in dried state. A) 3D printed inducible ELM in wall hook form assembled after obtaining 3D wall hooks pieces. 3D printing allows fabrication in customized size. Wall hooks induced with Cu (II) for betaxanthin bioproduction to improve mechanical stiffness. B) Examples of how wall hook can carry different items of varying masses (scissors: 58 g, wrench: 128 g, keychain: 108 g) C) At the end of usage period, wall hooks induced with galactose at pH 4 for proteinase A production to start degradation. All scale bars represent 1 cm.