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Engineered Living Materials II

Mapping the ELM Field from Biogenic Content to Fabrication

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Summary

Building on the foundations, classifications and future potentials established in the Nano-Trust-Dossier 64 "Engineered Living Materials I", this dossier explores the ELMs landscape in more detail, introducing the Biogenic Composition Ratio (BCR) as a categorisation scheme and providing an in-depth review of ELMs types ranging from fully biogenic to bioinspired materials. Additionally, production methodologies such as lamination, bioprinting, and electrospinning are discussed in ELMs fabrication. This dossier serves as a transition towards examining ELMs properties across length scales. It addresses the complex challenges inherent in designing and scaling ELMs, which are elaborated upon in the concluding part.

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Introduction

The NanoTrust dossier 64 presented an exhaustive survey of Engineered Living Materials (ELMs), establishing a taxonomy based on the organisms utilised for the ELM as composite material components or base material producers[1]. The diverse functions facilitated by these organisms were elucidated in the antecedent publication through illustrative instances from referenced research endeavours. The current dossier extends the taxonomic framework by introducing additional categorisation dimensions, encompassing material type and production methodology.

The integration of ELMs manifests in diverse forms, giving rise to hybrid materials with unprecedented functionalities. These integrations span from ELMs incorporating living organisms to cultivated ELMs and to those emulating living behaviour.

The production methods of ELMs cover a spectrum of techniques, ranging from the in-function growth of living organisms to traditional methodologies such as the laminating processes rooted in industry. A nuanced understanding of and comparison between these methodologies are imperative for advancing the field and unlocking the full potential of each approach.

As we continue this exploration of ELMs, the primary objective is to elucidate the field's current state and provide insights that will drive future advancements. This dossier offers an overview of the discernible challenges in active ELMs research through a comprehensive examination of material types and production methods, introducing categorisations based on biogenic content and production technique. It is part of a series that constitutes the foundational basis for interpreting the current status of the ELMs domain and lays the groundwork for future developments. In the subsequent part, we will conduct an in-depth investigation into functional material properties and hierarchical structuring, concluding the series.

Horizon Scanning: Emerging ELMs

ELMs ordered by Biogenic Composition Ratio

The expansive domain of ELMs presents a diverse landscape, and a nuanced perspective can be gained by considering the Biogenic Composition Ratio (BCR). The BCR serves as a categorisation for understanding the involvement of living components within various ELM formulations. Some ELMs intricately integrate living organisms into their structure, while others selectively employ living cells exclusively during the material synthesis and formulation stages. Certain ELMs are derived from perished organisms shaped during growth processes. Recent advancements in the field extend to ELMs designed to emulate living organisms, such as the Venus fly trap biomimetic structure[2], which may operate without direct interaction with living cells. This nuanced classification emphasises the spectrum of approaches within the ELM landscape, catering to diverse research interests and needs.

ELMs composed of living organisms

The most apparent category within the ELMs spectrum is characterised by the direct integration of living organisms into their composition, or by the exclusive utilisation of living entities to construct the material. The distinction between such ELMs and naturally occurring living organisms lies in the intentional engineering by humans for specific functionalities or purposes, contrasting with the evolution of organisms shaped by their environment. While this differentiation may encounter complexities under rigorous evaluation, it is a pragmatic guideline for categorising materials within the ELMs domain.

Living organisms incorporated in ELMs

Microbes have been utilised in medical applications since antiquity for their antibiotic properties[3] and, in more contemporary contexts, technological applications for processes involved in their metabolism, exemplified by activities ranging from the recycling of plastics[4] to the fixation of radioactive waste[5]. Integrating microbes within ELMs facilitates material production, organisation and maintenance across various length scales while providing a means to sense changes in the material's environment.

Bacteria have been employed extensively in biotechnology because of their fast proliferation and easy genetic tractability (amenable to genetic manipulation). Spore-producing bacteria exhibit the unique capability to survive without moisture as spores (in a dormant state), enabling survival over extended periods in harsh environments. Bacterial spores revert to their vegetative state upon a triggering circumstance, such as contact with water. Spores of calcium carbonate-producing bacteria have been used as additives in concrete to impart self-healing properties to concrete structures. When micro-cracks in concrete materialise, water can infiltrate the cement through those cracks, initiating the vegetative state of the dormant bacteria and starting the repair process by filling the cracks with calcium carbonate[6]. This innovative approach to concrete repair extends the lifetime of the material, contributing to the sustainability of concrete structures.

Full biogenic ELMs

The living root bridges in India and Indonesia are some of the oldest ELMs that still exist today, as some of them are hundreds of years old[1, 7]. These bridges are made of aerial roots of the tree *Ficus elastica* wound around a frame of dead wood to direct their growth across rivers or canyons. The bridges strengthen due to the inosculation of the individual roots, gaining more load-bearing capacity over time. While timber bridges would rot and disintegrate in such wet environments within a few years, using living wood guarantees the long-term stability of the bridges[8, 9]. They represent an ancient ELM consisting entirely of living organisms.

Cultivated ELMs, utilised lifeless

Cultivated ELMs are defined as ELMs that do not incorporate living organisms in the end material, as the living component does not survive past the production process. The differentiation from current biogenic materials (such as dead wood or bone) is achieved through the engineering step crucial to the production process. These ELMs integrate growth into the engineering/construction process to achieve specific functions or properties.

Biofabricated bricks via microbially induced calcite precipitation

Microbially induced calcite precipitation (MICP) is a process in which microbes secrete urease, an enzyme that can generate CO_2 from urea and catalyse calcite formation[6]. Calcite serves diverse applications as the most stable manifestation of calcium carbonate. *Biomason*™[10] utilizes MICP to create cement bricks grown for multiple weeks in a brick-shaped mould under ambient conditions. The calcite formed by the microbes acts as a scaffold for the other constituents of the final bricks. This process produces a fraction of the greenhouse gas emissions than the conventional production of bricks, as those are usually burned at high temperatures to facilitate the desired strength.

Cultivated biofilms

Biofilms are interconnected communities of microorganisms that adhere to surfaces and produce a slimy, protective matrix of extracellular substances that form the extracellular matrix (ECM). This matrix allows the microorganisms within the biofilm to interconnect with each other and adhere to surfaces, forming a structured and organized community. The ECM of bacterial biofilms consists primarily of polysaccharides (such as cellulose), nucleic acids and proteins[11]. Besides structural support, ECMs offer protection from the environment and support homeostasis[12]. Bacterial cellulose (BC) (see Sections *"Bacterial-based materials"* and *"Evolution of ELMs: Historical and Contemporary Applications"* in[1]) is an example of a cultivated ELM made from the biofilm produced by specific bacteria species.

Non-biogenic ELMs mimicking living behaviour

Biomimetic materials can be classified as ELMs without incorporating living parts in any step of production, savie for the design process. Such biomimetic materials can possess features and abilities associated with living organisms, such as self-healing properties.

Incorporating self-healing properties into a material can drastically reduce its environmental impact by extending its lifespan. Self-healing can be introduced in materials in various ways, such as via intrinsic features, extrinsic features and additives. Intrinsic self-healing of polymers relies on the reversibility of chemical bonding to repair the damage done to the material on the molecular and microscale. This strategy allows self-repair without introducing external materials. Intrinsic self-healing needs some physical stimulus, such as heat, exposure to light, or mechanical movement, to initiate the healing process.

Self-healing strategies relying on extrinsic features depend on healing agents different from the material incorporated into the material during production. The healing agent can, e.g. be present in capsules, nanoparticles, or vascular networks mixed with the raw material[13]. The healing agent is released into the damaged region, where a crack or rupture materialises in the material, which damages the containers of the healing agent. This damage releases the agent; in the case of liquid agents, this release can be amplified via capillary forces[14].

Hygroscopic motions, which are movements caused by the absorption or desorption of moisture, can be achieved in biomimetic materials by incorporating cellulose. An example of this can be seen in the development of intelligent textiles and actuators. When cellulose is integrated into a biomimetic material, it enables the material to respond to changes in humidity. This response is due to the natural ability of cellulose fibres to swell and contract with varying moisture levels[15].

ELMs interfacing living organisms

Biomimetics draws on technological advancements to address challenges for which nature has already devised solutions throughout its evolution. ELMs additionally utilise nature by directly integrating living components into technological materials, thus creating novel materials to address technical dilemmas.

Cell-polymer composites with hydrogels

Hydrogels are utilised for scaffolding in biomedical applications[16] like tissue engineering and cell or drug delivery[17]. Their high biocompatibility makes hydrogels a promising platform for incorporating engineered cells to create living, functional materials.

The weak bonding of hydrogels to other materials is the primary challenge for their usage in ELMs. Early hydrogels exhibit low mechanical toughness and strain before breakage[18]. In the last decade however, various hydrogels were developed with clearly improved mechanical properties[19] as well as improved binding properties[20].

A mechanically robust, biocompatible hydrogel-elastomer hybrid containing multiple genetically engineered bacterial cells is the basis for a stretchable ELMs sensor[21]. This sensor can respond to several chemicals with interactive genetic circuits. Such composites enable the creation of novel chemical sensors that can take a manifold of shapes and deform without losing function.

Biorobots with muscle cells

In soft biorobot research, mammalian muscle cells are intriguing, enabling linear actuators to perform walking, swimming, or gripping tasks. Cardiomyocytes are heart muscle cells that exhibit spontaneous contraction without the need for external stimuli and are, therefore, often employed in fabricating biorobots[22, 23]. A robotic system incorporating living cells for actuation represents an illustration of a functional composite ELM (see Sections "*ELMs ordered by Biogenic Composition Ratio*" and *"Composite materials"* in[1]). Cardiomyocyte cultivation provides various challenges because of the unique requirement of these cells to undergo mechanical load and stress during their development[24].

Previous research has conceived different designs for such biorobots, such as walking biological machines that can move in an aqueous medium by locomotion on a surface[25]. Other examples are biorobots capable of gripping, pumping, walking, swimming, and creating propulsion like jellyfish[26, 27].

ELMs sorted by production method

The construction of ELMs differs from classical materials because the living component needs certain minimum conditions to survive the assembly procedure. These conditions also allow us to sort ELMs by the possible production methods. The following examples showcase the exciting possibilities that already established conventional production methods offer for ELMs production.

Laminating

Lamination (Figure 1a) generally refers to producing material by fabricating different layers and fusing them in a specific way. Laminates are used all around us, for example, in wood products, where laminates allow larger uniform pieces of wood than would otherwise be possible, textiles and clothing to impart water-repellent properties, or identification and credit cards.

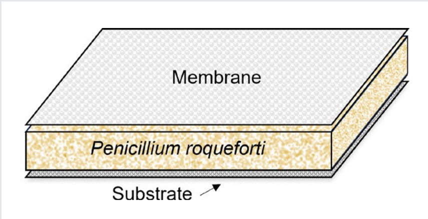
In ELMs, laminates can either consist of layers produced by living organisms, or incorporate layers of a living organism. By combining a supportive layer on which fungi grow protected by a nanoporous top layer that allows the transport of nutrients and gases, surfaces exhibiting self-cleaning[28] and the release of antibiotics[34] have been demonstrated. Notably, the pore diameters of the top layer were small enough so that the fungi could not grow out of their habitat between the two artificial layers. These surfaces display striking differences from traditional constructions: The antibiotic one could, in principle, keep on releasing antibiotics indefinitely as long as nutrients are supplied, yielding an on/off switch for functionality.

In-function growth

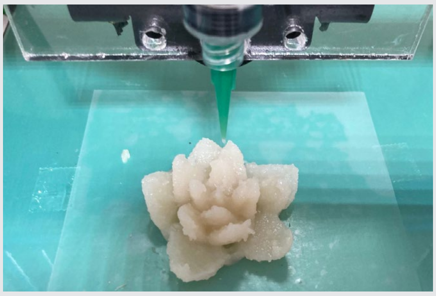
ELMs exhibit the potential unique capability of directing growth into predefined shapes and functions tailored for specific applications, streamlining the production process in contrast to conventional engineering methods. This approach leverages nature's inherent ability to create hierarchically structured and complex materials, presenting challenges to traditional production mechanisms[35, 36]. A classic plant-based example of in-function growth is the generation of compressed and tensioned wood in trees to control the required strength (Figure 1c).

Biofilm growth

Biofilms are conventionally harnessed for their pollutant-conversion capacity in applications like wastewater treatment. In typical decontamination scenarios, biofilms are cultivated on bio-carriers such as synthetic polymer structures de-

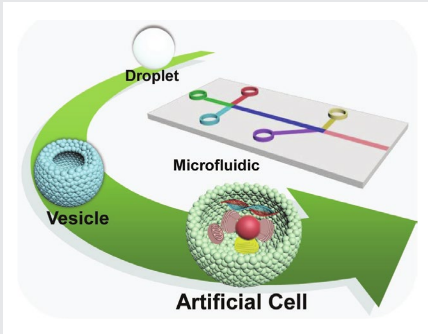


(a) Composite structure: A laminate comprising a substrate (90 µm), a living layer composed entirely of a food spill-digesting fungus (300 µm), and a nanoporous membrane atop (10 µm)[28].

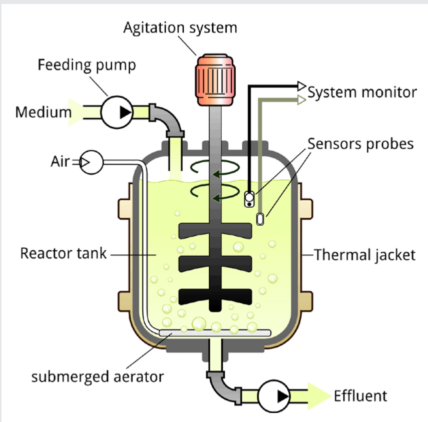


(b) A flower-shaped object's 3D ELM printing process that utilises microalgae bio-ink with an adapted FDM printer. The illustrated flower has a diameter of 25 mm[29].

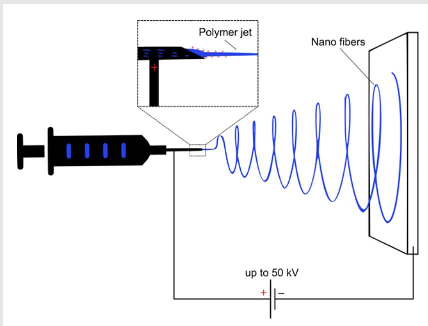
(c) Cross-sectional view of a Silver Linden tree, exhibiting a diameter of 190 mm, employing both compression and tension wood regulating and optimizing structural strength[30].



(d) Microfluidic-assisted generation of droplets and vesicles to fabricate artificial cells[31].



(e) Schematic Representation of a Continuous Stirred-Tank Bioreactor[32].



(f) Schematic Illustration of the Electrospinning Process: By ionizing the materials in solutions, melts or suspensions in a syringe, with the needle connected to high voltage and accelerating it toward the target via electromagnetic forces to form (nano) fibres[33].

Figure 1: *Diverse Production Methods for ELMs. Representative examples illustrate the range of production approaches for ELMs, including (a) layer-by-layer laminating, (b) 3D bioprinting, (c) controlled growth, (d) bioreactor-based cultivation, (e) synthetic biology-cell synthesis, and (f) electrospinning. These methods highlight the technological and biological diversity involved in ELM fabrication..*

signed for cell attachment. Conversely, in ELMs production, the focus shifts from the biofilm's pollutant-conversion capabilities to its intrinsic biomass. Rather than relying on bio-carriers, biofilms are cultivated directly in moulds. The material can be harvested when the ECM envelops the entire mould. This innovative approach finds application in endeavours such as *Biomason*™'s production of calcite bricks (see Section *"Taxonomy Categorizing ELMs Based on Biological Constituents"* in[1]).

Directed growth of plants

A more macro-sized approach to the in-function growth of ELMs consists of plant shaping: Over millennia, humans have manipulated trees to grow in desired shapes, usually for aesthetic or functional reasons. Trees can be grown in functional shapes such as the form of furniture or for usage in architecture. The company *Full Grown*™ grows chairs and other furniture from trees that can be harvested like crops. Even though the resulting furniture product is made from dead wood, it was produced by directing the growth of a living organism that continues to live after harvesting the product[37]. India's and Indonesia's tree bridges (paragraph *"Full biogenic ELMs"*) are a classic historical example of an ELM grown in function by directed plant growth.

Bioprinting

3D printing (Figure 1b) has become extremely widespread in recent years in applications and availability. A particular variant of 3D printing (Fused Deposition ModelingFDM) is bioprinting, where living cells are integrated with high spatial precision in scaffolds that offer conditions for cell viability. In that way, three-dimensional non-homogeneous living composite materials are constructed in a bottom-up approach in arbitrary forms[29, 6, 38].

Light-based lithographic techniques (Liquid Crystal DisplayLCD and Digital Light Processing DLP) may fix bioprinted cells at specific places in the scaffolding material via focal cross-linking of scaffolding material molecules. That way, bacteria can be encapsulated to limit their mobility. At the same time, the transmission of biologically active chemicals, such as antibiotics or quorum-sensing signals through the capsule boundaries, is still possible. Such an approach allows investigations into the relationship between function and structure in microbial communities[39]. Investigations in this direction may enable the design of ELMs with embedded microbe populations tailored to specific problems via the 3D organisation of microbes in the material.

Another use case for bioprinting is tissue engineering in medicine. Bioprinted tissue classifies as ELM in this category. The possibilities range from creating artificial blood vessels to constructing artificial bone replacements and possibly even whole organs[40].

Bioreactors

Bioreactors are widely used in biology, biochemistry research, and industries, such as in wastewater treatment (Figure 1e)[41]. In bioreactors, biofilm growth can be influenced in various ways, such as by controlling the influx of nutrients, the pH value of the surrounding medium, or the inclusion and structure of a biofilm carrier. Bioreactors offer unique possibilities for developing microbially produced ELMs through this tuning of biofilm growth. BC (see Sections *"Taxonomy Categorizing ELMs Based on Biological Constituents"* and Section *"Bacterial-based materials"* in[1]) is an example of this method of ELMs production, as it is a refined material grown by living organisms with minimum processing necessary before it can be applied in a wide range of applications.

Electrospinning

Electrospinning (Figure 1f) is a process through which materials such as organic polymers can be spun into thin fibres. It works by electrifying the liquid material, generating a jet[42]. Electrospun fibres feature diameters in the order of micro- and nanometres, though fibres with diameters below one nanometer have been reported[43]. Electrospun fibres are applied in various fields, from biomedical applications to air and water filtration and fabric production.

Trapped yeast cells in an electrospun polymer shell were still active in producing ethanol and biodegradation of polyphenols, illustrating the possibility of utilising such constructs as ELM-based dynamic filtration systems[44].

Further examples of novel materials with exceptional properties using this production method are *Micrococcus luteus* and *Nitrobacter winogradskyi* bacteria trapped in electrospun fibres. These can be used to create meshes that enable the sequestration of gold and the removal of nitrate from solutions, hinting at exciting future applications of ELMs[45].

Microfluidic-generation of synthetic cells

Synthetic biology enables the design of artificial cells that serve as micro-scale factories for ELMs (see Figure 1d). Built using Bottom-up Synthetic Biologys approaches (BUSBs), these cells are assembled from biological components such as lipids, proteins, and nucleic acids. Microfluidic systems are crucial here, allowing precise control over the structure and function of these synthetic cells[31]. Synthetic cells offer advantages over traditional cell factories, including modularity and improved stability. These synthetic cells can be tailored to produce materials, catalyse reactions, or respond to environmental signals using multicompartment vesicles and droplet-based methods[46, 47]. When embedded in material scaffolds, these synthetic cells enable creating responsive ELMs.

Acronyms

BC......... Bacterial cellulose
BCR...... Biogenic Composition Ratio
BUSB.... Bottom-up Synthetic Biology
DLP....... Digital Light Processing
ECM...... Extracellular matrix
ELM ...... Engineered Living Material
FDM...... Fused Deposition Modeling
LCD ...... Liquid Crystal Display
MICP..... Microbially induced calcite precipitation

Glossary

biocompatibility: Biocompatibility specifies the ability of a material to interact harmoniously with living organisms without causing harm or any other adverse reactions.

biomimetic: Biomimetics is the ingenious emulation of nature's designs, processes and systems to engineer innovative solutions for human challenges. This field draws inspiration from the intricacies of biological structures, functions, and strategies, seeking to replicate nature's efficiency, resilience and elegance across diverse disciplines. Biomimetic approaches aim to harness the profound wisdom encoded in the natural world to advance human technology and design[35, 48, 49].

hydrogel: A hydrogel is a mixture of a porous material, such as a porous polymer matrix, consisting at least of 10% interstitial fluid, and is mainly composed out of water[50].

inosculation: Two physically connected trees, where the outer bark was removed by friction at the connection, started producing callus tissue that grows outward, thereby merging the two trees.

Conclusion

This NanoTrust Dossier, "Engineered Living Materials II", has elaborated on the spectrum of biogenic integration and examined diverse fabrication approaches; a deeper exploration of structural properties and functionality is essential. The final part of this dossier series will address these aspects in detail, including ELMs classification by features, hierarchical length scales, and critical challenges such as scalability, containment, and bio-integration.

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Declarations

All authors declare that they have no conflicts of interest.

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