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Hydrogel platforms for engineered live biotherapeutics: materials, microbial integration and clinical potential

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Engineered living materials (ELMs), which integrate live microorganisms into biocompatible matrices, are emerging as powerful platforms for therapeutic applications. Among these, hydrogels encapsulating engineered live biotherapeutic products (eLBPs) offer enhanced microbial stability, targeted delivery, and functional versatility for treating human disease. By protecting microbes from environmental stress and immune clearance while supporting nutrient diffusion and activity, hydrogel systems address key challenges in microbial therapeutic delivery. This review highlights recent advances in hydrogel-based delivery of eLBPs, focusing on material design, microbial engineering, and performance metrics critical for clinical translation. We provide a framework for designing next-generation living materials for human health, emphasizing opportunities and challenges in bringing these systems from bench to bedside.

1. Introduction

Recent advances in materials science and microbial engineering have given rise to a novel class of materials known as engineered living materials (ELMs) – composite systems composed of matrices embedded with live microorganisms. These materials exhibit dynamic, responsive, and often regenerative properties, enabling applications across biotechnology and medicine.[1,2] ELMs offer vast potential in medicine, with applications ranging from probiotic bacteria-loaded microparticles for gut microbiome modulation, to microalgae-loaded patches for wound healing, and engineered yeast-loaded hydrogel biosensors for disease detection.[3–5]

Parallel to this, the field of live biotherapeutic products (LBPs) has rapidly evolved. LBPs are microorganisms such as bacteria or yeast that confer health benefits to humans, either through their natural biological activity or *via* engineering.[6] Probiotics represent a subset of LBPs that have demonstrated health-promoting effects in various clinical and wellness contexts including gut health, psychological disorders, and infection.[7–9] In contrast, engineered LBPs (eLBPs) are microorganisms that have been genetically modified to perform specific therapeutic functions.[10] These include the production of recombinant proteins or metabolites for disease treatment, as well as acting as biosensors for disease diagnosis.

While LBPs and eLBPs are promising new therapeutic modalities, their *in vivo* efficacy can be limited due to poor delivery efficiency or delivery challenges.[11–13] One promising strategy to overcome this challenge is encapsulation of LBPs and eLBPs within protective materials.[14] Inspired by natural biofilms, these matrices allow for nutrient and metabolite diffusion while containing the microbes.[2] Encapsulation also shields microbes from immune attack and prevents their escape, enhancing both safety and efficacy.[15] Compared to free cells, immobilized microbes exhibit greater resistance to environmental stresses such as pH, temperature, and mechanical forces.[16,17]

Of the materials to deliver these therapeutic microbes, hydrogels stand out due to their biocompatibility, high water content, and tunable mechanical properties.[5,11,18] Hydrogels can support the diffusion of small molecules and metabolites, maintain structural integrity, and can be engineered for specific biomedical applications.[5] Microencapsulation of microbes within hydrogels is a widely employed strategy to enhance microbial stability and facilitate targeted delivery to sites of disease.[19] These approaches can enable higher cell densities, improved productivity, and enhanced protection during delivery compared to free cells.[20,21] For example, immobilized microalgae demonstrate superior oxygen generation compared to free cultures.[22] Microencapsulation has also been used to add new functions to microbial cells by coating them with hydrogels. This coating can include

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components such as magnetic nanoparticles which give the cells new capabilities, such as being guided with magnets, without the need to genetically engineer the cells themselves.[23,24] Additionally, hydrogel encapsulation can prolong storage stability and post-storage viability.[25]

While significant progress has been made in hydrogel-based delivery of probiotics and LBPs, eLBPs expand the potential use of engineered live materials and their applications. Existing reviews have addressed probiotics in hydrogels, cell–material interactions, and LBP delivery, but a focused discussion on eLBP-loaded hydrogels is still lacking.[19,26,27] These systems hold broad potential across medical applications from wound healing, where hydrogels provide moisture and protection, to targeted delivery in the gastrointestinal tract, cancer therapy, and beyond. The field is rapidly expanding, as evidenced by increasing publications, market growth, and clinical trial activity.

Despite this momentum, no FDA-approved LBP hydrogel currently exists, even with growing research and clinical interest. This review aims to provide a comprehensive framework for designing eLBP-loaded hydrogels, covering materials and fabrication methods, microbe selection, and key considerations for therapeutic efficacy. We focus specifically on systems intended for human health applications, excluding environmental uses and non-engineered probiotics, which are covered in other reviews.[28–31]

2. Engineering the system: materials and microbial design

A. Material properties and fabrication

I. Material selection for desired system properties. The choice of hydrogel material is foundational to the design of engineered living systems, as it directly influences microbial viability, therapeutic delivery, and host integration. Hydrogels used in these systems can be broadly categorized into natural and synthetic materials, each offering distinct advantages and trade-offs in terms of biocompatibility, mechanical properties, and tunability (Fig. 1A). This section provides a general overview of hydrogel materials relevant to engineered living systems. For a more comprehensive understanding of hydrogel chemistry, fabrication techniques, and application-specific performance, readers are encouraged to consult specialized reviews and primary literature that explore these topics in greater depth.[32–36]

Biopolymer-based hydrogels, derived from biological sources such as plants, algae, and animals, are generally favored for their inherent biocompatibility, biodegradability, and renewability.[37–39] Polysaccharides are a category of biopolymer that have been used extensively in hydrogel fabrication because of their functionalization groups, natural bioactivity, and tunable mechanical properties.[37,40] They are specifically beneficial as eLBP delivery systems because many can be crosslinked using mild, cell-friendly conditions, are hydrophilic, and non-toxic to cells.[19] Their ability to absorb high water content and porosity allows for the diffusion of molecules, like secreted biologics produced by eLBPs, through the construct while protecting loaded cells from osmolar stress and the surrounding microenvironment.[41] Some polysaccharides like alginate, chitosan, and hyaluronic acid, are pH responsive and can be designed to degrade or swell to release contents during pH changes such as during gastrointestinal transit or during wound healing.[42] Many polysaccharides also contain some inherent mucoadhesion which can be beneficial for drug delivery.[19]

Among polysaccharide-based hydrogels, alginate is one of the most widely used. It forms hydrogels in the presence of divalent cations like calcium under mild gelation conditions and is generally recognized as safe (GRAS) by the FDA.[43] Alginate hydrogels are sensitive to pH and chelating agents, offering excellent biodegradability.[44] Chitosan, a positively charged polysaccharide, has demonstrated natural mucoadhesive and antibacterial properties.[37] Due to its positive charge, it can form complexes with anionic materials to form multifunctional constructs.[45] Hyaluronic acid (HA) is a polysaccharide naturally found in the extracellular matrix (ECM) that has cell surface receptors for improved cell interactions, making it a useful material in tissue engineering and drug delivery.[37] While these materials are well suited for biodegradable applications, their limited mechanical stability under physiological conditions restricts their value in long-term applications requiring durability.[46,47] Other polysaccharides, such as cellulose, xanthan gum, and resistant starch, exhibit greater tolerance towards pH changes and enzymatic activity; however, proper clearance of materials and drug release from these more stable constructs must be considered.[19,48] For information about other polysaccharide materials, reference these other reviews.[49–51]

To address the poor stability and enhance the tunability of polysaccharide-based hydrogels, researchers have developed a range of strategies to engineer hydrogel properties for specific applications.[46] The incorporation of additional crosslinking methods can help improve hydrogel stability but the use of added crosslinkers or solvents may reduce the system's biocompatibility.[46] Alternatively, composite hydrogels can be engineered by introducing polymers with improved stability, such as synthetic materials, into the polysaccharide matrix, to form interpenetrating networks (IPN) or semi-interpenetrating networks (sIPNs) that exhibit enhanced mechanical integrity and biocontainment.[46,47,51]

Protein-based hydrogels represent another class of natural materials, often preferred for biomedical applications due to their biocompatibility, biodegradability and functional versatility.[52] They can be made from either extracting and processing protein-based polymers from natural plant and animal sources, such as collagen, or from constructing synthetic polypeptide polymer chains of unique amino acid sequences.[53] A major benefit of protein-based hydrogels is their inherent bioactivity and modularity which allows bioactive domains to be incorporated for specific functionalities such as cell binding, controlled degradation, and cell signaling.[53] These

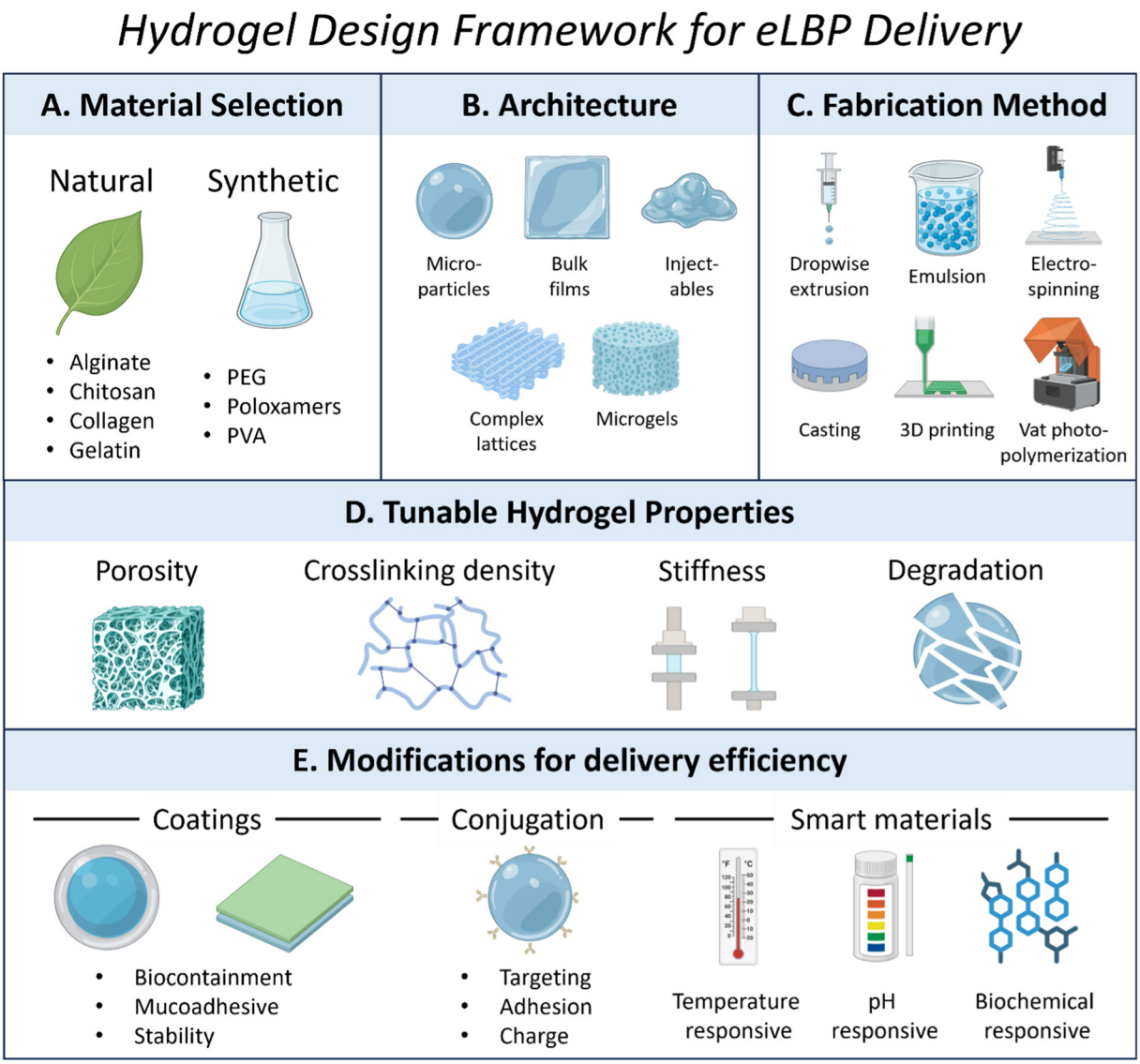

Fig. 1 Material considerations for loading engineered living biotherapeutic products. (A) Hydrogel material selection based on naturally-derived or synthetically-derived polymers with examples of the most commonly used materials in live cell loading for each. (B) Choice of hydrogel architecture with examples of common designs to consider. (C) Fabrication methods used to synthesize cell-loaded hydrogels. (D) Important hydrogel properties to consider and fine-tune for desired application. (E) Different methods to modify materials to achieve various material properties to improve delivery.

properties make protein-loaded hydrogels favorable as delivery vehicles but also offer benefits specifically for loading live cell cargo. Their biocompatibility, easy assembly, simple crosslinking, and tunable mechanical properties facilitate methods of hydrogel construction optimized for cell viability and proliferation within constructs.[52] Further, bioactive motifs on protein biopolymer chains used to interact with the extra-hydrogel milieu can also be applied to modulate the function of internal eLBPs.[52] Protein-based hydrogels can be integrated with growth factors, binding agents, or signaling moieties to affect microbial cell growth within hydrogels. For example, some peptide sequences can act as prebiotics or growth factors that enhance microbial cell viability and growth.[55] Proteins such as mucin can be also engineered into constructs to promote mucoadhesive properties for enhanced eLBP retention and delivery efficiency at target sites.[54] Although not studied, there is opportunity to engineer both protein-based biopolymers and LBPs to interact or support each other; for example, having cells express a binding motif and engineering the hydrogel with the corresponding sequence.

Of the protein-based hydrogel materials, the most common are gelatin and collagen, both derived from animal sources.[56] Collagen-based hydrogels self-assemble at physiological temperatures, are highly degradable in the body due to collagenase enzymes, and can be further crosslinked to improve stability in physiological environments.[57] As a component of natural extracellular matrix, it is especially useful in regenerative medicine and wound healing applications.[57] Gelatin, derived from

denatured collagen, is able to thermo-reversibly crosslink when heated which can be beneficial during manufacturing but increases its degradability in the body.[57] Gelatin can be crosslinked with agents like glutaraldehyde or modified with methacrylate groups to form gelatin methacrylate (GelMA), a popular material in photopolymerization-based bioprinting.[57–59] Both materials can be useful for encapsulating eLBPs because they can be fabricated in mild and cell-safe conditions. Silk fibroin hydrogels are biocompatible, biodegradable, and offer high mechanical strength compared to other protein-based hydrogel materials.[57] Their solubility and drug release profile in physiological environments can be extended with additional crosslinking such as using horseradish peroxidase (HRP).[57] Bovine serum albumin (BSA) protein-based materials have also been used for cell loading contexts as they can be designed to be mechanically robust while remaining enzymatically degradable.[1] They can exhibit lower immunogenicity due to their similarities to albumin naturally found in the body.[60] Synthetic protein-based hydrogels are polymeric materials composed of precisely engineered amino acid sequences that retain biocompatibility and biodegradability.[61,62] While they have the benefit of customizable design for specific functionality, this can increase manufacturing cost and complexity.[56]

Although protein-based materials are biocompatible and biodegradable, their instability in physiological environments due to enzymatic degradation, pH sensitivity, and heat responsivity can be a significant limitation to their success as drug delivery systems.[57] Their degradation can be improved by adding crosslinkers but many crosslinkers or their byproducts are toxic to cells.[56] Additionally, naturally-derived sources can exhibit batch-to-batch inconsistencies that cause irregularities in constructs and potential immunogenicity.[57]

In contrast to natural hydrogels, synthetic hydrogels provide greater control over mechanical properties, degradation rates, and functionalization, though they can lack the inherent biocompatibility of natural materials.[37,63,64] While they are less utilized for cell loading applications, there are synthetic polymeric hydrogel materials that can be used for eLBP loading. Poly(ethylene glycol) (PEG)-based hydrogels are commonly used due to their hydrophilicity, low cytotoxicity, and their FDA-approved status.[65,66] There are a variety of PEG-based derivatives that can be used as hydrogel materials such as PEG-diacrylate (PEGDA), a common photopolymerization material.[65] While they can be engineered with fine-tuned properties like porosity and mechanical strength, LBP cell viability must be considered as both the materials used and the fabrication processes needed to form gels can affect living cells.[65] Poloxamers, or PEO-based block copolymers, are a common PEG derived hydrogel material used for loading cells.[65,67] Pluronic F127 (Poloxamer 407) is a thermosensitive, biodegradable poloxamer commonly used for 3D bioprinting and injectable hydrogel systems.[65] Its ability to self-assemble into a hydrogel matrix in mild conditions allows for successful eLBP loading; however, Poloxamer 407 hydrogels have low stability *in vivo*, and cell viability is a concern in more stable composite systems.[67,68] Poly(vinyl alcohol) (PVA) is another synthetic polymer that can physically crosslink without additional agents.[66] It offers high mechanical strength and can support cell viability, although toxicity and lack of biodegradability can limit its effectiveness in eLBP applications.[65,69]

Other synthetic hydrogel materials have been used to load live cells, such as polyacrylamides, but issues with cell viability and toxicity remain with these materials.[66] To retain the vast functionality of synthetic hydrogels while protecting cell viability, composite materials can be made to achieve the benefits of both.[63,66,70] Additionally, systems can be designed with compartmentalized materials, allowing cells to mainly interact with biocompatible materials, while structurally supportive but less biocompatible materials provide support or containment for the system without cell interactions.[71,72]

II. Constructing living hydrogel systems to suit different engineering needs. Hydrogels can be fabricated into various geometries and structures to support and protect embedded microbes, tailoring their structure for both biological function and environmental interaction (Fig. 1B). Important geometrical considerations for eLBP-loaded hydrogel constructs include ensuring mechanical and structural compatibility with the intended application, optimizing the surface area to volume ratio, and achieving precise control over cell loading and distribution.

One of the simplest configurations is spherical hydrogel microparticles, which are commonly used in oral and systemic drug delivery applications due to their high surface area to volume ratios and minimal aggregation.[73] Spheres can provide vehicles for homogenous cell loading and uniform drug delivery of eLBPs to their target site.[73] A key consideration for eLBPs is the distance between encapsulated cells and the particle surface, as this spatial parameter governs the diffusion of therapeutic drugs, sensing molecules, and nutrients.[54] This diffusion distance varies depending on the size of the microspheres and the spatial distribution of cells within them, whether concentrated in the core or uniformly dispersed throughout the matrix. Core shell microparticles in which a spherical core material is surrounded by another shell material can be beneficial when separation between cells and the physiological environment is critical to their viability or function but can be less useful when cell interactions with the outside environment is important.[74] For example, Lewińska *et al.* used a co-axial electrospray system to create core–shell particles composed of alginate-encapsulated microbes in the core surrounded by a synthetic polymeric shell (PES), with a glycerol layer in between to act as a protective buffer.[75]

Films and patches are another commonly used system, especially useful for local therapeutic delivery, offering the advantage of a large surface area for interactions between loaded cells and tissue environments.[34,76] These can be bulk gels or lattices with defined geometries, such as cubes and hollow pyramids, to enhance diffusion and nutrient transport.[77] Advances in 3D bioprinting methods allow for high resolution, complex hydrogel matrix architectures loaded with live therapeutic cells.[78–82] The lattice geometry can be tailored

to meet the required mechanical properties of the desired delivery tissue for better patch application and healing.[35,79] Beyond tailoring matrix geometry to mimic target tissues, the ability to design complex architectures with high surface area-to-volume ratios offers significant advances for eLBP delivery and bioactivity.[78] In highly porous, interconnected structures, enhanced mass transfer and nutrient diffusion support more efficient microbial metabolism and therapeutic output compared to dense, non-porous constructs.[20,78,83] This high level of control over construct geometry allows for the spatial patterning of microbial cells using different inks, each loaded with a specific strain of microbe.[77] This is achieved by using separate ink cartridges, each loaded with a specific strain, and depositing them in a precise arrangement. For example, Liu *et al.* printed a sensor system in which three microbe strains were engineered to secrete GFP in response to a chemical inducer, either AHL, Rham, or IPTG.[77] These strains were printed in a specific conformation that was able to create spatially organized logic gates for cell-based complex biosensing.

Multi-layered hydrogel constructs can also be generated using additive manufacturing techniques to produce asymmetric systems with distinct surface functionalities. These designs are particularly useful in biomedical applications requiring targeted adhesion or directional drug release.[71] For instance, gastrointestinal capsules can be designed with an adhesive tissue-interfacing layer on one side and an eLBP-loaded layer on the other, enabling targeted adhesion and drug delivery or sensing.[84] Similarly, Rivera *et al.* employed extrusion-based 3D printing to produce tablet-shaped drug delivery devices with distinct layers, including a physical barrier to control unidirectional drug release.[85]

Hydrogel constructs made from a combination of these structures can also be used. One such strategy involves embedding cell-loaded microspheres into bulk hydrogel film matrices to provide physical separation between cell populations and other materials within the matrix.[15] Another embeds cells first in microspheres then encapsulates these microspheres in capsules.[86] This modular design approach can improve both viability and function by creating microenvironments tailored to specific tasks.

Finally, in applications where permanent matrix geometry is less critical, injectable hydrogels offer a convenient alternative. These systems consist of thermo-responsive polymers that crosslink rapidly *in situ* upon exposure to body temperature, enabling minimally invasive administration and localized treatment.[34] These materials are valuable in applications requiring the filling of cavities to enhance structural integrity, such as in cases of tissue damage.[87] For instance, Lu *et al.* describe a hydrogel formulation that transitions from liquid to gel within seconds of injection, providing both structural support and microbe encapsulation at the target site.[88] These can act as *in situ* drug depot or molecule sensing sites that can be implanted in harder to reach locations in a minimally invasive manner.[89] These systems provide benefits for eLBP delivery, including shorter processing time from cell culture to implantation in the crosslinked hydrogel and reduced cell damage during the preparation process.[90,91] Additionally, because these constructs conform to the shape of the cavity into which they are injected, homogeneously embedded cells within the ink remain closer to the target tissue, potentially improving drug delivery and metabolite sensing.[90] However, injectable eLBP hydrogels face the challenge of balancing injectability with mechanical robustness and stability in physiological conditions.[91–93]

III. Fabrication methods of living hydrogels. Living hydrogels can be fabricated using a variety of crosslinking strategies, including ionic, covalent, thermal, and physical interactions. Each of these methods influences the structural, mechanical, and biological properties of the resulting hydrogel.[32,94] For instance, covalent crosslinking tends to produce more mechanically robust materials, while ionic and physical crosslinking are often favored for their simplicity and biocompatibility.[41] A common covalent crosslinking method used for cell encapsulation in hydrogels is photocrosslinking and is often applied as a curing agent either as the primary crosslinking method or a secondary reinforcement for already crosslinked structures.[95] It has been used for a variety of constructs such as microparticles, 3D-printed patches and injectable hydrogels. Materials such as gelatin methacrylate (GelMA), hyaluronic acid methacrylate (HAMA), and Pluronic F127 modified with diacrylate groups (PluDA) are frequently used for cell applications as they crosslink in response to specific wavelengths of light and are biocompatible.[15] One study found that photocrosslinked PluDA hydrogels loaded with engineered bacteria were more mechanically robust and higher resolution than non-photocrosslinked constructs while maintaining cell viability and the diffusion of small molecules.[77] Photocrosslinking offers excellent control over microstructure and avoids mechanical stresses to cells seen in extrusion-based methods but may require careful optimization to avoid phototoxic effects on encapsulated cells.[95] The choice of crosslinking strategy is tightly linked to the intended application and directly shapes the fabrication process, which in turn impacts the functional performance of the hydrogel.[35,96]

There are a variety of fabrication methods for hydrogels that have been reviewed in more detail in these articles, we will focus on methods most suited for cell encapsulation.[35,38,45,73] For particles, common fabrication methods include extrusion, electrospraying, emulsion, microfluidics and for films, patches and more complex architectures electrospinning, casting, and three-dimensional printing are commonly used for cell encapsulation (Fig. 1C).[19,35,73,97]

One of the simplest ways to develop eLBP loaded microspheres is through simple extrusion methods in which cells are loaded into pre-crosslinked solutions and extruded through a needle into a crosslinking solution. For example, engineered *E. coli* was successfully loaded into chitosan microspheres with a diameter of ~1000 μm using extrusion.[98] Particles were able to keep cells contained for at least 2 weeks during which therapeutic proteins were produced and secreted *in vivo*. This method is valued for its simplicity and mild fabri-

cation conditions although there are limitations in the range of particle size able to be achieved and slow throughput.[19]

More advanced encapsulation techniques, such as electrospraying where a voltage is applied to extruded samples, can achieve a smaller particle size faster while maintaining cell viability during the process.[97,99] Li *et al.* developed electrosprayed alginate methacrylate microspheres ranging from 100–300 μm by applying a voltage of 1–6.5 kV to an eLBP-loaded alginate solution extruded into a $CaCl_2$ bath followed by photocrosslinking.[100] Bacteria maintained high viability inside microspheres and were able to detect target molecules in the supernatant and respond with a high fluorescent signal. Co-axial needle setups can be used to create core shell particles of different materials for added protection for cells and separation between components of the microparticles and the environment. In one example core–shell alginate microparticles were constructed using co-axial electrospray to encapsulate *E. coli* engineered as a sensor for colon inflammation.[101] Resulting particles were able to contain viable and active cells inside cores for improved cell viability and separation from the gastrointestinal environment while allowing the diffusion of biomarkers inside cells for successful detection by engineered cells.

Another common fabrication method is emulsion which creates droplets by mixing oil and aqueous phase solutions.[95] This method can create particles quickly but suffers from limited control over particle polydispersity and the materials required can be cytotoxic.[97,102] These limitations can be improved by using biocompatible oils and surfactants in the process and applying microfluidic techniques to control particle size, although this increases method complexity.[97] In one instance, *L. salivarius* bacteria was encapsulated in alginate and soy protein isolate particles prepared by oil-in-water emulsion. Cells were protected from the conditions of the gastric environment and retained their functional activity of lectin binding on their surface after release from particles.[103]

Widely used methods to manufacture hydrogel films, patches and other complex scaffolds and architectures include electrospinning, casting, and three-dimensional printing. Electrospinning involves applying a high voltage to a polymer solution extruded onto a collecting plate forming a porous film.[104] Cells can either be seeded onto films after fabrication or extruded in the polymer solution.[31] While electrospinning can produce scaffolds that mimic the extracellular matrix and support cellular activities, traditional electrospinning is generally unsuitable for encapsulating live cells due to harsh fabrication conditions, including high voltage and dehydration forces.[105] However, modified approaches like cell-electrospinning have been developed to address these limitations, enabling the incorporation of viable cells into micro/nanofibrous meshes under more cell-friendly conditions. Cell-electrospinning (C-ES) has emerged as a promising technique for incorporating live cells into fibrous scaffolds by modifying traditional electrospinning parameters to create a more cell-compatible environment.[106] Unlike conventional methods that involve high voltages and cytotoxic solvents, C-ES employs lower voltage settings—typically below 10 kV—to reduce electrical stress on cells. It also utilizes aqueous or biocompatible solvents, such as culture media or phosphate-buffered saline, to avoid chemical damage.[107] Flow rates are carefully optimized to minimize shear stress during extrusion, and the process is conducted in a humidified chamber to prevent cell dehydration. Additionally, temperature control ensures that fabrication occurs at physiological or room temperatures, further preserving cell viability. Biocompatible polymers like gelatin, alginate, and collagen are commonly used to support cell adhesion and function, making C-ES a viable approach for creating cell-laden scaffolds for tissue engineering.[106] This hydrogel fabrication method allows for a high level of control over film mechanical properties, including porosity beneficial for biomolecule exchange, but can be limited in its encapsulation efficiency and cell viability.[29,31,108] Cell viability during film fabrication can also be improved by adding cryoprotectants to pre-cursor solutions.[109] Co-axial electrospinning can be used to create core shell fibers to help protect live cells from environmental influences such as harsh gastric conditions.[110] Feng *et al.* created fibers with probiotic-loaded alginate cores surrounded by PVA/alginate shells which helped improve both resistance to harsh gastric conditions and thermal stress.[110]

Casting techniques involve creating a mold of the desired scaffold and adding cell-loaded solutions before crosslinking to encapsulate cells.[104] Molds are commonly made using materials such as polydimethylsiloxane (PDMS) that are easy to separate from casted materials and can create a range of geometries from simple rectangle films and disks to more complicated structures like microchannels.[111–113] This fabrication method is well-suited for loading live cells, as gels can be formed under mild conditions. Casting is particularly advantageous when simple geometries are required. In one example, flat sheets were prepared by casting silk hydrogels into well-shaped molds and allowing them to crosslink with horseradish peroxidase for 5–15 minutes.[112] By changing the mold geometry, researchers were able to improve total engineered bacteria growth and protein production inside hydrogels. Photocrosslinking after casting hydrogel solutions in PDMS or other transparent molds allows for faster and more comprehensive crosslinking. Dhakane *et al.* created bilayer PluDA hydrogel disks with eLBP bacteria loaded cores surrounded by a protective shell by designing two PDMS molds for the inner and outer portions of the disks then photocrosslinking each layer for 1 minute.[113] Nutrients were able to diffuse through hydrogels to support cell growth and protein production inside the inner core while the protective layer prevented leakage of bacteria over 15 days. While this method is simple, cell viability and construct porosity depend on crosslinking methods and structures are limited in their three-dimensional complexity.[71,112]

Additive manufacturing techniques, such as three-dimensional printing, enable the fabrication of more precise 3D hydrogel structures through layer-by-layer material deposition, allowing for greater structural complexity. It offers several

advantages, including high spatial resolution, design flexibility, and the ability to create architectures with high surface-area-to-volume ratios that enhance nutrient diffusion and promote protein expression, while maintaining cell viability.[72,77,114] The most commonly used fabrication method, extrusion-based 3D printing, involves a hydrogel precursor solution that is extruded through a needle into a geometry that is then crosslinked with a variety of crosslinking methods and agents. For example, alginate and gelatin solutions loaded with LBPs were heated and deposited into a 3D architecture that was first physically crosslinked as the solution cooled, followed by ionic and covalently crosslinking through the addition of calcium and genipin.[114] Dual crosslinked hydrogels were more stable and resistant to swelling than single crosslinked gels and these bioprints were able to support cell growth and sustained release for 28 days *in vitro*. Bioinks for extrusion-based 3D printing can be uncrosslinked hydrogel solutions or can be composed of crosslinked microparticles that can be crosslinked together as a scaffold, known as microgels.[99] LBPs can be loaded into microparticles then formed into a gel which can create flexible, fine-tuned microporous structures where cells are adequately protected and contained in their exact patterned location.[72]

Another 3D bioprinting method suitable for LBP encapsulation is vat photopolymerization where a photocrosslinkable resin is exposed to a UV light mask to crosslink an entire layer of material at once, then the construct is moved to crosslink the next layer and so on.[1] Altin-Yavuzarslan *et al.* used vat photopolymerization to create PEGDA-based hydrogels loaded with either engineered bacteria or yeast.[1] They were able to construct a variety of construct geometries loaded with viable and metabolically active engineered cells that continuously produced therapeutic compounds over 5 days.

A major advantage of the high spatial resolution possible with 3D bioprinting methods is the ability to control where different eLBP strains and materials are deposited, allowing for contained, multi-strain hydrogels.[115] Engineered microbes can be arranged to act as spatiotemporally responsive sensors or can create logic gates for more complex sensing and response capabilities.[77] In a study by Usai *et al.*, 3D printed alginate and gelatin hydrogels were seeded with engineered bacteria that produced either red (RFP) or green (GFP) fluorescent protein. These strains were printed in separate sections of the hydrogels and were found to be contained within their respective locations in the gel as very low (0.1%) cross-contamination between strains was observed after overnight incubation.[116]

Despite its strengths, extrusion-based bioprinting faces challenges, including a reduction in cell survival due to mechanical shear forces and the high cost of specialized equipment.[83,117] Reported cell survival rates following extrusion vary depending on the bioink and printing parameters, though optimization can help preserve cell function. In one case, 3D printed, photocrosslinked PluDA hydrogels loaded with engineered bacteria were developed with mild conditions (90 kPa pressure and 365 nm light cured for 5 minutes) and biocompatible materials (Pluronic F127-DA material with Irgacure 2959 photoinitiator) to protect cells and >95% cell viability was achieved after 24 hours loaded into hydrogels.[77] Each fabrication method brings its own set of benefits and drawbacks related to complexity, scalability, biocompatibility, and control over structure and function that must be considered and chosen based on desired applications and outcomes.

IV. Mechanical tuning to influence delivery characteristics and cell behavior. The physical and chemical properties of biomaterials can significantly affect how cells interact with and respond to their environment. By adjusting material parameters such as porosity, crosslinking density, and stiffness it is possible to direct cell behavior and optimize conditions for LBP growth, activity, and viability (Fig. 1D).[70]

Porosity can influence microbial mobility within biomaterial constructs and their access to essential nutrients which can influence LBP viability, metabolic activity and their ability to efficiently deliver therapeutic compounds and respond to their environment.[118] For example, in one study bacterial cells were microencapsulated in alginate with the addition of Fe_3O_4 nanoparticles, which increased the roughness and porosity of the hydrogel shell.[23] This modification resulted in higher cell viability compared to constructs without nanoparticles, likely due to increased nutrient diffusion. Similarly, when nutrient distribution within gels was varied, cell viability was highest in regions with greater nutrient availability, underscoring the strong dependence of microbial survival on local nutrient access.[112] Porosity can also influence how cells grow in scaffolds as materials with larger pores allow pockets for cell growth, migration and communication.[119] Porosity changes based on the material and fabrication method used but can also be controlled by adding temporary materials to bulk hydrogels that create specific sized pores that are later removed.[120] For example one study created alginate-based scaffolds but mixed in methylcellulose during fabrication intended to be released during the final crosslinking step to make macropores for microalgae cells to proliferate inside.[120] Pore size can be highly controlled in microgels where bioinks are made of pre-fashioned microparticles of an exact size that when extruded as a bioink and crosslinked contains micropores between particles.[99]

Crosslinking density changes materials mechanical stability, elasticity, and swelling behavior as increasing crosslinking density increases material stability but decreases elasticity and swelling degree. The degree of crosslinking can be changed by adjusting material parameters like polymer and crosslinker concentration or by controlling fabrication parameters, such as the type of crosslinking employed. Bhusari *et al.* found that in purely physically crosslinked hydrogels, bacteria colonies were able to grow extensively and exert forces of up to ~10 kPa on the surrounding matrix, causing noticeable gel deformation.[2] In contrast, introducing chemical crosslinks restricted colony expansion and significantly increased resistance to mechanical deformation. A higher concentration of PluDA was used to increase the degree of covalent crosslinking

and colonies within highly crosslinked hydrogels tended to grow more slowly, were smaller in size, adopted a more spherical shape, and displayed a more uniform morphology. These effects appeared to result primarily from physical confinement imposed by the dense network rather than nutrient limitation. Protein production initially increased with greater mechanical restriction but declined once the restriction became too severe, as cellular growth was inhibited. Swelling of hydrogels, which is inversely related to crosslinking degree, can have both beneficial and detrimental effects on encapsulated cells. On the one hand, excessive swelling can destabilize the matrix, leading to particle degradation and the premature release of encapsulated microbes.[11] One the other hand, moderate swelling can enhance nutrient and therapeutic diffusion and allow for deeper penetration into the matrix, thereby promoting increased cell growth and activity.[5]

LBPs loaded into hydrogels have mechanosensing abilities that change their growth and behavior depending on matrix stiffness. Stiffer materials are more resistant to deformation caused by expanding cell clusters and can limit their expansion, causing cells to change their morphology and colony structures.[121] Additionally, bacterial cells show reduced growth and a lower delivery efficiency when placed in hydrogels with a higher stiffness.[122] A higher hydrogel stiffness can also increase the rate of protein production which is an important consideration for eLBPs.[123]

V. Controlled degradation for timed release and clearance. Degradation rate is another critical factor that must be tuned to be aligned with the specific requirements of the intended application. For instance, degradable hydrogels may be preferred for quickly delivering cells or therapeutic cargo at a target location to minimize patient exposure to materials, while slower or non-degradable hydrogels are advantageous for sustained drug delivery or sensing capabilities of LBPs while maintaining their biocontainment in constructs.[54,100] Degradation can be influenced by hydrogel porosity, crosslinking density, materials, and LBP cargo.

Pore size plays a significant role in degradation dynamics, with larger pores generally accelerating the breakdown of the hydrogel network. In a study by Ou *et al.*, hydrogel microparticles of varied sizes were fashioned into a bioink for extrusion-based 3D printing to control micropore size of the printed scaffold. When exposed to a degradative enzyme, the bulk hydrogels degraded slower than those containing micropores with degradation kinetics increasing as pore size was expanded from small to medium to large.[72]

Increasing crosslinking density and using covalent crosslinking methods can resist degradation in hydrogel matrices.[100,124] For example, alginate hydrogels ionically crosslinked with calcium can disassociate in the presence of cation scavengers within the body. Incorporating covalent crosslinking, such as photocrosslinking, can increase their stability in physiological environments. Photocrosslinked alginate methacrylate microbeads loaded with LBPs demonstrated significantly enhanced resistance to degradation compared to ionically crosslinked alginate. The improvement in stability was achieved without compromising permeability, allowing engineered cells to retain their viability and sensing abilities within the more robust constructs.[100]

While natural hydrogel materials are often more sensitive to enzymatic and chemical degradation *in vivo*, the relative stability compared to synthetic materials and hydrogels is dependent on how the synthetic polymers are designed. Natural polymers, such as gelatin, hyaluronic acid, and collagen, are susceptible to enzymatic degradation in physiological environments. However, their degradation rates can be tuned through strategies such as incorporating additional components or altering crosslinking methods.[41,53,54,119,125] For example, alginate-protamine microcapsules loaded with *E. coli* MG1655 enabled localized delivery and colonization in the proximal colon by leveraging trypsin-triggered protamine degradation in the small intestine.[126]

Further, natural hydrogels can undergo degradation in response to chemical stimuli such as pH, redox potential, and the presence of divalent cation chelators.[41,53,54,127] To leverage the pH responsiveness of polysaccharides, *L. plantarum* was encapsulated within a carboxymethyl cellulose core and an outer layer formed by covalently cross-linking carboxymethyl chitosan molecules using dialdehyde alginate. In the small intestine, deprotonation of the carboxymethyl groups combined with imine bond breakage triggered capsule degradation and bacterial release.[128] In another study, oxidized konjac glucomannan (KGM) functionalized with cysteine was used to form bacteria-loaded microspheres stabilized by Fe^{3+} crosslinking. Once in the intestine, iron hydroxide formation ruptured the hydrogel, enabling disulfide bond interactions between the probiotic surface and mucus to increase intestinal colonization of the bacteria.[129]

Introducing cells to hydrogels can also influence degradation. Higher cell loading can make hydrogel materials more vulnerable to degradation because colony structures can apply pressure to the surrounding gel material, leading to breakdown.[130] In one example, an increase in cell loading density caused an increase in the speed of degradation of GelMA-based hydrogel patches.[82] Some microbes can also consume or break down hydrogel materials leading to increased degradation rate.[131]

VI. Material modifications for enhanced delivery. Hydrogel material can be physically and chemically modified to expand their functionality, address performance limitations, and expand their applications (Fig. 1E). One common strategy involves adding coatings or additional layers to hydrogel constructs. These modifications can improve properties such as biocontainment, mechanical stability, and targeted adhesion.[132] For example, since many hydrogels are highly porous and present challenges in biosafety and cell leakage, applying protective coatings can significantly enhance containment.[96] One study found that coating alginate particles with polyacrylamide completely prevented eLBP escape for at least 72 hours without reducing their engineered sensing capabilities.[96] Notably, this containment persisted even under com-

pressive stress, highlighting the mechanical resilience imparted by the coating.

Adhesive coatings represent another modification strategy, especially valuable for applications requiring hydrogel attachment to tissues, such as skin in wound healing or mucosal surfaces for GI-related applications. Materials like chitosan and its derivatives are commonly used as biocompatible, adhesive coatings due to their natural origin and mild gelation.[133] Yao *et al.* encapsulated therapeutic bacteria in layers of alginate and chitosan for improved mucoadhesion following oral administration and found coated bacteria were more resilient in gastrointestinal conditions and more mucoadhesive which improved therapeutic outcomes as these coated groups showed improved intestinal barrier recovery following DSS-induced colitis.[133]

In addition to bulk coatings, surface chemical modifications can impart specific functionalities to hydrogel materials.[119] For example, sulfhydryl groups can be grafted onto hydrogel polymers forming mucoadhesive disulfide bridges, enabling strong and targeted adhesion to the intestinal epithelium.[134] Sulfhydryl hyaluronic acid-based microparticles were loaded with bacterial LBPs to serve as an orally delivered, mucoadhesive colon chemotherapy system. They found modified HA delivery systems increased cell viability and H_2S scavenging ability, increased colon residence time, and inhibited tumor progression. Due to the abundance of functional groups on the surface of many hydrogel materials it is also possible to conjugate targeting moieties for improved delivery. Yang *et al.* functionalized alginate/chitosan microparticles with a small intestine targeting peptide, Pept1, for small intestine specific delivery of eLBPs for the treatment of IBD.[135]

Hydrogel materials can also be engineered to respond to external stimuli, often referred to as "smart" or stimuli-responsive materials.[136] These materials undergo physical or chemical changes in response to cues such as temperature, pH, and biochemical stimuli.[85] Temperature responsive materials such as gelatin and Pluronic F127 are commonly used in injectable hydrogels to encapsulate live cells into precursor solutions. These materials then undergo physical crosslinking upon injection into the body, enabling cell-safe *in situ* hydrogel formation. However, additional crosslinking methods, such as photocrosslinking, may be required to achieve sufficient mechanical stability in physiological conditions.[91,93] Chemically-responsive materials can also be used for hydrogels to respond to chemical stimuli changes during gastrointestinal delivery, in tumor microenvironments, and in infected wounds.[42,137] For example, an ROS-responsive hydrogel was used to deliver *L. reuteri* to sites of inflammation in a DSS-induced colitis murine model. The hydrogel, composed of crosslinked methacrylated hyaluronic acid and thiolated thioketal, bound to positively charged inflamed colonic tissue. At these sites, excessive reactive oxygen species (ROS) production selectively cleaved thioketal linkages, triggering hydrogel degradation and localized probiotic release.[138]

Further, smart materials can respond to biochemical stimuli, enabling highly selective activation or transformation. For example, Rivera-Tarazona *et al.* developed a 3D-printed hydrogel system incorporating auxotrophic yeast strains, each requiring specific amino acids to grow.[85] Upon exposure to the corresponding amino acid, only the targeted yeast strain proliferated, resulting in a change in shape and size of its specific compartment. In another design, a drug-loaded capsule was secured with a gel layer containing one such auxotrophic yeast. Exposure to its biochemical trigger led to localized growth and deformation of the hydrogel, ultimately releasing the encapsulated drug cargo. These material modifications, ranging from structural coatings to smart, responsive properties, greatly enhance the versatility and performance of hydrogel systems for microbial delivery, drug release, and sensing applications.

Lastly, hydrogel materials can be loaded with live cargo that can change their mechanical properties either consistently for the duration of drug delivery or in response to external stimuli.[139] For example, Altin-Yavuzarslan *et al.* designed microbes able to secrete L-DOPA to enhance the mechanical stiffness of BSA-based hydrogel constructs.[1] Hydrogels loaded with L-DOPA-producing LBPs resulted in a 20% higher compressive modulus than non-cell hydrogels. In the same study, yeast cells were engineered to produce betaxanthins to resist microbial degradation. It was found that eLBP hydrogels were found to resist degradation for >38 days while non-LBP hydrogels were completely degraded at this time.

B. Microbial selection and integration

I. Choice of organism: commensal *vs.* engineered strains. The choice of eLBP organism falls into three categories: bacteria, fungi (primarily yeast) and microalgae (Fig. 2A). They can be probiotic, offering inherent benefits to humans in addition to their engineered therapeutic capabilities, or inert with only engineered functions.[140,141] Each group offers distinct advantages and limitations depending on the target application, with variations in biocompatibility, genetic tractability, and metabolite production.[140,142] All three microorganisms have been loaded into a broad range of hydrogels and have shown good survival after hydrogel fabrication.[142]

Bacteria are the most utilized microbial chassis for eLBP development.[142] This is largely due to their robustness, ease of cultivation, and well-established genetic engineering tools.[112] A diverse array of bacterial strains have been utilized, including both probiotic strains and synthetic chassis.[143] Probiotic commensal strains such as *Escherichia coli* Nissle 1917 (EcN) and *Lactobacillus reuteri* have been extensively studied for their natural benefits to humans such as immune regulation and gut barrier integrity, reduced low immunogenicity as commensal strains, and their potential in clinical applications, including ongoing clinical trials for multiple diseases.[7,144,145] Other probiotic strains, like *Lactococcus lactis*, are frequently employed for engineered LBP constructs due to their safety profile and ease of genetic manipulation.[21,88] Additionally, probiotic *Bifidobacteria*, such as *B. longum*, *B. animalis*, *B. breve*, and *B. adolescentis*, are emerging chasses due to their ability to metabolize diverse carbohydrates through the bifid shunt, reduce inflammation, and compete with pathogens in anaerobic niches.[146] Commensal strains, like

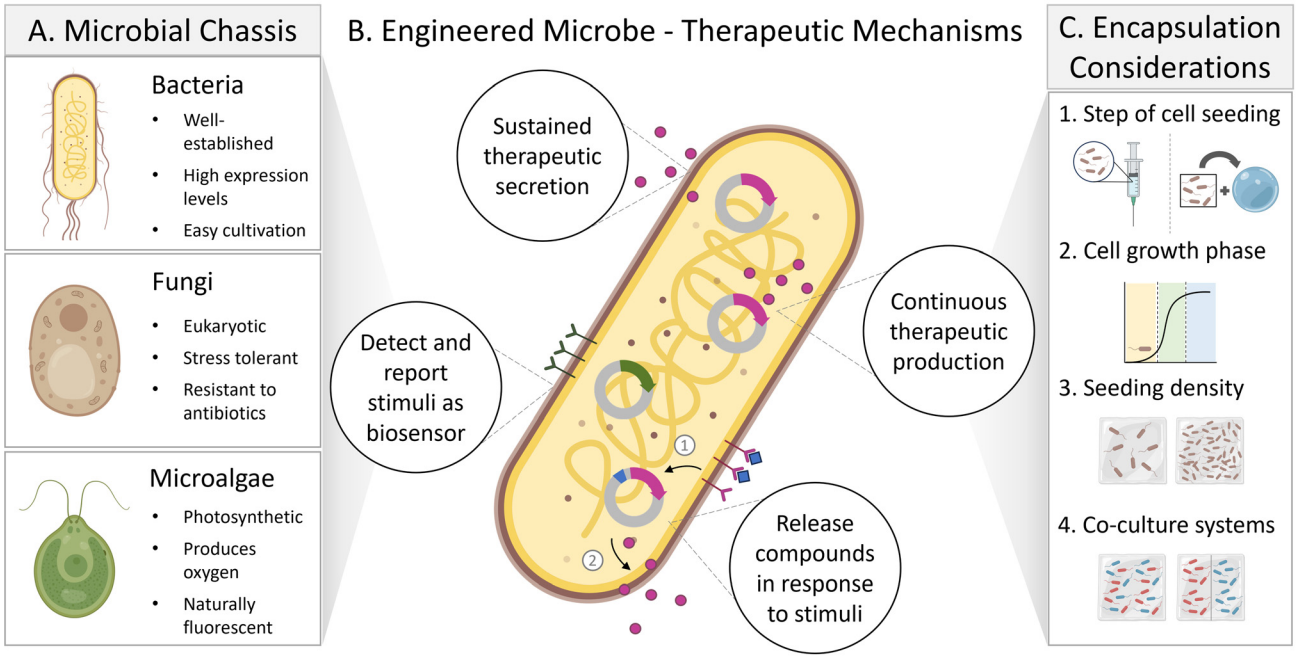

Fig. 2 Considerations for microorganism selection and potential functionalities. (A) Benefits of each cell type (bacteria, fungi, or microalgae) for therapeutic efficacy and engineering potential. (B) Potential therapeutic functions of genetically engineered live biotherapeutic products. (C) Important factors to consider when loading eLBPs into hydrogel materials.

Bacteroides spp., have been utilized as chassis organisms for genetic engineering, despite lacking inherent probiotic functions, owing to their stable colonization and compatibility with the host gut environment.[147] Synthetic strains, some derived from those above, can also be utilized for eLBP formation with additional benefits like genetic stability, safety, and flexibility.[148] Anaerobic bacteria offer benefits as eLBPs in certain disease contexts, such as cancer, where low-oxygen conditions persist. Their ability to selectively target and proliferate within tumor microenvironments makes them particularly effective for localized therapeutic delivery.[149] The Gram status of bacteria can also influence their efficacy as eLBP chassis as this can affect protein secretion, immunogenicity, and robustness.[123]

Other bacteria, such as the photosynthetic cyanobacteria, can convert light energy into oxygen with nearly 100% internal quantum efficiency, making them attractive for oxygenation-based therapies.[142,150] Some strains of bacteria also have the inherent ability to scavenge specific compounds or other microorganisms. For example, *Thiobacillus denitrificans* exhibit innate biochemical functions like sulfide oxidation, which is valuable for treating diseases involving hydrogen sulfide accumulation, such as colon cancer.[134] Some other bacterial strains can inherently secrete valuable biomolecules, such as cellulose or hyaluronic acid, which can reinforce hydrogel structure or confer additional benefits.[20,115] Further, *Actinomycetes*, traditionally used in industry to manufacture a diverse range of natural products, also hold promise as eLBPs for *in situ* synthesis of antibiotics, vitamins, and immunomodulators, as well as for pesticide degradation. *Streptomyces* spp. has been identified in low quantities in the human gastrointestinal tract and have previously demonstrated success in preventing disease in aquaculture and poultry. These findings have prompted scientists to consider *Streptomyces* as a potential chassis for eLBPs aimed at treating gastrointestinal infections in humans.[151]

Predatory bacteria, such as *Bdellovibrio bacteriovorus*, has demonstrated the ability to selectively lyse and kill other bacterial species without harming eukaryotic cells, suggesting utility in microbiome modulation or pathogen targeting.[152]

Using bacteria as an organism in eLBP-loaded hydrogel drug delivery systems has the advantages of well-established genetic engineering practices in many species. Despite their many advantages, bacterial systems also have limitations. These include potential immunogenicity, difficulty maintaining engineered functions *in vivo*, the risk of horizontal gene transfer to native microbiota, and regulatory hurdles associated with genetically modified organisms.[145,153] Strain selection must therefore be carefully tailored to the specific use case.

Yeast, particularly *Saccharomyces cerevisiae* (*S. cerevisiae*), are another promising eukaryotic chassis for eLBP development. These single-celled fungi are generally recognized as safe (GRAS), are well characterized, and can be easily genetically modified.[5] Like bacteria yeast grow quickly in mild culture conditions, but they also exhibit enhanced tolerance to environmental stress compared to bacteria, including pH, temperature and oxidative conditions, which can improve their viability after fabrication into hydrogel matrices.[154,155] As yeasts are eukaryotic, they offer benefits as eLBPs due to similarities between yeast and human cell secretory systems. Yeasts can

perform post-translational modification allowing them to secrete more clinically relevant biopharmaceutical compounds.[155] Yeast cell walls are rich β-D-glucans, which have antioxidant and ROS scavenging capabilities, aiding their therapeutic potential.[156] Yeasts are also resistant to antibiotics and can be used alongside them for dual therapy, a functionality that bacterial constructs do not possess. Probiotic strains of yeast, most commonly *Saccharomyces boulardii* (*S. boulardii*), have shown anti-inflammatory and immune regulating effects.[157] Some strains secrete therapeutically relevant compounds that can be beneficial to humans. For example, *Kluyveromyces lactis* secretes lactase, an enzyme that can cleave lactose and that can be used to manage lactose-intolerance.[11,158] Despite these advantages of yeasts as eLBP systems, limitations like lower gene expression levels compared to bacterial systems, and greater complexity in large-scale genetic manipulation must be addressed for clinical success.[155,159]

Lastly, microalgae can be used as an eLBP chassis and are valued for their photosynthetic capabilities.[18] Microalgae represent the largest group of auxotrophic oxygen-producing organisms on earth.[22] Many microalgae, such as *Chlamydomonas reinhardtii* (*C. reinhardtii*) and *Spirulina platensis* (*S. platensis*), are capable of photosynthesis and naturally produce large amounts of oxygen, making them highly attractive for wound healing, infection, and myocardial infarction applications.[18,120,160,161] A detailed overview of their therapeutic utility can be seen in other reviews.[161] Chlorophyll can act as a photosensitizer that produces reactive oxygen species in response to laser irradiation and can be used as a method of pathogen removal.[162] Chlorophyll can also be used as a fluorescence marker to visualize algae growth without adding exogenous compounds.[22] In addition to oxygen production, microalgae can be cultured in simple conditions and have a short growth cycle.[22] Like bacteria and fungi, algae secrete unique metabolites that can be beneficial to humans.[22] Additionally, some strains of microalgae have shown anti-tumor effects.[158] Despite these advantages, algae-based eLBPs face challenges such as potential immunogenicity, low product yield, high harvesting costs, and limited genetic tools compared to bacterial or yeast systems.[22,161]

Each microbial chassis – bacteria, fungi, and microalgae – offers unique advantages for developing eLBPs embedded in hydrogels. Bacteria are widely used due to their versatility and engineering ease; yeasts provide added resilience, the ability to produce complex proteins, and unique bioactive properties; and algae introduce novel photosynthetic capabilities and metabolic outputs. The optimal chassis depends on the intended therapeutic goal, required stimuli responsiveness, and desired secretion profile. Readers are directed to existing literature that provide comprehensive discussions of chassis and eLBP properties.[148,163,164] As this field matures, we expect to see further diversification in microbial strains and broader application areas.

II. Genetic engineering for therapeutic production, biosensing, or self-regulation. Traditional drug delivery systems often exhibit poor efficacy due to low stability of biologics, off-target delivery, and limited communication with the host environment.[165,166] These limitations can be addressed by leveraging eLPBs, which can be modified for local production of therapeutics and equipped with environmental sensing capabilities for the prevention and treatment of human diseases.[166] Broadly, eLBP applications can be grouped into three categories: (1) treatment of disease through production of therapeutic compounds, (2) diagnosis of disease through biosensing, and (3) combined diagnostic and therapeutic functions, known as "theranostic" applications (Fig. 2B).

To achieve new therapeutic functions, LBP chassis can be genetically engineered using plasmids, genomic integration, or a combination of both for a balance between genetic stability, flexibility, and protein production levels.[167–169] Common microbial engineering techniques include plasmid transformation, homologous recombination, CRISPR-directed gene editing, and phage-mediated DNA delivery and recombination.[170,171] Antibiotic resistance markers or nutrient auxotrophy are routinely employed to engineer microbes and select for transformants; however, it is important that antibiotic resistance genes are not maintained in eLBPs for therapeutic applications due to the risk of horizontal gene transfer and spread of antibiotic resistance.[172–174]

When designing eLBPs for encapsulation within hydrogels, key considerations must be made regarding the compatibility of the eLBPs, hydrogel, and extra-hydrogel environment. Factors such as pore size, crosslinking density, and degradation rate determine how effectively nutrients, inducers, and signaling molecules reach cells for stimuli responsive eLBP activity, as well as how efficiently therapeutic products diffuse out of the hydrogel.[175] For example, hydrogel microspheres composed of an alginate core and polyacrylamide-alginate shell were engineered to selectively filter compounds that passed in and out of the bead. The alginate core was loaded with nutrients and auxotrophic *E. coli* to sustain cell viability for up to two days while the shell (5–50 nm pores) prevented the escape of cells and DNA into the surrounding media. Importantly, the beads allowed diffusion of anhydrotetracycline (aTc) into the core of a "sender" bead where a quorum sensing molecule, acyl homoserine lactone, was produced and secreted into the media to be taken up by a "receiver" bead that triggered GFP production.[176] Further, eLBPs and hydrogels can be engineered to work synergystically. EcN engineered to secrete keratinase encapsulated within keratin hydrogels achieved degradation in the colon where EcN was delivered with higher viability compared to free cells. In the colon, EcN provided inherent probiotic activity while keratin-derived small peptides generated by the recombinant keratinase invoked antimicrobial activity in a DSS-induced colitis model.[177] To further optimize eLBP functionality within hydrogel matrices, future research is needed into streamlined methods such as directed evolution or adaptive evolution of eLBPs under hydrogel-constrained conditions.[178–182] These strategies could enhance cellular activity and therapeutic production in hydrogels for enhanced therapeutic production along with improv-

ing the tolerance of chassis to toxic crosslinkers or byproducts present during the formation of select hydrogels.

At the genetic level, eLBP functionality within hydrogels is dictated by how recombinant expression systems are designed and regulated. eLBPs can be engineered to secrete therapeutic compounds constitutively or in response to stimuli. Recombinant DNA can be organized into simple expression cassettes comprised of a constitutive promoter, gene of interest, and terminator for continuous therapeutic production. Yuan *et al.* engineered *Pichia pastoris* (*P. pastoris*) yeast to produce and secrete recombinant proteins with various molecular weights (60–150 kDa) like α-amylase and anti-HER2 antibody.[183] Engineered yeasts loaded with Pluronic F123-BUM hydrogels were able to maintain their protein production capabilities following encapsulation, transiently secrete proteins outside of hydrogels after production, and retain high protein production levels after lyophilization and storage, unlike liquid cultures tested in the study. Moreover, advanced regulatory genetic frameworks can be assembled using components such as inducible promoters, logic gates arranged in gene circuits, and riboregulators.[184–187] These more sophisticated designs are especially useful for stimuli-responsive applications where signals originating from the extra-hydrogel environment, hydrogel matrix, or intra-hydrogel microbial niche, such as quorum sensing molecules, can be used to control therapeutic production for site or event specific delivery.[165] EcN was engineered to respond to elevated nitric oxide levels (NO) at bone fracture sites to release bone morphogenetic protein 2 (BMP2) in a sustained and responsive manner that mirrored the rate of healing.[188] These were encapsulated in GelMA microspheres then embedded in HAMA matrices where they were able to respond to NO signals in surrounding media and maintain their viability while remaining biocontained for the experiment's duration. These smart, responsive eLBPs can also be used for externally triggered protein production. In one example, *E. coli* was engineered to release deoxyviolacein when exposed to light by using an optogenetic protein expression plasmid, pDawn.[189] Cells were loaded into agarose hydrogel and maintained light dose-dependent secretion of the therapeutic protein for over 42 days.

The ability to sense and genetically respond to multiple forms of stimuli in a dose-dependent manner and the ability to retain genetic memory allows eLBPs to act as long-term diagnostic tools for human disease.[96,145] A simple example involves engineering cells to express fluorescent protein, such as GFP, in response to external signals, enabling quantification of stimulus intensity. In one study, orally delivered microparticles were loaded with EcN engineered to secrete GFP upon detecting thiosulfate, a biomarker of gut inflammation.[101] This study found that biosensing capabilities were preserved following eLBP encapsulation, and the system was able to accurately quantify the degree of epithelial damage in a DSS-induced model of colitis in rats. It is also possible to use eLBPs for longitudinal sensing using genetic memory systems where responses to stimuli can be detected through sequencing even after they have been removed from the stimuli or after cell death. Tang *et al.* were able to engineer *E. coli* to edit their gene expression in response to exogenous biochemical cues.[96] Engineered cells were loaded into hydrogel capsules and showed successful genetic memory in response to cues outside of particles, even using robust particles with 100% biocontainment.

Developments in genetic engineering of LBPs has also led to the development of tools capable of simultaneous monitoring and treatment of disease, termed "theranostics". EcN was engineered as a bioluminescent monitor of heme, a biomarker of IBD, and a responsive producer of therapeutic AvCystatin, an immunosuppressive drug for IBD treatment.[190] eLBPs were loaded into alginate-based mucoadhesive microparticles and dosed orally to mice, where they produced AyCytavin in a heme concentration-dependent manner. This system generated sufficient luminescence to enable accurate monitoring of disease severity through heme detection.

While eLBPs offer many benefits as genetically engineered smart therapeutics for disease diagnosis and treatment, several limitations must be considered. One challenge lies in engineering plasmids for stable secretion, as they are vulnerable to gene loss and instability over time.[144] Biosafety of these genetically engineered microbes is another concern, which can be mitigated by engineering circuit-based control kill switches into constructs to prevent unintended microbial spread or colonization post-treatment.[165] There can also be high costs associated with more complex genetic engineering approaches that could limit their use clinically.[165]

III. Encapsulation strategies to maintain viability. There are multiple factors to consider when loading eLBPs into hydrogels to maintain their viability and function (Fig. 2C). One consideration is whether cells are seeded into hydrogel precursor solutions and encapsulated into matrices during crosslinking or if cells are added to hydrogels after crosslinking.[28] When cells are added to pre-crosslinked solutions, they typically become homogeneously distributed within gels but can be harmed by harsh crosslinking conditions.[2] These harsh conditions include but are not limited to toxic reagents, extrusion forces, heat, and UV light.[191,192] Some reduction in cell viability during live cell fabrication can be mitigated by employing a cultivation step where cells are allowed to proliferate in favorable conditions for an amount of time after fabrication to recover cells lost during the process before application or testing.[75] These studies contained outgrowth steps of around 12–24 hours after fabrication and found this time was sufficient to recover most cells. Despite these disadvantages, loading cells into solutions pre-gelation may be preferred for more homogenous distribution within the constructs, better encapsulation efficiency, and faster fabrication.[193] In contrast hydrogel constructs with cells loaded post-crosslinking can have higher viabilities and expand the materials able to be used since harsh fabrication methods are less of a factor, but poor cell penetration or adhesion can hinder their effectiveness.[44] Introducing eLBPs after hydrogel gelation are less common in therapeutic applications since many require biocontainment or high encapsulation efficiency.[194] Liu *et al.* fab-

ricated alginate/polyvinyl alcohol hydrogel constructs and subsequently introduced bacteria, a strategy that proved effective due to the small size of the bacterial strain, the intentionally large hydrogel pore size, and the design goal of rapid microbial release.[152]

Another consideration is the growth phase of cells at the point of encapsulation as this can change how they proliferate and metabolize inside hydrogel constructs especially immediately following fabrication. The final density of microbes loaded into hydrogels is typically independent of the growth phase of starting cultures but their biochemical reaction rates and therefore therapeutic activity varies post-encapsulation until they reach maximal density.[101] One study loaded EcN into alginate-based microparticles at either exponential (OD_{600} = 0.1) or stationary phase (OD_{600} = 1).[101] They found exponential phase bacteria had a longer growth period and steady state period of therapeutic activity. Additionally, incorporating spores of engineered microbes into hydrogels is a promising way to enhance cell viability and storage. Gonzalez *et al.* demonstrated that *Bacillus subtilis* spores were able to better tolerate heat shock (75 °C), survive dehydration of the hydrogel, and achieve an even distribution of GFP production throughout an agarose hydrogel compared to non-sporulated *B. subtilis*. The ability of spores to respond more efficiently after environmental stress was demonstrated in nine *Bacillus* strains, indicating that spores are robust for hydrogel integration.[195]

Cell loading density can also affect eLBP cell viability, growth patterns and therapeutic activity when encapsulated in hydrogels. The rate of cell growth is largely dependent on nutrient access and spatial constraints, so cells loaded into hydrogels typically experience rapid growth and metabolic activity until they reach a certain density saturation.[131] Lower initial cell densities result in faster growth and more metabolic activity initially than higher cell densities before slowing down at saturation.[122,196] For example, algae at a high seeding density grew slowly, moved less, and secreted less metabolites than cells loaded in smaller amounts which exhibited faster growth and increased metabolism.[22] While this high level of growth and activity can be beneficial, it can also lead to faster breakdown of hydrogel carriers due to metabolic byproducts and pressure of microbial colonies on hydrogel materials though this can be mitigated through engineered hydrogels with more stable crosslinking or with robust coatings to mechanically limit cell growth.[131]

IV. Co-culture systems for synergistic effects or multi-functionality. Another consideration is single strain loading *versus* multi-strain loading into gels. Incorporating additional strains of engineered microbes can add functionality to the system, but can increase complexity of engineering, as each strain may have distinct nutritional needs or interact in unpredictable ways. However, co-culture systems within hydrogels tend to perform better than those in free cultures and suspension.[21] Since hydrogels allow for the diffusion of small molecules, different microbial populations can communicate through hydrogels even if they are spatially confined.[72] Multiple microbial strains can be added either together in a homogeneous mixture or spatially separated and confined in parts of the gel system. The success of these methods can depend on the relationship between microbe strains loaded. For strains that have a mutually beneficial relationship, loading both together in the same ink can offer benefits over individual loading while strains without a mutually beneficial relationship can benefit from separation. *P. putida* and *B. subtilis* were both loaded into a hydrogel for combined phenol degradation and cellulose production.[115] They found that both phenol degradation ability and cellulose production remained present when these bacteria were loaded into hydrogels.

Other studies have found more success when separating cell strains using hydrogel encapsulation as they are hindered less by competition. Johnston *et al.* created a three-layer hydrogel in which each layer contained a different type of microbe (bacteria, yeast, and algae).[197] They found that cells remained in their printed region and did not migrate. *C. vulgaris* and *B. subtilis* can form a bioremediation cycle where *C. vulgaris* can undergo photosynthesis to convert carbon dioxide into oxygen and *B. subtilis* can respirate this oxygen and emit carbon dioxide. The study found that bioremediation rates were lower when these two species were mixed into single particles compared to a mixture of particles containing only one species. This proved that these cells could interact when separated in hydrogel particles but interacted negatively when placed in gels together due to competition due to confinement. In these situations, nutrients can be a limiting factor and can result in the faster growing strain having a competitive advantage and limiting the other. This can be mitigated by designing a system in which cells are loaded into gels separately and at different ratios to control growth. Liu *et al.* printed a sensor system in which three strains were engineered to secrete GFP in response to a chemical inducer, either AHL, Rham, or IPTG.[77] These strains were separated into separate inks, printed in a specific conformation that was able to create logic gates for complex sensing.

3. Ensuring success: key performance metrics

To engineer a successful eLBP-loaded hydrogel, the system must meet these requirements: (1) deliver therapeutic cargo to the target location, (2) support cell viability and growth during transit or during application, (3) allow cells to complete their functional and metabolic activities for sufficient therapeutic action, and (4) degrade at an application-relevant time scale without leaving significant byproduct materials (Fig. 3A). These metrics are discussed in more detail below.

A. Delivery efficiency

Delivery location and method are important to consider when designing eLBP hydrogel constructs. Oral delivery is the most common drug delivery method, valued for its ease of administration, patient compliance and safety.[26,198] Oral delivery can

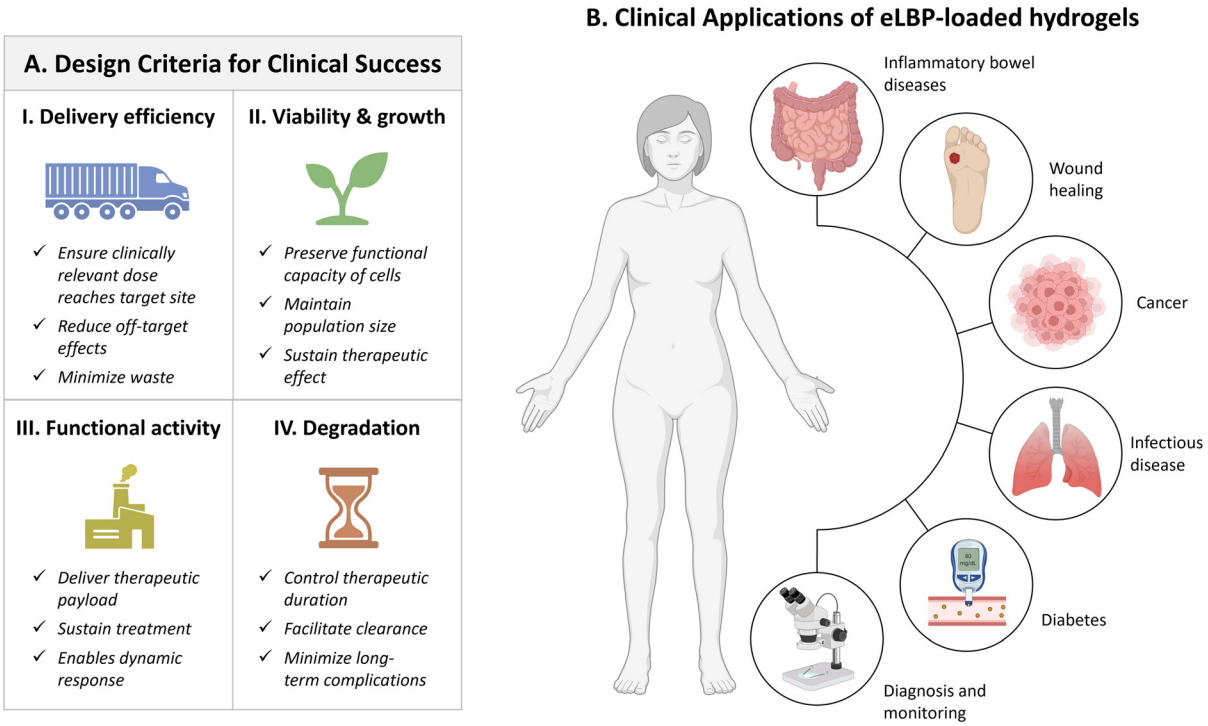

Fig. 3 Design criteria of eLBP-loaded hydrogels for clinical success and potential clinical applications. (A) Four essential design criteria of eLBP-loaded hydrogels needed to ensure translational success and why each factor is important to consider. (B) Preclinical disease contexts where eLBP-loaded hydrogels have been explored as potential treatments.

be a challenge for delivering live cells due to the harsh pH, digestive enzymes and mechanical stresses encountered during gastrointestinal transit.[199] Different materials can be used to target specific regions of the GI tract through pH-responsive dissolution like alginate and chitosan.[198] Additional considerations for oral delivery include swallowability and good flow properties to prevent dislodgement which are most commonly achieved using uniform spherical or cylindrical geometries for hydrogel constructs.[200,201] To improve patient compliance, capsules must be small but need to hold sufficient therapeutic cargo to reduce the number of doses needed to be effective.[200] This can be achieved by engineering highly effective eLBPs that do not require frequent dosing and by increasing retention in the GI tract to expand the therapeutic window using mucoadhesive materials and coatings, like chitosan and thiolated biopolymers.[19,199]

Local delivery by applying eLBP-loaded hydrogels directly onto the disease area can have improved delivery efficiency compared to other methods since it has less obstacles to overcome to reach its therapeutic site of action but there are some considerations to improve the delivery efficiency of these systems.[202] The main consideration for these constructs is adhesion to their applied surface as this determines the length of possible therapeutic activity.[80] For application on dynamic organs like the skin and lungs, tuning hydrogel mechanical properties and geometry to match that of native tissues can improve adhesion and prevent further injury to tissues during healing and therapeutic application.[79,81] Additionally, eLBP cells can be affected by mechanical forces imparted on them through adhesive but non-compliant hydrogels. Matching hydrogel mechanics to that of the target organ could reduce negative affects to growth and activity caused by these unwanted forces.[21]

For all delivery methods and location, eLBP-loaded hydrogels can improve delivery efficiency and reduce off-target effects through stimuli-responsive materials as discussed above. Here, eLBP cells can preserve their therapeutic function until they reach the desired delivery location where they can be activated by both endogenous and exogenous cues like biochemical markers, light, pH and temperature.[165] A unique advantage of eLBP-loaded hydrogels is the ability of live cells to modify materials during delivery to improve efficiency by secreting products to improve hydrogel stiffness and regenerative hydrogel matrix materials *in vivo*.[1,139]

B. Viability and growth

Cell viability typically depends on access to nutrients and spatial confinement. In three-dimensional hydrogel matrices, access to nutrients can vary based on density and distance to the outside environment. In areas of higher nutritional access in hydrogels, more live, active cells were found than in areas of lower nutritional access. Hydrogel geometry can be designed to improve overall cell viability by using lattice designs or surface channel designs with a higher surface area to volume

ratio compared to bulk designs to shorten the distance between cells and nutrients outside of hydrogels.[77,196] Spatial confinement of cells in hydrogels, determined by stiffness and crosslinking density, can limit their growth and methods to reduce these can improve cell viability, although stability must be taken into account.[2]

With increasing cell growth or increasing confinement, 3D colony structures appear in some hydrogel constructs, and their appearance can change based on a variety of factors like nutrient access and cell morphology.[197,203] Many bacteria and yeasts form biofilms, or 3D dimensional structures of adhesive cells and secreted extracellular polymeric substances (EPS), as an adaptive mechanism of survival in stress conditions.[204] Biofilms allow cells to organize and communicate for protection, nutrient provision, and stability for enhanced survival.[204] As biofilms can enhance cell viability and growth in stressful conditions like hydrogel encapsulation, a potential therapeutic avenue could be explored where colony formation is encouraged or engineered as a method of preserving eLBP viability in hydrogels during transit or application for improved therapeutic efficacy although little research in this field has been completed so far.

Another method of increasing eLBP cell viability and growth when encapsulated in hydrogels is the addition of nutrients to supplement cells when access is limited during application.[142] Nutrients can include individual essential nutrients like sugar and nitrogen, cell culture media, or prebiotics, classified as components beneficial for microbial growth but not digestible by humans.[8] In one study, alginate core shell particles were developed containing engineered *E. coli* LBP and cell culture medium in the core, surrounded by a protective shell that prevented leakage while maintaining cell viability.[96] Similarly, Liu *et al.* designed alginate microcapsules with a core of bacteria and resistant starch prebiotic, encapsulated within a protective shell to enhance the delivery of viable therapeutic bacteria to the colon.[3]

C. Activity and metabolism

In many cases, an increase in microbial cell growth rate increases metabolic activity and subsequent therapeutic activity like protein production and sensing.[72] For example, a study found that increasing crosslinking density decreased yeast metabolic activity by limiting cell growth.[72] The efficacy of biomolecule diffusion through hydrogel matrices is limited by material pore size and should be considered when engineering eLBP hydrogel systems.[183] Cell viability is not the only determinant of metabolic activity and other environmental and encapsulation factors can change cell's functional activity. A study by Bhusari *et al.* varied the degree of chemical crosslinking in gels and found that although cells grew slower in high crosslinking situations, protein production was more efficient compared to protein production from fast growing cells in low crosslinked gels.[2] As the rate of nutrient diffusion in these gels did not differ, it was concluded that varying crosslinking degrees alone was able to modulate metabolic activity. It was also concluded that cell density does not impede cell metabolism as long as nutrient access remains the same.[20] In some cases, encapsulation can improve activity compared to cells in bulk solutions. In cells performing photosynthesis, they show a higher efficiency when immobilized because of the difference of light transmission compared to cells in bulk solutions.[22] One study found that cells continued their photosynthetic activity when encapsulated in an alginate-based matrix, indicating suitable cell conditions in the biocompatible construct.[120]

Hydrogel geometry design can increase eLBP metabolic activity and protein production similarly to methods used to improve viability. Designing constructs with higher surface area to volume ratios can improve metabolic activity by increasing access to nutrients and decreasing the distance between cells and their environment.[156] This can increase the rate of protein release from hydrogels as diffusion through the matrices becomes easier.

D. Degradation and clearance

The rate of degradation and clearance of eLBP-loaded hydrogel materials is a critical parameter in influencing their therapeutic suitability and is highly dependent on their application. Hydrogels can be degraded through naturally occurring elements such as ions, temperature, and biomolecules or on-demand in response to stimuli such as light and mechanical force.[136] Cell-loaded hydrogels can be engineered with a range of degradation profiles, from fast degradation at the target site for immediate release of therapeutic cargo, to sustained degradation for prolonged delivery, or even non-degradable systems for long-term structural support. Slow degrading systems can allow for longer therapeutic windows, reducing the need for reapplication or dosing, but maintain a level of biodegradation for proper clearance of materials. For example, gelatin and alginate-based 3D prints loaded with therapeutic bacteria were engineered through optimization of crosslinking methods to degrade over 28 days in an *in vitro* model of the vaginal epithelium without harming cells.[114] Non-biodegradable systems are common for engineered biosensors intended for collection after application and in oral delivery systems where clearance can be achieved naturally through stool passage.[101]

Additionally, biodegradation and complete clearance from the body must be considered as some materials can disassociate to release cargo but non-biodegradable materials remain as byproducts that can be harmful when not cleared.[57] Hydrogel materials exhibit diverse biodegradation profiles depending on their source. Protein-based materials typically degrade rapidly, while polysaccharide-based materials tend to degrade more slowly but often produce non-toxic byproducts. In contrast synthetic hydrogel materials generally have minimal biodegradability, though they can be engineered for breakdown in physiological environments.[119]

4. Therapeutic applications of engineered living materials

Engineered LBP-loaded hydrogels represent a transformative platform in therapeutic drug delivery, offering spatially controlled, biocompatible environments for the survival, delivery, and function of beneficial microbes. These biomaterials

enable control over cell viability, metabolic activity and delivery directly to sites of injury or disease. By encapsulating live engineered microbial cells within hydrogels, researchers have developed systems capable of sustained therapeutic production and secretion, biosensing for disease diagnostics and monitoring, and stimuli-responsive actions. This section explores the use of eLBP hydrogels across various human diseases, highlighting how hydrogel design and microbial function are tailored to meet the unique challenges of each condition (Fig. 3B). A summary of examples of eLBP-loaded hydrogels for human disease that have been studied preclinically are displayed in Table 1.

Gastrointestinal conditions like inflammatory bowel diseases and colon cancer are common applications for eLBP-loaded hydrogels as they are derivatives of probiotics which have been extensively used in these applications.[54] eLBP hydrogels can be engineered to survive gastrointestinal transit and adhere to the mucosa to prolong therapeutic action and the use of probiotic eLBPs can offer additional benefits of modulating the gut microbiome.[140,205] Inflammatory bowel diseases are a group of conditions whose hallmark is chronic inflammation of the gastrointestinal tract.[41] Current treatments aim to reduce inflammation and symptoms of disease but many show poor stability.[41] As an IBD diagnostic, bacteria engineered to sense indomethacin as a marker of intestinal bleeding was loaded into a gut-retentive hydrogel and was able to successfully sense bleeding in mice *in vivo*.[206] In another example, bacteria engineered to sense thiosulfates, another biomarker of inflammation, were successfully loaded into alginate capsules and were effective at quantifying the level of disease severity after oral administration.[101] eLBP-loaded hydrogels have also been successfully used for oral delivery of therapeutics and for detecting biomarkers associated with inflammatory bowel disease. Responsive eLBP hydrogels have been used as therapeutics for IBD like *E. coli* engineered to secrete transforming growth factor-β1 (TGF-β1), an immunosuppressant immune factor, in response to light and EcN engineered to both secrete AvCystatin, a protein immunosuppressant drug, and display bioluminescence for detection, in response to heme, a biomarker of IBD.[135,190]

Wound healing is another common application of both hydrogels and LBPs and represents a field that could highly benefit from eLBP-loaded hydrogels. Hydrogels have been used frequently as a wound healing tool due to their ability to provide a biocompatible, moist environment for tissue repair.[15] The wound healing process can be hindered by per-

Table 1 Current Clinical Applications of eLBP-loaded hydrogels

Application	Hydrogel material	Architecture	Strain	Engineered function	Therapeutic outcomes	Ref.
Inflammatory bowel disease	Polyvinyl alcohol (PVA)	Disk-shaped capsule	EcN	Detect indomethacin (biomarker of intestinal bleeding)	Significant increase in bioluminescence in treatment group *versus* control after oral delivery	206
	Alginate	Micro-particle	EcN	Detect thiosulfates (biomarker of inflammation)	Thiosulfate detection following oral delivery correlated with disease progression	101
	Chitosan-alginate	Micro-particle	*E. coli*	Produce TGF-β1 in response to light (anti-inflammatory cytokine)	Prevented and reduced inflammation in IBD mouse model following oral delivery	135
	Alginate (polyserine modified)	Micro-particle	EcN	Dual production of AvCystatin (immunosuppressant) and detection of heme (IBD biomarker)	Prevented and reduced disease state following oral delivery, dose-dependent protein production with biomarker detection	190
Wound healing	Poloxamer (heparin modified)	Injectable	*L. lactis*	Produce VEGF (promotes angiogenesis)	Promoted vascularization and accelerated wound healing following local injection	88
	PluDA	Bilayer patch	*E. coli*	Produce QK (VEGF peptidomimetic)	Induced angiogenic differentiation *in vitro*	113
Dermal biosensor	Alginate	Microgel patch	*E. coli*	Detect IPTG (model biochemical cue)	Concentration-dependent sensing on skin mimic	207
	PluDA	Microgel patch	*E. coli*	Detect AHL, IPTG, Rham, aTc (model biochemical cues)	Spatial sensing of multiple biochemical cues after application on human skin	77
Cancer	Chitosan-alginate	Micro-particle	*L. lactis*	Produce IFN-γ in response to light (antitumor cytokine)	Significantly reduced tumor volume following oral delivery	135
	Alginate	Inject-able	*S. typhimurium*	Bioluminesce with D-luciferin exposure	Inhibited tumor metastasis and prevented tumor challenge post-injection	210
Infection	BSA	Patch	*E. coli*	Produce melanin (photothermal therapy)	Treated bacterial infection in mouse wounds after topical application	211
Diabetes	Chitosan	Micro-particle	*E. coli*	Produce exendin-4 (GLP-1 agonist)	Reduced blood sugar in diabetic rats for 2 weeks following subcutaneous administration	98

sistence of inflammatory cells that deplete oxygen and nutrients required for angiogenesis and proper healing.[88] Lu *et al.* engineered *L. lactis* to produce and secrete vascular endothelial growth factor (VEGF), an angiogenic wound healing therapeutic, and loaded these eLBPs into a poloxamer-based hydrogel.[88] The results showed a 5-fold increase in angiogenesis, reduced inflammation, faster wound healing (90% healed by day 12 compared to 30–50% healed in control) in the treatment group in a mouse model of diabetic wound healing compared to untreated controls. Another study engineered bacteria to secrete a growth factor-like peptide in response to light for on-demand, controlled drug delivery that was able to enhance angiogenesis in a light-dependent manner over 9 days.[113]

The skin is a common target for biosensors because of the ease of application and the ability to measure a range of biomolecules non-invasively.[207] Engineered LBPs offer benefits over traditional small molecule biosensors as they are more sensitive, can respond to multiple types of stimuli in one system, and can provide long term continuous sensing.[207] Allen *et al.* synthesized hydrogel "dermal tattoos" loaded with engineered bacteria able to sense both biochemical and biophysical cues after application on the skin.[207] Liu *et al.* designed a multifunctional sensor system of bacteria able to sense three different signals.[77] These microbes were loaded into Pluronic F127-DA hydrogels and printed onto a transparent, multi-layer elastomer sheet capable of being placed on the skin for biosensing.

Cancer could also benefit as a therapeutic target from eLBP-loaded hydrogels since many therapeutic agents for cancer have limited stability and long-term local delivery can improve outcomes.[208] Additionally, some bacteria can specifically colonize tumor microenvironments, while others can generate oxygen to counteract tumor-induced hypoxia, offering a novel therapeutic strategy.[209] Yang *et al.* engineered *L. lactis* bacteria to produce interferon-γ (IFN-γ), a cytokine known to promote antitumor activity, in response to light and loaded these engineered LBPs into chitosan-alginate microparticles for oral delivery.[135] This system was able to reduce tumor volume in mice in both prevention and treatment experiments.

Photodynamic therapy (PDT) is a non-invasive, clinically approved method to induce pro-inflammatory cell death by exciting photosensitive entities with light to produce ROS that kill cells.[210] Bioluminescent bacteria can be engineered to continuously generate light which can be combined with photosensitizers and loaded into hydrogels for bioluminescence-triggered PDT.[210] This eLBP hydrogel system was found to exhibit high antitumor immunity in large *in vivo* tumors following hydrogel injection to the tumor site.[210] Photodynamic therapy can also be used in tangent with eLBP hydrogels as a treatment of infection to kill pathogenic bacteria. Xu *et al.* engineered bacteria to produce melanin granules for photothermal therapy and conjugated a photosensitizer on its surface for photodynamic therapy. This combination approach was successful in treating infection in response to light in mice after encapsulation and local delivery in BSA-based patches.[211]

Metabolic diseases, like diabetes, are another potential target of eLBP hydrogels.[9] In one study, *E. coli* LBPs were engineered to secrete exendin-4 (Ex-4), a glucagon-like peptide-1 (GLP-1) receptor agonist to encourage the release of insulin as a treatment for diabetes.[98] These LBPs were loaded into chitosan microcapsules, and a single dose was delivered subcutaneously in mice. After 2 weeks, they found Ex-4 levels and resulting insulin levels in the blood to be higher than untreated controls. This same study also tested a self-assembled protein nanovaccine, in which LBPs were engineered to produce distinct components of the system (bacterial microstructures and SIINFEKL peptide derived from the ovalbumin antigen) to generate an immune response against B16-OVA tumor cells.

5. Conclusions and future directions

A. Challenges, limitations, and future directions

Engineered live biotherapeutic products encapsulated within hydrogel systems represent a rapidly advancing frontier in the treatment of human disease, offering a unique convergence of synthetic biology and biomaterials engineering. While these platforms have demonstrated significant promise in enabling localized, tunable, and sustained delivery of therapeutics along with biosensing capabilities, challenges in the field remain that have prevented the FDA approval of any LBP loaded hydrogel technology.

A primary challenge in eLBP–hydrogel systems is balancing microbial activity with biomaterial performance for efficacy and reproducibility. Hydrogels must provide sufficient mechanical stability and containment while simultaneously permitting nutrient transport, waste removal, and diffusion of inducers and therapeutic products necessary for sustained eLBP function.[97] Predicting chassis compatibility with specific hydrogel polymers and fabrication methods remains challenging due to the lack of comparative studies on encapsulating diverse microbial species within different hydrogel chemistries. This gap underscores the need for systematic research to establish design principles that account for factors such as nutrient accessibility, material degradation, and processing constraints in eLBP–hydrogel systems.

Additionally, while natural hydrogels offer biocompatible and highly tunable platforms for live cell delivery, they often suffer from limited stability and mechanical strength. This can result in swelling under physiological conditions and material degradation that can affect degradation kinetics and therapeutic release profiles.[11,31] Although synthetic hydrogels offer higher mechanical integrity and stability, these materials are frequently subject to lower biocompatibility.[44] Hybrid hydrogel formulations provide a promising route to integrate the advantages of both material classes but require careful optimization.

The biosafety and biocontainment of engineered LBPs is another significant challenge. According to the NIH, less than

one in 100 million cells may escape the biocontainment platform.[212] eLBPs can be engineered with genetic kill switches to stop their activity in response to stimuli or after use.[98] For example, Han *et al.* engineered *E. coli* with a production-lysis circuit in which cells were programmed to lyse once protein production density reached a controlled limit. Additionally, hydrogel constructs can be designed with higher degrees of containment for loading cells with biosafety concerns.[59]

Another hurdle with eLBP-hydrogel drug delivery systems is the precise storage conditions required to keep constructs fresh and active. For example, Liu *et al.* found a decrease in activity after exposure to air for 24 hours compared to freeze-dried samples.[152] While lyophilization can be a great solution to storage issues, it can also change construct geometry and function when re-hydrated. This can be mitigated in samples where microbes are added later by introducing a freeze–thaw cycle to hydrogels before the addition of microbes.

While hydrogels provide biocompatible and tunable constructs for live cell and drug delivery, they exhibit limitations that can hinder their success for drug delivery. A major limitation is the complexity of factors related to cell viability, hydrogel mechanics, and the host environment. Based on the dynamic relationship of living hydrogel materials, it can be challenging to achieve reproducibility between batches regarding polymer configuration, cell viability, eLBP distribution, and microbial activity in hydrogels. This variability has the potential to cause PK/PD profiles in hosts that are difficult to predict and replicate. Recent developments in the fields of machine learning are emerging as useful tools for predicting these interactions for better screening.[112] For example, Martineau *et al.* used machine learning to identify a hydrogel composition for optimal gelation time to balance microbial cell viability with crosslinking speed.

Immunogenicity represents a significant limitation of eLBP–hydrogel systems. Degradation of certain hydrogel materials can generate byproducts that trigger local inflammation or immune clearance.[213] Natural polymers may contain residual bioactive motifs that activate innate immune responses, whereas some synthetic polymers and crosslinkers can induce cytotoxic or pro-inflammatory effects.[57,214,215] In addition, encapsulated eLBPs may elicit host immune recognition through microbial-associated molecular patterns or secreted products that diffuse beyond the hydrogel matrix. Strategies such as optimizing degradation profiles, minimizing immunogenic crosslinking chemistries, incorporating immunomodulatory materials, and engineering eLBPs with reduced immunogenic signatures or immune-evasive circuits are therefore critical to improving biocompatibility and therapeutic persistence *in vivo*.[44]

In summary, the integration of engineered live biotherapeutic products into hydrogel-based delivery systems represents a transformative approach to treating a wide range of human diseases. By combining the dynamic functionality of living microbes with the structural and tunable properties of hydrogels, these platforms offer unprecedented opportunities for localized, responsive, and sustained therapeutic interventions. While technical and translational challenges remain, continued interdisciplinary innovation is rapidly advancing the field toward clinical realization. As our understanding of host–microbe interactions deepens and biomaterial technologies evolve, LBP-loaded hydrogels are poised to become a cornerstone of next-generation precision medicine.

Conflicts of interest

There are no conflicts to declare.

Data availability

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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