

Engineering PEG-based living hydrogels for controlled transport and bacterial
microenvironments

by

Jeffrey Alexander Reed

B.S., Kansas State University, 2021

AN ABSTRACT OF A DISSERTATION

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DOCTOR OF PHILOSOPHY

Tim Taylor Department of Chemical Engineering
Carl R. Ice College of Engineering

KANSAS STATE UNIVERSITY
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Abstract

Engineered living hydrogels (ELHs) are an emerging class of biomaterials that integrate living microorganisms within hydrogel matrices to create dynamic materials capable of sensing, responding to, and modifying their surrounding environment. Bacteria are attractive as living components because of their genetic programmability, metabolic diversity, rapid growth, and ability to produce a wide range of functional biomolecules. Hydrogels are three-dimensional, water-swollen, crosslinked polymer networks that provide a hydrated environment for cell encapsulation while allowing molecular transport throughout the material, making them well suited as the structural component of engineered living hydrogels. ELHs have been investigated for applications including therapeutic delivery, biosensing, bioelectronics, and environmental remediation. However, as this novel field has emerged, quantitative relationships between hydrogel architecture, molecular transport, and bacterial function remain poorly understood, limiting the rational design of these systems. This dissertation addresses these challenges through the development of experimental platforms and poly (ethylene glycol) (PEG)-based ELHs. Collectively, these studies establish quantitative design principles for engineering bacterial behavior and the local microenvironment within engineered living hydrogels.

The first portion of this dissertation focuses on establishing quantitative relationships between hydrogel network architecture and molecular transport in PEG-based hydrogels. Because molecular transport depends on both hydrogel properties and the physicochemical characteristics of diffusing molecules, quantitative characterization is necessary to predict solute transport and engineer hydrogel function. Experimental methods were developed to quantify diffusion coefficients, partition coefficients, and hydrogel mesh size, providing complementary descriptions of transport behavior and network architecture. Together, these measurements establish a

quantitative framework for characterizing molecular transport and hydrogel network architecture in PEG-based hydrogels that are used throughout the remainder of this dissertation.

The second portion of this dissertation applies the quantitative transport framework established in the first portion to develop increasingly sophisticated PEG-based ELHs. A photodegradable PEG hydrogel platform integrated within a microfluidic device was first developed to investigate how controlled hydrogel degradation influences bacterial migration, chemotaxis, and cellular delivery. Controlled degradation regulated bacterial speed and directionality, enabling selective delivery of motile bacteria to a defined location while excluding nonmotile particles. PEG molecular weight was then systematically varied to engineer hydrogels with distinct mesh sizes, transport properties, and mechanical characteristics. Hydrogel network design and the resulting nanoscale confinement regulated bacterial growth and biomolecule production, while encapsulated endospores created a resilient material that maintained bacterial function following dehydration and temperature stress. These hydrogel design principles were then integrated with enzyme immobilization to engineer the local biochemical microenvironment surrounding encapsulated bacteria. A reaction-diffusion model predicted hydrogen peroxide profiles guided the selection of catalase loadings, which maintained bacterial viability, recovery, and function following oxidative stress.

The final portion of this dissertation discusses several remaining challenges that limit broader implementation of engineered living hydrogels. These include integrating multiple biochemical functionalities, developing increasingly sophisticated hydrogel architectures, extending predictive transport models to dynamic environments, and validating these materials under application-relevant conditions. Addressing these will support the continued development of engineered living hydrogels for biomedical, environmental, and industrial applications.

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Dedication

To my beloved wife,

Taylor Reed

Chapter 1 - Introduction

1.1 Engineered Living Materials (ELMs)

Engineered living materials (ELMs) have emerged as a novel class of biomaterials that integrate living cells with non-living structural components to create dynamic systems with programmable functions.^{1,2} By incorporating living organisms, ELMs can actively sense, respond to, and interact with their surrounding environment, allowing their function to evolve over time.³ Among the available cellular components, bacteria have become particularly attractive due to their genetic programmability, metabolic diversity, rapid growth, self-assembly, and ability to continuously produce a wide range of functional molecules.^{2,4} These characteristics have enabled ELMs to address engineering challenges across the environmental, industrial, and biomedical sectors.⁵ To maintain microbial activity and function, and modulate the microenvironment, bacteria are commonly encapsulated within hydrogels. These systems, referred to as engineered living hydrogels (ELHs), are three-dimensional crosslinked polymer networks with high water content and tunable physicochemical properties that promote cell viability, while allowing diffusion of nutrients, metabolites, and other small molecules.^{6,7} Unlike conventional, non-living hydrogels, ELHs maintain biological activity after fabrication, allowing material function to adapt over time in response to changing environmental conditions. Because living cells remain metabolically active after encapsulation, ELHs can perform functions that extend well beyond the capabilities of conventional biomaterials.

1.1.1 Sensing applications

Environmental sensing and bioremediation represent some of the earliest applications of ELHs by leveraging the natural ability of encapsulated bacteria to detect and respond to environmental stimuli. ELHs have been developed as living biosensors capable of

detecting a wide variety of contaminants and toxins. For example, Tang et al. developed a hydrogel-based platform that enabled engineered bacteria to detect cadmium in water from the Charles River while maintaining bacterial viability, physical containment, and protection from environmental stressors.⁸ This system also incorporated a genetically-encoded memory circuit capable of recording chemical exposure, allowing the bacteria to retain sensing information after sampling. Beyond sensing, immobilized microorganisms in ELHs can remediate contaminated environments through pollutant degradation or metal sequestration, as demonstrated by Knierim et al., who developed a synthetic biofilm system capable of removing nitrite and sequestering high-value materials like gold.⁹

1.1.2 Remediation applications

While environmental sensing and remediation leverage the ability of microorganisms to respond to their surroundings, ELHs have also been engineered to use microbial metabolism for sustainable energy conversion and resource recovery. Many environmental systems and biological processes generate waste streams containing chemical energy and carbon that can be harnessed rather than traditionally discarded. ELHs can capture these resources, continuously converting them into electrical energy, chemical feedstocks, and other value-added products. In microbial electrochemical systems, hydrogel-based bioanodes have been used to keep electroactive microorganisms at the electrode surface, improving cell retention and, in some designs, extracellular electron transfer.^{10, 11} While these coatings are closely related to ELHs, they are primarily designed as bioelectrochemical interfaces rather than standalone living materials. Extending beyond energy recovery, Zheng et al. developed a hybrid electro-biosystem coupling electrochemical CO₂ reduction with engineered yeast fermentation, converting electrochemically generated acetate into glucose, fatty acids, and other value-added chemical products.¹² More

recently, these principles have been translated into living bioelectronic materials, with Wang et al. developing 3D-printable living hydrogels containing electroactive bacterial biofilms that function as self-charging biobatteries that power nerve stimulation for blood pressure regulation.¹³ The ability of encapsulated microorganisms to continuously consume substrates and produce bioactive molecules extends the function of ELHs beyond environmental and industrial applications, enabling living materials that can directly interact with biological systems.

1.1.3 Biomedical applications

By maintaining viable microorganisms within engineered materials, ELHs can establish sustained, dynamic interactions with surrounding tissues by responding to physiological signals over extended periods.^{7, 14} Harimoto et al. developed a robust implantable living material that confined engineered bacteria within a hydrogel designed with high stiffness to regulate bacterial proliferation and high toughness to prevent bacterial escape due to material fracture. Upon sensing pathogen-derived quorum-sensing molecules, the encapsulated bacteria underwent programmed lysis to release an antimicrobial payload, providing localized treatment of prosthetic joint infections. Additionally, this material maintained complete bacterial containment over a 6-month period, demonstrating sustained interactions between living microorganisms and the host environment.¹⁵ Beyond interaction with pathogenic microorganisms, living materials have also been engineered to establish prolonged interactions with specific host tissues. Duraj-Thatte et al. engineered self-regenerating bacterial hydrogels that enabled persistent adhesion to the gastrointestinal mucosa, maintaining a living coating despite continual mucosal turnover.¹⁶

Because encapsulated microorganisms remain viable and metabolically active, ELHs can serve as renewable production platforms capable of sustained, programmable production of proteins, enzymes, antimicrobial compounds, and other biologically active molecules.^{17, 18} Unlike

traditional biomaterials, which often serve as passive scaffolds or temporary drug reservoirs, ELHs enable continuous, localized production of functional biomolecules. Guo et al. developed a programmable living material in which engineered *Escherichia coli* were retained within a large-pore hydrogel, enabling the producer cells to remain localized within the material while allowing continuously secreted lysostaphin to diffuse into the surrounding environment.¹⁹ Similarly, Ben David et al. encapsulated *Bacillus subtilis* within PVA microparticles to create an active wound dressing that continuously produced and secreted the antimicrobial lipopeptides, surfactin and fengycin, directly at the wound site, providing a sustained source of antimicrobial compounds with relying on preloaded antimicrobial agents.²⁰ These diverse capabilities ultimately depend on a material platform that can simultaneously maintain microbial viability while regulating the surrounding physicochemical environment.

1.2 Material Selection for Hydrogels

Hydrogels have emerged as the dominant biomaterial platform for engineered living materials because their physicochemical properties can be independently tuned across multiple design dimensions, including chemistry, network architecture, mechanics, degradation, and transport.^{2, 6} This level of control allows for precise material properties that can be tailored to meet the functional requirements for the embedded cells and the surrounding environments, rather than serving simply as passive carriers for encapsulated microorganisms.⁸ In bacterial ELHs, these tunable material properties collectively define the cellular microenvironment. By modulating nutrient and analyte diffusion, metabolite exchange, cellular confinement, and protection from the surrounding environment, hydrogels establish the physicochemical conditions that govern bacterial growth, metabolism, and function. Although hydrogel properties can be tuned across multiple design dimensions, they are not independent. Changes in network architecture

simultaneously influence mechanical strength, molecular transport, swelling, and degradation. For example, increasing crosslink density generally increases stiffness and decreases swelling and molecular transport. Consequently, hydrogel design requires balancing these interdependent properties to achieve the desired function.²¹⁻²³

1.2.1 Natural hydrogels

Naturally derived hydrogels provided some of the earliest and most widely used matrices for ELHs due to their ability to provide hydrated, biocompatible environments support living cells, combined with their abundance and low cost.^{2, 6} Common examples include alginate, collagen, gelatin, hyaluronic acid, cellulose, and chitosan, which are most commonly used and have favorable biocompatibility, biodegradability, and relatively mild gelation conditions that facilitate cell encapsulation. Their natural compositions can also provide bioactive cues that support living-cell function without extensive material modification.²⁴ However, these same features can limit engineering control. Natural hydrogels may vary by source or batch, have heterogeneous structures, and contain bioactive components that introduce unwanted interactions between the material and encapsulated cells. As a result, it can be more difficult to reproducibly tune encapsulated bacterial behavior through material design.^{6, 25}

1.2.2 Synthetic hydrogels

Synthetic hydrogels have become increasingly important in ELHs and address many of the limitations associated with natural hydrogels. With chemically defined compositions, synthetic hydrogels provide greater reproducibility and tailored chemistry for well-controlled, tunable material properties, controlled degradation and biofunctionalization.^{6, 26} Unlike naturally derived hydrogels, synthetic materials generally provide fewer intrinsic biological cues and often require additional engineering to incorporate bioactivity, tuned degradability, or other biological

functions.²⁴ Material selection therefore represents more than a choice of polymer platform; it determines whether hydrogel function is derived primarily from intrinsic material bioactivity, or from deliberate network design and functionalization.

Because their precursor chemistry and network formation can be systematically engineered, synthetic hydrogels enable well-defined structure–property relationships that allow material behavior to be designed more predictably than in naturally derived systems.²¹ Synthetic hydrogels enable a wide range of crosslinking strategies and functional modifications that further expand hydrogel design beyond the capabilities of many naturally derived materials. These capabilities have led to the widespread use of polymers such as poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), and poly(acrylic acid) (PAA) as versatile platforms for ELHs.⁶ Among these materials, PEG has become one of the most widely used model hydrogel systems primarily in tissue engineering and cell encapsulation. It offers simple, well-defined chemistry to enable systematic investigation of network formation, degradation, transport, and biofunctionalization.^{22, 27, 28} The widespread use of PEG arises because it is chemically inert, biologically compatible, and has versatile routes for chemical functionalization.⁶ PEG resists nonspecific protein adsorption and cell adhesion. Its macromers are commercially available across a wide range of molecular weights and reactive end-group chemistries, enabling straightforward incorporation of bioactive or stimuli-responsive functionalities.^{21, 26} As a result, PEG has become a model hydrogel platform for systematically study of how network structure governs material properties, transport, and biological function.^{28, 29}

1.3 Hydrogel Network Formation

In addition to polymer selection, hydrogel properties are strongly influenced by the mechanisms used to form the polymer network. Network formation determines crosslink densities

and mesh size homogeneity, which in turn govern hydrogel stability, degradation, transport, and other physicochemical properties.²⁹ As a result, network formation represents a second major design variable in hydrogel engineering. Hydrogels are broadly classified as physically or chemically crosslinked depending on whether network formation occurs through reversible intermolecular interactions or covalent bonds.^{24, 25}

1.3.1 Physically Crosslinked Hydrogels

Physical crosslinking provides a simple strategy for hydrogel formation through noncovalent interactions rather than covalent bonds. Depending on the polymer chemistry, these interactions may include hydrogen bonding, electrostatic interactions, or hydrophobic interactions.²⁴ Because gelation often occurs under mild conditions with minimal chemical modification, physically crosslinked hydrogels are particularly attractive for biological encapsulation and remain widely used in naturally derived hydrogel systems. The dynamic nature of these interactions can enable desirable properties such as injectability and self-healing. However, these same interactions generally produce networks with lower long-term stability than covalently crosslinked hydrogels.²⁶

Different polymers achieve physical crosslinking through distinct mechanisms. Alginate forms hydrogels through ionic crosslinking with divalent cations such as calcium,³⁰ whereas gelatin forms thermoreversible gels through temperature-dependent hydrogen bonding between polymer chains.³¹ Electrostatic interactions are similarly used to form polyelectrolyte complexes between oppositely charged polymers such as alginate and chitosan.³² These mechanisms provide simple and cytocompatible routes to hydrogel formation, but because these interactions come from the intrinsic properties of the polymers themselves, opportunities to independently tune network architecture are more limited.

For many engineered living material applications, long-term regulation of molecular transport and cellular confinement requires hydrogel networks with reproducible structure and consistent material properties.⁶ Because physical crosslinking relies on reversible polymer interactions, modulating network architecture can be difficult and is often more limited compared to covalently crosslinked systems.²⁶ Although physically crosslinked hydrogels remain highly attractive for cell encapsulation, many engineered living hydrogels instead use chemical crosslinking to provide greater control over network architecture and material behavior when required.

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1.3.2 Chemical Crosslinked Hydrogels

Chemically crosslinked hydrogels are commonly formed through either chain-growth or step-growth polymerizations.²⁹ Although both produce covalently crosslinked networks, the polymerization mechanism strongly influences the overall network architecture, including network homogeneity, structural defects, and crosslink distribution, leading to important differences in swelling, degradation, mechanical behavior, and molecular transport.²¹

1.3.2.1 Chain-growth polymerization

Chain-growth polymerization is one of the most widely used approaches for chemically crosslinking hydrogels and is commonly achieved through free-radical photopolymerization of multifunctional macromers such as PEG-diacrylate (PEGDA).^{22, 33} Upon light exposure, photoinitiators generate reactive radicals that propagate through vinyl groups to rapidly form a covalently crosslinked polymer network.³⁴ The rapid reaction kinetics and precise spatial and temporal control of photopolymerization have made this approach particularly attractive for in situ hydrogel formation and cell encapsulation.^{35, 36}

Because polymer chains propagate from localized radical centers, chain-growth polymerized network architecture differs substantially from that produced by step-growth polymerization.²⁹ These network non-idealities arise from the stochastic nature of free radical chain propagation reactions and chain termination reactions to drive heterogeneous crosslink distributions and incomplete functional group conversion in the resulting hydrogels.³⁴ Oxygen inhibition can further reduce polymerization efficiency by scavenging propagating radicals and limiting functional-group conversion.³⁷ In cell-encapsulating systems, photoinitiator chemistry, free radical exposure, and initiator conditions (UV or visible light intensity, elevated temperatures) must also be carefully optimized to maintain cytocompatibility.³⁸ Although chain-growth polymerizations remain widely used due to its simplicity and photopatterning capabilities,³⁹ these limitations have motivated the development of step-growth polymerization strategies that produce more homogeneous hydrogel networks with improved tailoring over material structure and transport properties.⁴⁰

1.3.2.2 Step-growth polymerization

In contrast, step-growth polymerization forms hydrogel networks through reactions between complementary multifunctional macromers that contain reactive functional groups.⁴¹ Because crosslinks are formed directly between these multifunctional precursors, step-growth polymerization can provide greater control over the hydrogel network compared to chain-growth polymerization.²⁹ The final network architecture can be tailored through precursor functionality, molecular weight, concentration, stoichiometric balance, and reaction conversion, allowing hydrogel properties to be systematically tuned for specific applications.²⁷ Although network defects can still arise depending on precursor design and reaction conditions,⁴² hydrogels formed with step-growth polymerizations generally produce more homogeneous and predictable network architectures compared to hydrogels formed with chain-growth polymerization (Fig. 1.1).²⁹

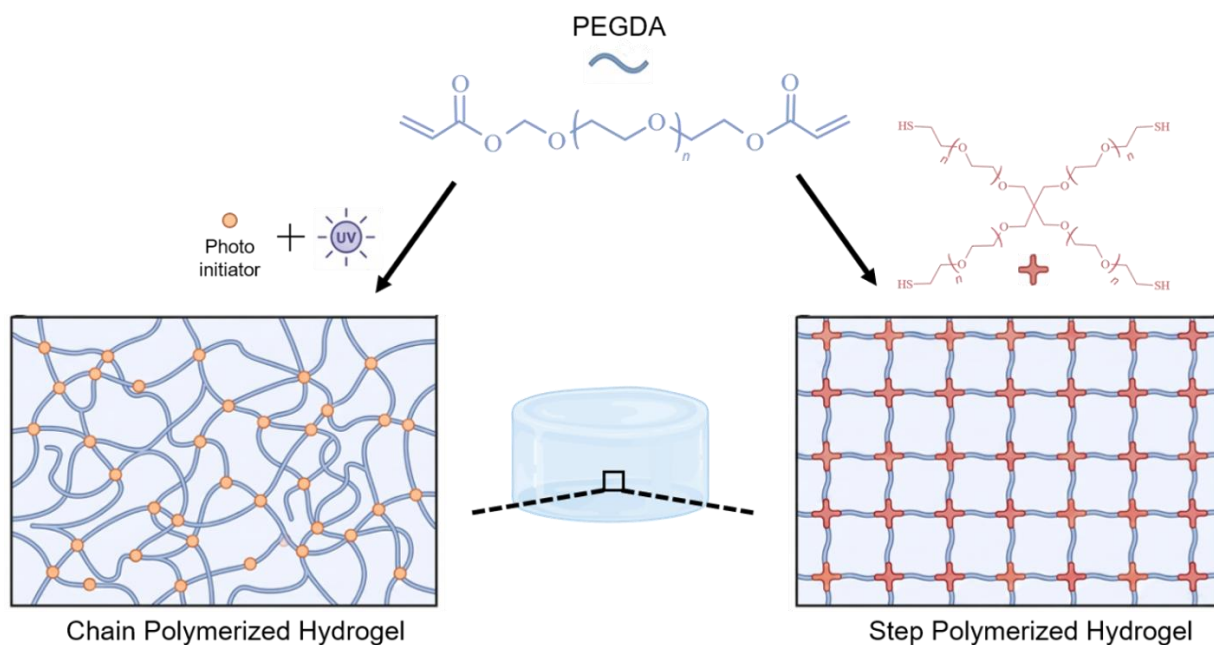


Figure 1.1. Chain vs. step growth polymerization

Among the various step-growth chemistries developed for hydrogel formation, click-type reactions have become widely used because they provide efficient and selective network formation under mild aqueous conditions.⁴³ Michael-type addition is one of the most widely used click-type reactions because it proceeds under mild conditions without radical initiators and therefore avoids the oxygen inhibition associated with free-radical polymerization.^{27, 44} For hydrogel formation, Michael-type addition most commonly occurs through the reaction of thiol groups with electron-deficient alkenes to form stable thioether linkages under alkaline conditions (Fig. 1.2).⁴³ Common Michael acceptors include maleimides, vinyl sulfones, acrylates, and acrylamides, each of which exhibits distinct reactivity and bond-linkage stability that can be leveraged to tailor gelation behavior, degradation, and network properties.⁴⁵⁻⁴⁷

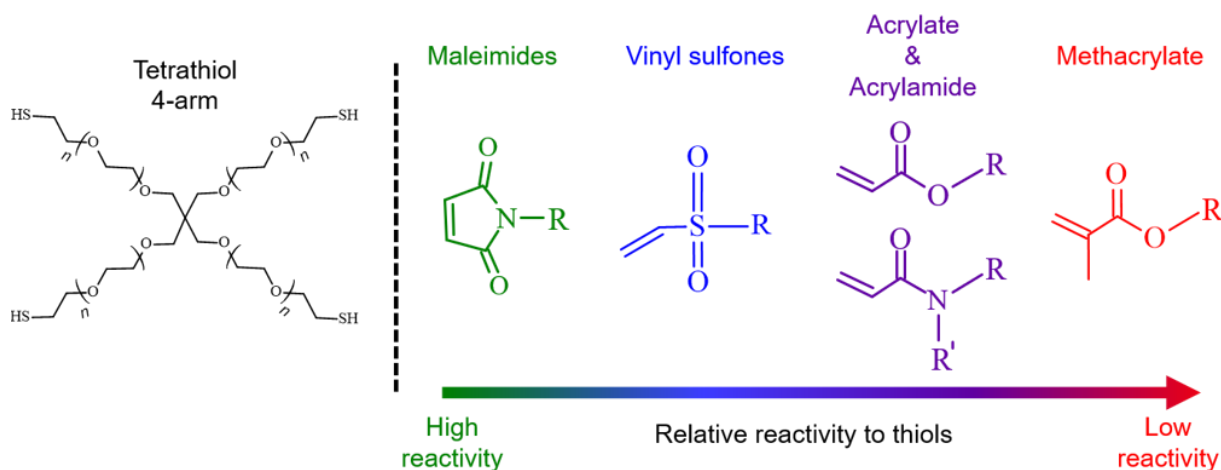


Figure 1.2. Common Michael acceptors for vinyl-functionalized PEG macromers

The combination of Michael-type addition and multifunctional PEG macromers establish a versatile platform for engineering hydrogel structure and function.²⁷ Because PEG is biologically inert, non-adhesive, and commercially available with a wide range of molecular weights and reactive end groups, its chemical composition and network architecture can be systematically engineered while minimizing unintended biological interactions.⁴⁸ Rather than relying on the

intrinsic bioactivity of naturally derived polymers, biological function can instead be introduced through the incorporation of bioactive ligands, degradable peptide crosslinkers, and other functional molecules.⁴³ One of the earliest demonstrations of this approach was reported by Lutolf and Hubbell, who used multiarmed PEG–vinyl sulfone macromers and bis-cysteine peptide crosslinkers to establish a modular PEG hydrogel platform with systematically tunable physicochemical properties through precursor design.⁴¹ Building on this platform, subsequent work demonstrated that cell-material interactions within PEG hydrogels could be programmed through rational molecular design, enabling cell-mediated remodeling and tissue regeneration.⁴⁹ This work helped establish PEG hydrogels formed through Michael addition reactions for cell-responsive synthetic extracellular matrices rather than simply structural biomaterials.⁵⁰ Because both the polymer chemistry and precursor design are well defined, PEG hydrogels formed through Michael-type addition provide precise control over crosslink density and mesh architecture, allowing swelling, degradation, and molecular transport to be systematically engineered.^{27, 48}

1.4 Mass Transport in Hydrogels

Hydrogels are uniquely suited for engineering biological microenvironments because they simultaneously regulate molecular transport and cellular confinement. With nanoscale mesh sizes, hydrogel networks can be designed to allow the diffusion of oxygen, nutrients, metabolites, signaling molecules, and therapeutic agents while retaining encapsulated cells. This provides the molecular exchange required for sustained biological activity within the material.⁸ In engineered living hydrogels, transport is coupled to cellular activity because encapsulated microorganisms continuously consume nutrients and oxygen while producing metabolites, signaling molecules, enzymes, and other functional products (Fig. 1.3).^{4, 6} Hydrogel function depends not only on the

movement of molecules through the polymer network but also on the dynamic interactions between the material and its encapsulated cells.⁵¹

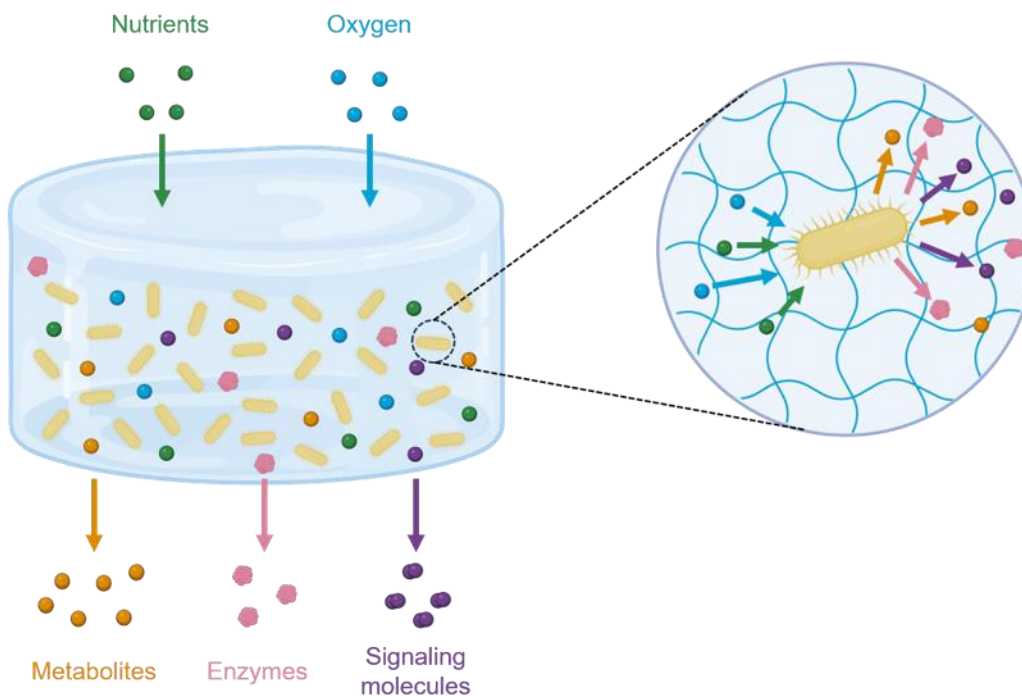


Figure 1.3. Molecular exchange within engineered living hydrogels

Mass transport in hydrogels is governed by the structure of the polymer network.^{52, 53} Specifically, network characteristics including crosslink density, polymer volume fraction, swelling, degradation, and network heterogeneity collectively influence the movement of molecules through the hydrogel.⁵⁴ Mesh size has become one of the most useful parameters for relating hydrogel structure to molecular transport because it provides an estimate of the effective spacing within the hydrogel network.⁵⁵ The influence of mesh size on the effective diffusivity of a molecule depends strongly on the dimensions of the transported species.⁵⁶ Oxygen, nutrients, metabolites, and signaling molecules are typically much smaller than the characteristic mesh size of most hydrogels and therefore experience relatively little steric hindrance during diffusion.

Instead, their transport is primarily influenced by the polymer volume fraction and the hydrated polymer network through which diffusion occurs.^{55, 56} As molecular dimensions increase and approach the characteristic mesh size, steric hindrance becomes increasingly important.⁵⁷ Rehmann et al. investigated the relationship between PEG hydrogel mesh size and protein transport, showing that proteins with hydrodynamic diameters well below the characteristic mesh size were rapidly released, whereas proteins with dimensions approaching the mesh size exhibited progressively slower release. In contrast, proteins substantially larger than the mesh size remained effectively entrapped within the hydrogel network.⁵⁸ While these trends are not absolute, the ability to tailor mesh size provides a powerful strategy for regulating transport across a broad range of biological length scales.^{54, 56}

Microorganisms are typically much larger than the characteristic mesh size of hydrogel networks and can therefore be physically confined within the material.⁴⁹ This selective permeability enables hydrogels to support long-term microbial cell viability while maintaining cellular localization, making them attractive platforms for engineered living hydrogels.⁸ Compared to passive molecular diffusion, cellular confinement is not static. Encapsulated cells continuously consume nutrients and oxygen while producing metabolites, signaling molecules, enzymes, therapeutic proteins, and other functional products, generating local concentration gradients that influence both cellular behavior and material performance.^{6, 51} As encapsulated cells proliferate and accumulate biomass within the hydrogel network, growing colonies can exert enough pressure to fracture the surrounding network and create pathways for cell release. The amount of microbial release can then be controlled by varying the initial cell loading and hydrogel stiffness.⁵⁹ More broadly, hydrogel swelling and degradation can alter network architecture and molecular transport over time, while cell-secreted proteases can enzymatically cleave degradable crosslinks within

PEG hydrogels, creating localized pathways for cell migration.^{48, 57} This dynamic relationship between network design, transport, and cellular activity provides opportunities to actively engineer hydrogel function after fabrication.

1.5 Responsive and Functional Hydrogels

1.5.1 Hydrogel Additives

Hydrogel performance can be enhanced by incorporating functional components directly into the polymer network. Rather than modifying network architecture alone, these approaches introduce additional biological, chemical, or physical functionality directly within the network that expands the capabilities of the material. Depending on the intended application, these functional components can be physically encapsulated or covalently immobilized within the hydrogel to provide sustained or localized activity or engineered for controlled release to deliver encapsulated payloads over time. Nanoparticles, enzymes, and other bioactive molecules have been incorporated into PEG hydrogels for a wide range of biological, chemical, and physical functions, including antimicrobial activity, catalytic function, and controlled release.^{60, 61} These functional modifications transform PEG hydrogels from passive structural matrices into active materials capable of regulating their local chemical, physical, and biological microenvironment.

Silver nanoparticles have been widely incorporated into PEG hydrogels to impart intrinsic antimicrobial activity. Fullenkamp et al. developed a mussel-inspired PEG hydrogel in which catechol groups simultaneously initiated hydrogel crosslinking while reducing silver ions to form silver nanoparticles *in situ*. By integrating nanoparticle synthesis directly into hydrogel formation, the resulting material combined sustained silver release, antimicrobial activity against both *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*, antifouling behavior, and mammalian cytocompatibility within a single PEG network.⁶²

Nanoparticle functionalization was extended beyond antimicrobial activity to generate additional electrical conductivity. Zhou et al. incorporated graphene oxide nanosheets into an oligo(PEG fumarate) hydrogel, producing a conductive biomaterial that improved electrical signal propagation, calcium coupling, and cardiac function following myocardial infarction. This study demonstrated that nanoparticle incorporation could enable PEG-derived hydrogels to electrically communicate with surrounding tissues while maintaining the desirable properties of the hydrogel network.⁶³ Beyond chemical and electrical functionality, magnetic nanoparticles have further expanded the capabilities of PEG hydrogels by enabling remote mechanical actuation. Steinbeck et al. embedded aligned magnetic nano spindles within PEGDA microgels, allowing the hydrogels to be remotely positioned, rotated, and actuated under externally applied magnetic fields. When incorporated into microfluidic devices, these magnetically responsive hydrogels generated localized fluid mixing, illustrating how nanoparticle incorporation can transform PEG hydrogels from passive materials into remotely engineered systems capable of performing mechanical work.⁶⁴ Nanoparticles therefore provide one strategy for introducing function that cannot be achieved through the polymer network alone.

A related approach is to directly incorporate biological catalysts capable of modifying the local biochemical environment. Enzyme incorporation represents one of the earliest and most common strategies for functionalizing PEG hydrogels because it transforms the hydrogel from a passive structural matrix into a catalytically-active environment. Depending on the intended application, enzymes may be physically encapsulated to preserve their native structure or covalently immobilized to improve long-term retention within the material. Early studies focused primarily on preventing enzyme loss from the hydrogel. Andreopoulos et al. demonstrated that covalent immobilization of organophosphorus hydrolase substantially reduced enzyme leaching

and improved operational stability compared with physical entrapment, although catalytic activity was reduced.⁶⁵ Subsequent work shifted from retaining enzymes to preserving their biological function within the hydrogel microenvironment. To preserve enzyme activity while maintaining the ability to recover and deliver active biomolecules, Marco-Dufort et al. engineered dynamic PEG hydrogels containing reversible crosslinks that physically encapsulated enzymes within the hydrogel network. This protective microenvironment shielded enzymes from thermal denaturation while permitting on-demand recovery and release of active biomolecules through reversible network disassembly, demonstrating that PEG hydrogels could actively preserve biological function rather than simply retain enzymes.⁶⁶ More recently, PEG hydrogels have been engineered to spatially organize multiple enzymes within a single material, enabling coordinated enzymatic reactions within a defined hydrogel architecture. Mancini et al. immobilized glucose oxidase within a hydrogel core and horseradish peroxidase within a surrounding shell, creating a compartmentalized architecture in which the product of the first reaction was directly utilized by the second enzyme. By spatially separating yet functionally coupling the two enzymes, this design regulated the transport of reactive intermediates and improved the efficiency of sequential enzymatic reactions, demonstrating that hydrogel architecture can be engineered to precisely coordinate complex biochemical processes.⁶⁷ While these approaches permanently introduce new functions into the hydrogel network, many applications also require those functions and material properties to be dynamically regulated after fabrication.

1.5.2 Stimulus Responsive (SMART) Hydrogels

Once formed, the structural and transport properties of conventional hydrogels are largely determined by their initial composition and crosslinking conditions.^{48, 54} Although passive processes such as swelling and degradation can alter these properties over time, many biomedical

and engineered living material applications require additional control over network architecture, molecular transport, and mechanical properties.⁵⁷ To address this, modern hydrogel engineering has increasingly focused on designing materials that respond to external or internal stimuli (Fig. 1.4). These physical, chemical, and biochemical stimuli allow hydrogel properties to be selectively modified in response to changing environmental conditions or user inputs, transforming hydrogels from passive encapsulation matrices into dynamic biomaterials whose structure and function can be programmed throughout the material lifetime.⁶⁸⁻⁷⁰

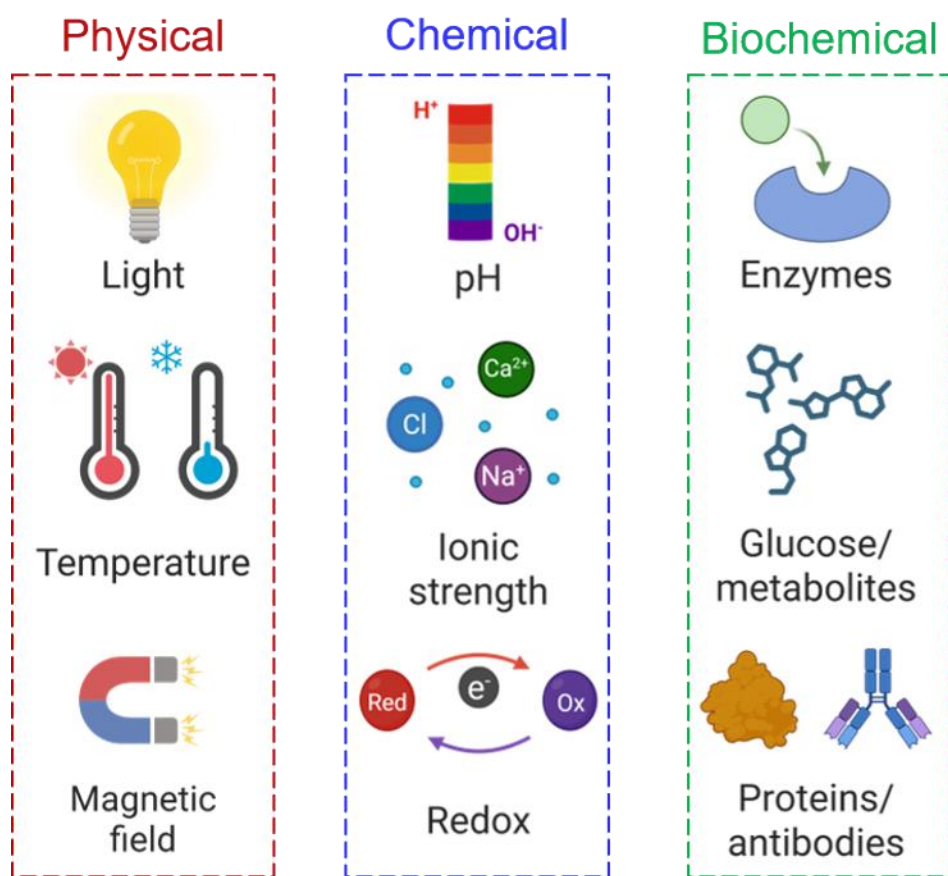


Figure 1.4. Examples of physical, chemical, and biochemical stimuli that can be used to regulate the behavior of stimuli-responsive polymers after fabrication

Among the various stimuli used to program hydrogel behavior, light has emerged as one of the most versatile because it provides remote, spatially, and temporally precise control over hydrogel properties after fabrication. Rather than serving only as an initiator for hydrogel formation, light can also be used to selectively modify the hydrogel network, enabling changes in crosslink density, mesh size, stiffness, degradation, molecular transport, and biochemical presentation on demand. Kloxin et al. established this concept through photodegradable PEG hydrogels, demonstrating that precise photocleavage of network crosslinks enabled user-defined changes in hydrogel architecture after gel formation. By dictating the location, timing, and extent of photodegradation, they dynamically regulated hydrogel mechanics, created microscale degradation patterns and migration channels, and directed cell morphology, migration, and differentiation within the material.⁷¹ Building on this platform, DeForest and Anseth developed orthogonal photochemical reactions that independently tuned hydrogel degradation and biochemical functionalization, enabling both the physical and biochemical properties of the cellular microenvironment to be modified without affecting one another.⁷² Subsequent studies further expanded these approaches to precisely regulate the timing and sequence of molecular release, extending programmable microenvironment beyond tailored hydrogel structure to the delivery of encapsulated biomolecules.^{73, 74}

Although light provides exceptional spatial and temporal control over hydrogel behavior, numerous other stimuli have also been used to dynamically regulate hydrogel properties after fabrication. Temperature- and pH-responsive hydrogels exploit changes in their local environment to regulate swelling, degradation, and molecular transport, enabling tailored drug delivery and tissue engineering applications.⁶⁹ Enzyme-responsive hydrogels similarly couple changes in hydrogel behavior to biological activity, allowing material properties to evolve in response to the

local cellular environment.⁷⁰ Other stimuli, including magnetic and electric fields, redox reactions, and specific biomolecular interactions, have likewise been incorporated to provide remote actuation, reversible changes in material properties, and environmentally responsive behavior.^{50, 75, 76} Collectively, these strategies illustrate how hydrogel behavior can be dynamically programmed after fabrication, providing the foundation for engineering living hydrogels that actively regulate bacterial transport, survival, and function in response to changing environmental conditions.

1.6 PEG-Based Hydrogel Design in ELHs

Additives within hydrogels can introduce protective, catalytic, or conductive functions, while stimulus-responsive chemistries allow transport, degradation, release, and mechanical properties to change after hydrogel formation in response to environmental conditions. Homogeneous step-growth PEG hydrogels provide precise control over crosslink density, mesh size, stiffness, swelling, and molecular transport, while functional additives and stimulus-responsive chemistries allow ELHs to be tailored for specific applications. In this dissertation, PEG-based hydrogels are used to characterize network structure and molecular transport, control bacterial transport and delivery, regulate bacterial growth and function, and engineer local bacterial microenvironments. By linking material properties to bacterial behavior and overall material performance, this work develops the fundamental understanding needed to guide the rational design of future ELHs across a wide range of applications.

Chapter 2 - Objectives

2.1 Background and Motivation

PEG-based ELHs combine functional microorganisms with a chemically-defined polymer network designed to regulate molecular transport, confinement, and cellular interactions with the surrounding environment.^{27, 77} PEG hydrogels are widely used in ELHs because their physical and chemical properties can be readily tailored for applications including biosensing, therapeutic delivery, bioproduction, environmental remediation, and living therapeutics.^{6, 7} Despite these advances, recent studies have continued to emphasize application-specific demonstrations, while the fundamental understanding of how PEG hydrogel properties influence encapsulated bacteria remains limited.^{15, 77, 78} In particular, the effects of hydrogel structure on molecular transport, bacterial movement, metabolic function, and environmental protection remain underexplored, making it difficult to establish quantitative design principles for PEG-based ELHs.^{8, 79, 80}

The studies presented in this dissertation investigate how PEG hydrogel architecture, nanoscale confinement, and incorporated functionality influence encapsulated bacteria. The first section of this dissertation establishes quantitative methods for characterizing transport and network architecture in PEG hydrogels by measuring hydrogel mesh size, diffusion coefficients, and partition coefficients across PEG formulations. The remaining studies build upon this framework to investigate how PEG hydrogel properties influence bacterial transport and delivery, how network structure regulates bacterial growth and function, and how incorporated enzymes can create protective bacterial microenvironments. Collectively, these studies provide a basis for designing ELHs with controlled transport, predictable bacterial function, and protective local microenvironments.

The objectives of this dissertation are summarized by the following specific aims:

Aim 1: Develop experimental methods for quantifying diffusion coefficients, partition coefficients, and mesh size in PEG hydrogels.

This study establishes quantitative methods for characterizing molecular transport and network architecture in PEG hydrogels. Diffusion coefficients, partition coefficients, and hydrogel mesh size are measured across varied PEG formulations to provide mass transport parameters used throughout the remaining studies.

Aim 2: Investigate how controlled photodegradation of PEG hydrogels influences bacterial transport and chemotaxis.

This study investigates how hydrogel architecture influences bacterial movement through photodegradable PEG hydrogels. A diffusion-based microfluidic platform combined with patterned photodegradation is used to evaluate bacterial chemotaxis under precisely controlled chemical microenvironments.

Aim 3: Determine how PEG hydrogel mesh size influences bacterial encapsulation, survival, growth, and surfactin production.

This study investigates how hydrogel mesh size influences bacterial encapsulation, growth, and surfactin production within PEG hydrogels. Hydrogel formulations with across a range of nanoscale mesh sizes were systematically varied and cellular behavior within these materials is evaluated by quantifying bacterial viability, colony morphology, cellular growth rates, and surfactin production following encapsulation.

Aim 4: Engineer enzyme-functionalized PEG hydrogels that protect encapsulated bacteria from oxidative stress.

This study investigates the use of enzyme-functionalized PEG hydrogels to protect encapsulated bacteria from oxidative stress. Cellular protection from hydrogen peroxide was achieved from hindered diffusion and hydrogen peroxide decomposition by catalase enzymes immobilized within the hydrogel. Hydrogen peroxide transport, decomposition by catalase enzymes, and resulting bacterial viability are quantified to evaluate the ability of catalase-functionalized hydrogels to preserve encapsulated bacterial under oxidative stress. A reaction-diffusion model that describes hydrogen peroxide toxin is developed for design of these hydrogels towards future applications.

Chapter 3 - Quantitative Measurements of Small Molecule

Diffusivity, Partition Coefficient, and Mesh Size in PEG-Based Hydrogel Networks

Overview

This chapter describes experimental methods developed to quantitatively characterize molecular transport and hydrogel network architecture in PEG-based hydrogel systems. Protocols are presented for measuring small-molecule diffusion coefficients, partition coefficients, and hydrogel mesh size. The first section describes experimental procedures for determining diffusion coefficients from one-dimensional release experiments and partition coefficients from equilibrium measurements. These methods have been applied in recent publications by Fattahi et al.⁸¹ and Reed et al.⁸² The second section presents methods for estimating hydrogel mesh size using equilibrium swelling theory across PEG hydrogels prepared from multiple macromer molecular weights. These methods have been applied in recent publications by Gutierrez et al.⁸³ and Reed et al.⁸⁴

*Manuscript: Fattahi, N.; **Reed, J.**; Heronemus, E.; Fernando, P.; Hansen, R.; Parameswaran, P. Polyethylene glycol hydrogel coatings for protection of electroactive bacteria against chemical shocks. *Bioelectrochemistry* 2024, 156, 108595. DOI: <https://doi.org/10.1016/j.bioelechem.2023.108595>.

*Manuscript: Gutierrez, M. M.; **Reed, J. A.**; McElroy, R. A.; Hansen, R. R. Bacteria encapsulation into polyethylene glycol hydrogels using Michael-type addition reactions. *MRS Advances* 2024, 9 (19), 1520-1526. DOI: 10.1557/s43580-024-00940-y.

*Manuscript: **Reed, J. A.**; Retterer, S. T.; Hansen, R. R. Photodegradable Hydrogel Matrices for Spatiotemporal Control of Bacteria Transport and Delivery. *ACS Applied Materials & Interfaces* 2025, 17 (37), 51919-51930. DOI: 10.1021/acsami.5c14670.

*Manuscript: **Reed, J. A.**; Gutierrez, M. M.; Dougherty, D. A.; Zaman, A. B.; Hansen, R. R. PEG-Based Living Hydrogels Engineered for Tunable Production of Bioactive Lipopeptides. *ACS Applied Polymer Materials* 2026. DOI: 10.1021/acsapm.6c00761.

3.1 Introduction

Quantitative characterization of molecular transport is fundamental to understanding and engineering hydrogel behavior.^{22, 58} Diffusion coefficients and partition coefficients govern the movement and equilibrium distribution of molecules within hydrogel networks and are therefore critical parameters for predicting nutrient availability, molecular retention, controlled release, and overall transport behavior within engineered living hydrogel material applications.^{85, 86} The effective diffusion coefficient describes the rate at which a solute moves through the hydrogel, whereas the partition coefficient describes its equilibrium distribution between the hydrogel and surrounding solution. Because these parameters describe different aspects of transport, one generally cannot be inferred from the other.⁸⁷ Hydrogel network architecture, particularly mesh size, is one of the most important structural characteristics because it strongly influences molecular transport, permeability, and many other physical properties of hydrogels.

Solute diffusion through hydrogels is controlled by several mechanisms, and their relative importance depends strongly on the size of the transported molecule compared to the hydrogel network. For molecules that are much smaller than the hydrogel mesh size, transport is primarily influenced by obstruction from polymer chains and chemical interactions with the polymer network. As the solute size approaches the mesh size, steric obstruction becomes increasingly important, and diffusion becomes strongly restricted by the surrounding polymer chains.^{58, 88} This size dependence has been demonstrated experimentally for protein transport through PEG hydrogels. Rehmann et al. showed that proteins substantially smaller than the hydrogel mesh size were released rapidly, whereas proteins approaching the mesh size exhibited slower transport and larger proteins remained entrapped within the network.⁵⁸ While these results demonstrate that mesh size strongly influences molecular retention, average mesh size does not represent a uniform

pore diameter or a sharp molecular cutoff.⁸⁹ Local heterogeneity in network structure or network disjunctions can allow some solutes to move through openings larger than the average mesh size.^{87,}

90

Several models have been developed to describe these different transport mechanisms. Hydrodynamic models describe reduced diffusion resulting from friction between the solute and the polymer-containing solvent, while obstruction models describe polymer chains as physical barriers that limit available transport pathways. Free-volume models instead describe diffusion as movement through temporary openings within the polymer network.⁸⁸ Axpe et al. combined these models to better describe solute diffusion in hydrogels. Using dextran transport through PEG hydrogels with different mesh sizes, they showed that diffusion is controlled by the combined effects of solute size, polymer volume fraction, and hydrogel network structure rather than by mesh size alone.⁵³ Because molecular transport is highly dependent on both hydrogel network architecture and solute properties, diffusion coefficients and partition coefficients are inherently system-specific. Variations in polymer chemistry, crosslink density, mesh size, polymer volume fraction, degree of swelling, solute size, and polymer–solute interactions can all significantly influence transport behavior.⁵³ Although PEG is often treated as relatively inert, solute-specific interactions with the polymer network can measurably alter diffusion and partitioning, particularly for proteins and charged molecules.⁵⁶ Consequently, transport parameters are often determined experimentally for individual hydrogel formulations and solutes rather than applied broadly across different material systems.

This chapter presents experimental methods for quantifying effective diffusion coefficients of small molecules within hydrogels (D_{eff}), partition coefficients (K_d), and hydrogel mesh size (ξ) in PEG-based hydrogel networks. These methods were initially developed to

characterize ammonium transport in PEG hydrogels,⁸¹ but are readily adaptable to a wide range of transported solutes and hydrogel systems and are applied throughout this dissertation. In addition, methods for determining hydrogel mesh size using equilibrium swelling theory are presented across a range of PEG macromer molecular weights. Together, measurements of diffusion coefficients, partition coefficients, and mesh size provide complementary quantitative descriptors of hydrogel transport behavior and network architecture that can be readily adapted to a wide variety of PEG-based biomaterial systems and can inform mass transport models to describe spatiotemporal chemical distributions throughout these materials.

3.2 Materials and Methods

3.2.1 Diffusion coefficient measurements

Diffusion coefficients were measured using cylindrical hydrogels formed at the bottom of glass cylinders, with release occurring into the solution above the hydrogel (Fig. 3.1). The impermeable glass walls and base prevented transport through the sides and bottom of the hydrogel, restricting diffusion to the exposed upper surface. Hydrogel volume and dimensions varied depending on the analyte and experimental application, but the same one-dimensional release configuration was used throughout.

Samples were collected from the surrounding solution at predetermined time intervals. Depending on the analytical method, the sampled volume was either replaced with an equal volume of fresh release medium, or a small sample volume was removed with the assumption that the total release-medium volume was considered unchanged. The concentration of the released analyte was quantified using a method selected based on its chemical and optical properties. For example, ammonium can be measured using ion chromatography or a colorimetric assay, hydrogen peroxide can be quantified using absorbance-based methods or commercially available peroxide

assays, and fluorescent molecules can be measured using absorbance or fluorescence spectroscopy. The same approach can also be extended to biomolecule release, including proteins, peptides, and nucleic acids, provided that an appropriate analytical method is available to determine the concentration of the released species.^{58, 91, 92}

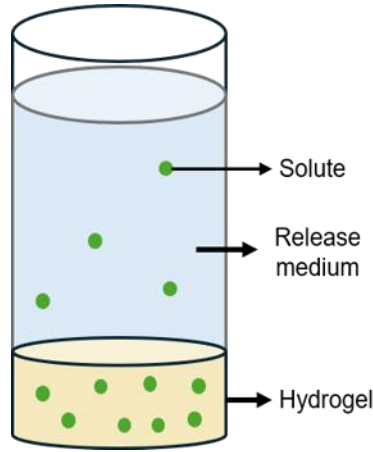


Figure 3.1. Experimental setup used for diffusion coefficient measurements

The experimental data were fit to the solution of Fick's second law⁹³:

$$\frac{C_t}{C_\infty} = 1 - \frac{8}{\pi^2} \sum_{n=0}^{\infty} \frac{\exp \left[-\frac{D(2n+1)^2 \pi^2 t}{L^2} \right]}{(2n+1)^2} \quad (1)$$

where C_t and C_∞ are the cumulative mass concentrations of the released molecule at time t and infinite time, respectively; L denotes the height of the cylindrical gel, and D is the diffusion coefficient of the molecule within the hydrogel. When $C_t/C_\infty \leq 0.6$, the early-time approximation can be used to calculate diffusivities according to⁹³:

$$\frac{C_t}{C_\infty} = 4 \left(\frac{Dt}{\pi L^2} \right)^{0.5} \quad (2)$$

3.2.2 Determination of diffusivities using microfluidic devices

Diffusivities can also be determined using microfluidic devices by monitoring the development of concentration profiles within a defined channel geometry. In the approach developed in this dissertation, a photodegradable PEG hydrogel was formed within a microchannel connecting source and sink wells. Alexa Fluor 594 was added to the source well, while phosphate-buffered saline (PBS) was added to the sink well, and fluorescence images were collected over time to monitor diffusion through the hydrogel and formation of the concentration gradient across the channel. Fluorescence intensity and position were normalized to the maximum intensity and total channel length, respectively.⁸² The transient fluorescence intensity at fixed positions along the channel was used to determine the effective diffusion coefficient. Similar to the release method described in Section 3.2.1, an early-time approximation was derived from the solution to Fick's second law and applied when $I_x(t)/I_{max} \leq 0.6$:

$$\frac{I_x(t)}{I_{max}} = 4 \left(\frac{D_{eff} t}{\pi x^2} \right)^{0.5}$$

where $I_x(t)$ is the fluorescence intensity measured at position x and time t , I_{max} is the maximum fluorescence intensity corresponding to the source concentration, and D_{eff} is the effective diffusion coefficient through the hydrogel. The transient intensity profiles measured at selected positions within the channel were fit to this expression to calculate D_{eff} .

Similar microfluidic approaches have also been used to determine diffusion coefficients. In hydrogels, diffusivity has been measured by monitoring changes in absorbance or fluorescence at a fluid-hydrogel interface and fitting the resulting concentration profiles to one-dimensional solutions of Fick's second law.^{94, 95} Diffusion coefficients in liquid systems have also been determined using laminar co-flow devices, where solute and buffer streams flow beside one another and diffusion is quantified from changes in the transverse concentration profile.⁹⁶

Although the specific device geometry and transport model depend on the application, these approaches provide spatially resolved measurements using small sample volumes and controlled boundary conditions.

3.2.3 Partition coefficient measurements

Partition coefficients were determined by equilibrating swollen hydrogels with a solution containing the analyte of interest. Hydrogels were first swollen in ultrapure water or an appropriate buffer for 24 h. The swelling solution was then removed and replaced with a known volume and concentration of analyte solution, and the system was allowed to equilibrate for 120 h. The initial and equilibrium analyte concentrations in the surrounding solution were measured using an analytical method appropriate for the analyte.

The hydrogel-solution partition coefficient, K_d , was calculated according to:

$$K_d = \frac{(C_0 - C_e)}{C_e} \cdot \frac{V_{sol}}{V_{hyd}} \quad (3)$$

where C_0 and C_e are the initial and equilibrium analyte concentrations in the surrounding solution, respectively, V_{sol} is the volume of the contacting solution, and V_{hyd} is the volume of the swollen hydrogel. This calculation assumes that the decrease in analyte concentration in the surrounding solution results from uptake into the hydrogel and that equilibrium is reached during the incubation period. For ammonium ion – nitrogen content ($\text{NH}_4^+\text{-N}$) measurements, the initial and equilibrium $\text{NH}_4^+\text{-N}$ concentrations were quantified using Hach TNTplus colorimetric test kits. Similar to diffusion coefficient measurements, other analytes can be quantified using an appropriate analytical method based on their chemical or optical properties. Partition coefficient measurements are performed in triplicate.

3.2.4 Hydrogel mesh size measurements

Hydrogel mesh size was estimated using equilibrium swelling theory according to the methods developed by Peppas and Merrill and later extended by Canal and Peppas.⁹⁷ This approach relates equilibrium swelling behavior to the molecular weight between crosslinks and ultimately to the characteristic mesh size of the hydrogel network. The mass swelling ratio was found by the ratio between the mass of a swollen hydrogel (M_s , $n > 3$) and the mass of the dry polymer (M_d , $n > 3$) shown as:

$$Q_m = \frac{M_s}{M_d} \quad (4)$$

The equilibrium volume swelling ratio was found by using the mass swelling ratio and the density of the polymer (ρ_{PEG} , 1.125 g/cm³) and the density of the solvent (ρ_s , 1.000 g/cm³ for water) and is shown as:

$$Q_V = 1 + \frac{\rho_{PEG}}{\rho_s} (Q_m - 1) \quad (5)$$

The molecular weight between crosslinks (M_C) was found using equation (6):

$$\frac{1}{M_C} = \frac{2}{M_n} - \frac{\bar{v}}{V_1} * \frac{\ln(1-v_2) + v_2 + \chi v_2^2}{v_2^{1/3} - \frac{v_2}{2}} \quad (6)$$

Where M_n is the average molecular weight of the un-crosslinked gel (molecular weight of 2-arm PEGDA plus 2 * (1/4) of the 4-arm Poly(ethylene glycol) tetra-thiol (PEGTT) molecular weight). For example, for PEGDA_{2k} – PEGTT_{5k} the M_n would be 2,000 + 2 * (1,250) = 4,500. $\bar{v}$ is the specific volume of the polymer ($\frac{\rho_{PEG}}{\rho_s}$, 0.8889), v_2 is the swollen polymer volume fraction (equal to the inverse of the volume swelling ratio), V_1 is the molar volume of the solvent (18.0 cm³/mol for water), and χ is the solvent-polymer interaction parameter; a constant value of 0.426 for water-PEG when the polymer volume fraction is in the range of 0.04 < v_2 < 0.2.^{22, 48}

The root-mean-square end-to-end distance of the polymer chain in the unperturbed state ($\bar{r}_0^2$) was given by equation (7):

$$(\bar{r}_0^2)^{1/2} = lC_n^{1/2}n^{1/2} \quad (7)$$

Where l is the average bond length (0.146 nm),⁴⁸ C_n is the characteristic ratio of the polymer (typically 4.0 for PEG), M_r is the molecular weight of the repeat unit (44 for PEG),^{22, 48} and n is the number of bonds in the crosslink, given by equation (8):

$$n = 2 \frac{\bar{M}_c}{M_r} \quad (8)$$

The mesh size for the hydrogel network is calculated using the equation where ξ is the mesh size (nm):⁹⁷

$$\xi = v_2^{-1/3}(\bar{r}_0^2)^{1/2} \quad (9)$$

3.3 Results and Discussion

The methods described in this chapter were applied across multiple PEG hydrogel systems to quantify solute diffusion, partitioning, and the influence of hydrogel structure on molecular transport. Initial measurements focused on $\text{NH}_4^+\text{-N}$ transport through PEG hydrogels (Fig. 3.2A). A partition coefficient, K_d , of 0.94 ± 0.08 was measured, indicating that $\text{NH}_4^+\text{-N}$ was only slightly favored in the bulk solution. Analysis of the early-time release profile yielded (Fig. 3.2B) an effective diffusion coefficient of $1.88 \times 10^{-6} \text{ cm}^2/\text{s}$, corresponding to an approximately 90% reduction relative to diffusion in water at the same temperature ($1.81 \times 10^{-5} \text{ cm}^2/\text{s}$)⁹⁸. To our knowledge, these values represent the first reported diffusion and partition coefficients for $\text{NH}_4^+\text{-N}$ in PEG hydrogels. The results are published in Fattahi et al.⁸¹

The same release-based approach was subsequently used to determine effective diffusion coefficients for other small molecules, including hydrogen peroxide and Alexa Fluor 488, demonstrating that the method can be adapted to solutes with different chemical and optical properties. Diffusivity was also measured using a microfluidic device by monitoring the development of an Alexa Fluor 594 concentration gradient through a PEG hydrogel. The transient

fluorescence data were fit to the early-time approximation of Fick's second law and used to calculate an effective diffusion coefficient of $2.7 \times 10^{-7} \pm 1.5 \times 10^{-7} \text{ cm}^2/\text{s}$, representing an approximately 88% reduction relative to diffusion in water.⁸² Together, these results demonstrate the use of complementary bulk-release and microfluidic approaches to quantify transport through PEG hydrogel networks. The resulting concentration profiles were also used to evaluate gradient formation across the channel and support subsequent modeling of glucose and tryptone transport.⁸²

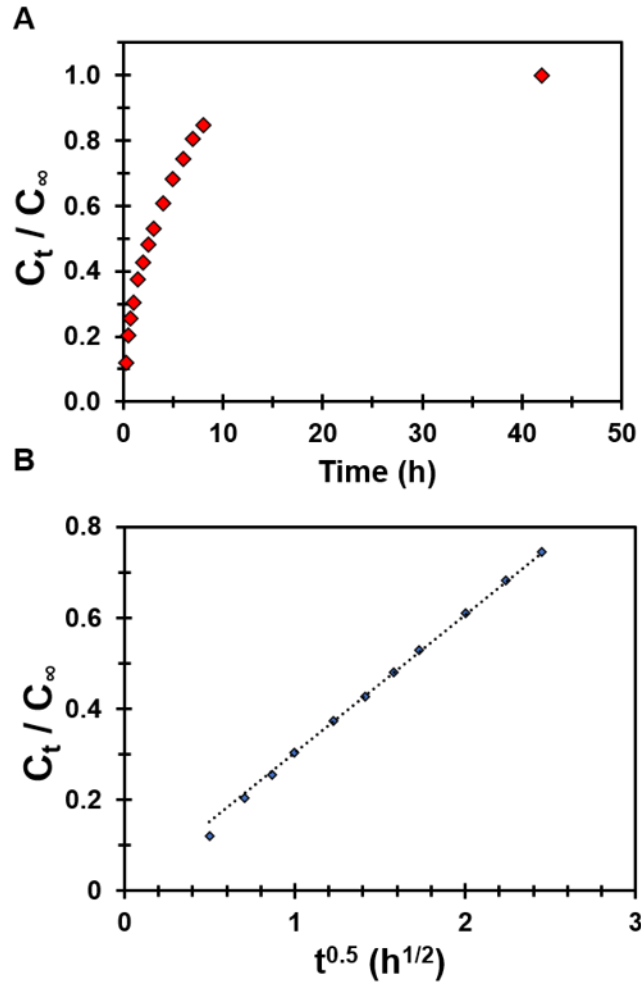


Figure 3.2. (A) Release kinetics of $\text{NH}_4^+\text{-N}$ from PEG hydrogels (B) Early time ($C_t/C_\infty \leq 0.6$) release profile used to determine DN, PEG using Equation (2). From Fattahi et al.⁸¹

For mesh size calculations, here the effect of varied molecular weight of the 2-arm PEG diacrylate crosslinker on the hydrogel mesh size (ξ , nm) was investigated using the equilibrium swelling theory described by Canal and Peppas.⁹⁷ All hydrogels used the same 10 kDa 4-arm PEGTT crosslinker with PEGDA molecular weights (2, 3.4, 5, and 10kDa). Mesh sizes were calculated to be 10.6 ± 0.3 , 11.7 ± 0.3 , 13.6 ± 0.3 , 14.7 ± 0.8 (Fig. 3.3). These values are consistent with previous reports of hydrogels with similar chemistries/molecular weights reported in literature.⁵⁸ These results demonstrate that equilibrium swelling theory provides a reliable method for estimating mesh size across a range of step growth PEG hydrogels formed by Michael type addition reactions. Because mesh size strongly influences molecular transport and permeability, this approach provides a useful tool for characterizing and comparing PEG hydrogel networks.

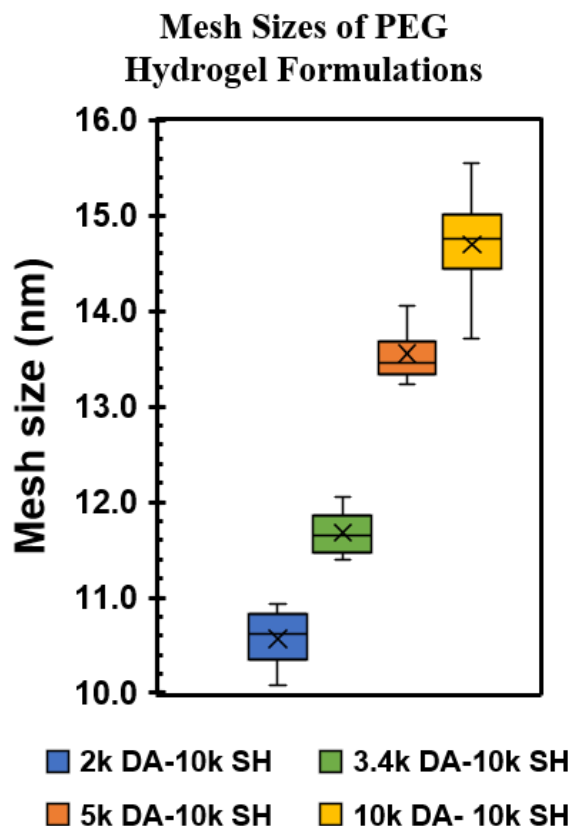


Figure 3.3. Mesh size calculation for 4-arm 10k SH and 2-arm 2k (blue), 3.4k (green), 5k (orange), and 10k (yellow) DA. Adapted from Reed et al.⁸⁴

3.4 Conclusions

The methods presented in this chapter enable experimental quantification of small-molecule diffusion coefficients, partition coefficients, and hydrogel mesh size in PEG-based hydrogel networks. Although initially demonstrated using $\text{NH}_4^+\text{-N}$, the diffusion and partitioning approaches are broadly applicable to a wide range of biologically relevant solutes, including nutrients, metabolites, signaling molecules, reactive species, and fluorescent probes. In addition, the equilibrium swelling approach provides a reliable method for estimating hydrogel mesh size across a range of PEG formulations. Together, these quantitative measurements provide complementary descriptors of hydrogel transport behavior and network architecture, enabling

systematic characterization of PEG hydrogels and supporting the rational design of engineered living hydrogel systems.

3.5 Acknowledgements

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Chapter 4 - Photodegradable Hydrogel Matrices for Spatiotemporal Control of Bacteria Transport and Delivery

4.1 Overview

Stimuli-responsive hydrogels that provide controlled degradation can be used as bacteria delivery systems for advanced therapeutic applications. Here, we report the first use of photodegradable hydrogels as materials that can direct bacterial movement, tune mean bacteria speed, and control bacteria delivery through spatiotemporal control of degradation. Hydrogels were formed using base-catalyzed Michael addition reactions between photodegradable polyethylene glycol (PEG) *o*-nitrobenzyl diacrylate macromers and PEG tetra-thiol crosslinkers within microfluidic channels. Nutrient gradients were generated across the channel, and micron-scale regions of the hydrogel were partially degraded by exposure to controlled doses (2.1-168 mJ/mm²) of patterned 365 nm light. Hydrogel degradation was then characterized *in situ* using fluorescence visualization of fluorescein-labeled hydrogels. Following characterization, *Bacillus subtilis* expressing green fluorescent protein was introduced into the device, and its movement up the nutrient gradient was monitored using time-lapse fluorescence microscopy to enable a systematic study of bacteria chemotaxis through the hydrogels at varied levels of degradation. *B. subtilis* showed minimal adhesion to partially degraded PEG hydrogels, and bacteria mean speed and mean directional change were tunable according to the level of hydrogel photodegradation, with a 2.6-fold difference in mean cell speed measured across the partially degraded hydrogel regions. Finally, the ability to alter bacteria speed and directionality through tunable degradation and without significant adhesion was used to achieve controlled release profiles of bacteria to delivery sites. These findings advance the use of PEG-based hydrogel materials as delivery

vehicles for bacterial therapeutic applications and other living material applications that require controlled bacteria transport.

KEYWORDS: hydrogels, biotherapeutics, living materials, photodegradation, chemotaxis, bacteria, polyethylene glycol

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4.2 Introduction

Bacteria can be used to treat human diseases, including cancer, gastrointestinal diseases, and metabolic disorders. For example, anti-cancer bacteria can destroy malignant tissues through exotoxin secretion, nutrient consumption, or immune cell activation, and can be engineered to carry therapeutic payloads and release them within the tumor environment.^{99,100} Further, bacteria can be engineered to sense and respond to biochemical cues from diseased cells, enabling chemotaxis to the disease site.¹⁰¹ However, a critical limitation is achieving targeted delivery of viable bacteria cells in a controlled manner to ensure efficacy while avoiding an immune response or pathogenic side effects.¹⁰² Hydrogel encapsulation for delivery is a promising solution, as these materials can provide a protective environment for bacteria and have the potential for controlled and localized release. Bacteria-encapsulated hydrogels have been demonstrated to improve efficacy and safety, particularly for probiotic treatments of gastrointestinal disorders.^{100, 102, 103} While the majority of hydrogels used to encapsulate bacteria consist of naturally occurring polysaccharide-based materials (alginate, chitosan, starch, etc.), encapsulation in synthetic hydrogels offers significant advantages. This includes design for response to environmental stimuli such as temperature, pH, and ionic strength for triggered and controlled release of cargo into a targeted environment.¹⁰⁴ While synthetic, stimuli-responsive hydrogels have found extensive use in biomolecular drug delivery,^{105, 106} they are currently underutilized in bacteria therapeutic applications.

Polyethylene glycol (PEG)-based hydrogels hold unique advantages for bacteria encapsulation and delivery, as PEG is chemically and biologically inert and non-adhesive to bacteria,^{107, 108} covalently crosslinked to provide stability, and can be chemically modified to

generate hydrogels that change physicochemical properties in response to a variety of stimuli. Further, PEG is one of the most versatile and adaptable biomaterials, with commercially available macromers available across a wide range of molecular weights for precise control of hydrogel pore sizes and mechanical properties. PEG is also available with a variety of end-group chemistries for selection of the polymerization chemistry used for encapsulation. Chain-growth polymerizations are commonly used for PEG hydrogel generation, but have drawbacks when used for cell encapsulation that include the generation of free radicals and hydrogel network heterogeneities.^{109,}
¹¹⁰ Step-growth polymerizations that use click chemistry avoid many of these limitations, as they can provide near-complete conversion of crosslinker molecules, do not require external initiators in some cases, do not generate reaction byproducts or free radicals,¹¹¹ and form hydrogels with less network heterogeneity at all length scales for higher control of mass transport.^{29, 40, 58, 112, 113} Given these advantages, we have recently used thiol-Michael addition reactions to encapsulate bacteria in PEG, demonstrating that these chemistries offer high cell viability and provide a stable matrix for 3D bacteria culture and control of mass transfer, making them useful as protective coatings against environmental toxins.^{81, 114}

Light holds unique advantages as a stimulus for bacteria-hydrogel systems, as it allows for spatiotemporal manipulation of bacteria cells. For example, Matsumoto *et al.* designed photoresponsive glycolipids that undergo reversible gel-sol/sol-gel transitions with light exposure to spatially localize bacteria.¹¹⁵ In recent years, we have added the photocleavable moiety *o*-nitrobenzyl (*o*-NB) to PEG hydrogels to yield photodegradable hydrogels that allow for spatial degradation with patterned light for selection and isolation of bacteria from heterogeneous cell mixtures,¹¹⁶⁻¹¹⁸ a key capability in the development of microwell arrays that screen and select bacteria with unique function from diverse microbial communities.¹¹⁹⁻¹²¹ While 365 nm light was

used here for degradation, this can be transitioned to near-infrared light with addition of up-conversion nanoparticles to these hydrogels to improve cytocompatibility and enable *in vivo* delivery applications.¹²²

Motivated by the need to develop materials that can control the release profile of bacteria for therapeutic efficacy,¹⁰² this study provides a systematic investigation of bacteria transport through PEG-based hydrogel materials at different stages of degradation in effort to control bacteria movement and release. Photodegradable thiol-acrylate polymerized PEG hydrogels are deposited within a microfluidic device and subjected to controlled levels of degradation using varied light doses. A nutrient gradient is then generated across the hydrogels to promote bacterial chemotaxis through the hydrogel. The uniform network structure of these hydrogels,²⁹ combined with spatiotemporal control of degradation and microfluidic control of the chemical environment, enables a highly systematic experimental approach. *Bacillus subtilis* was chosen as the focal species due to its unique potential in bacterial therapeutic applications.¹²³ This includes its designation as a generally recognized as safe (GRAS) bacterium, its ability to sporulate for long-term stability, its ease of genetic modification for drug delivery, and its natural production of bioactive secondary metabolites such as surfactin, with known antimicrobial and anticancer properties.¹²⁴ The results reveal that tunable degradation of PEG-based hydrogels can be used to control the speed, directionality, and release profile of bacteria, findings directly applicable to therapeutic bacteria delivery. Beyond biotherapeutics, this study broadly informs other engineered living material applications that rely on controlled microbial transport in hydrogels,¹²⁵ as well as antifouling approaches that employ PEG-based hydrogels with the pre-requisite of inhibiting bacterial infiltration,^{126, 127} and bioinoculant applications,¹²⁸ where hydrogel-microfluidic systems

can serve as well-controlled porous-media analogs to understand microbial transport in soils.¹²⁹⁻

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4.3 Results and Discussion

4.3.1. Concept and Device Characterization.

The strategy used to probe bacteria taxis through the partially degraded PEG hydrogels involved the deposition and patterned degradation of the hydrogel within a microfluidic channel (Fig. 4.1). Microfluidic devices were designed to contain a single channel (width = 250 μm , height = 8.3 μm , length = 3,000 μm) between a nutrient-rich media source and a nutrient-deficient sink well. The hydrogel was deposited by mixing the PEG tetrathiol (M_w 5,000 Da) and PEG-*o*-nitrobenzyl diacrylate (PEG-*o*-NB-DA, M_w 3,400 Da) macromers together in basic buffer (pH 8.0), then quickly loading this solution within the microchannel for formation of photodegradable PEG hydrogels (herein referred to as *pd*-PEG hydrogels), driven by thiol-acrylate addition crosslinking reactions. This precursor solution was developed and characterized in prior studies.^{117,}
¹³² It generates an average mesh size of 10 nm when fully swollen and has a gelation time of ~25 min, providing adequate time to load the liquid precursor solution within the microchannel. After hydrogel formation, addition of nutrient-rich media into the “source” well and a nutrient-deficient solution into the “sink” well on either side of the channel generated a chemical driving force for passive nutrient diffusion, forming a nutrient gradient across the hydrogel. Subsequent exposure with user-defined, micron-scale light patterns enabled spatially-controlled degradation of the hydrogel within the desired region of interest (ROI) at the end of the channel. Inoculation of a dilute suspension of *B. subtilis*-GFP into the nutrient-deficient sink positions the cells in an environment that promotes chemotaxis across the hydrogel barrier.

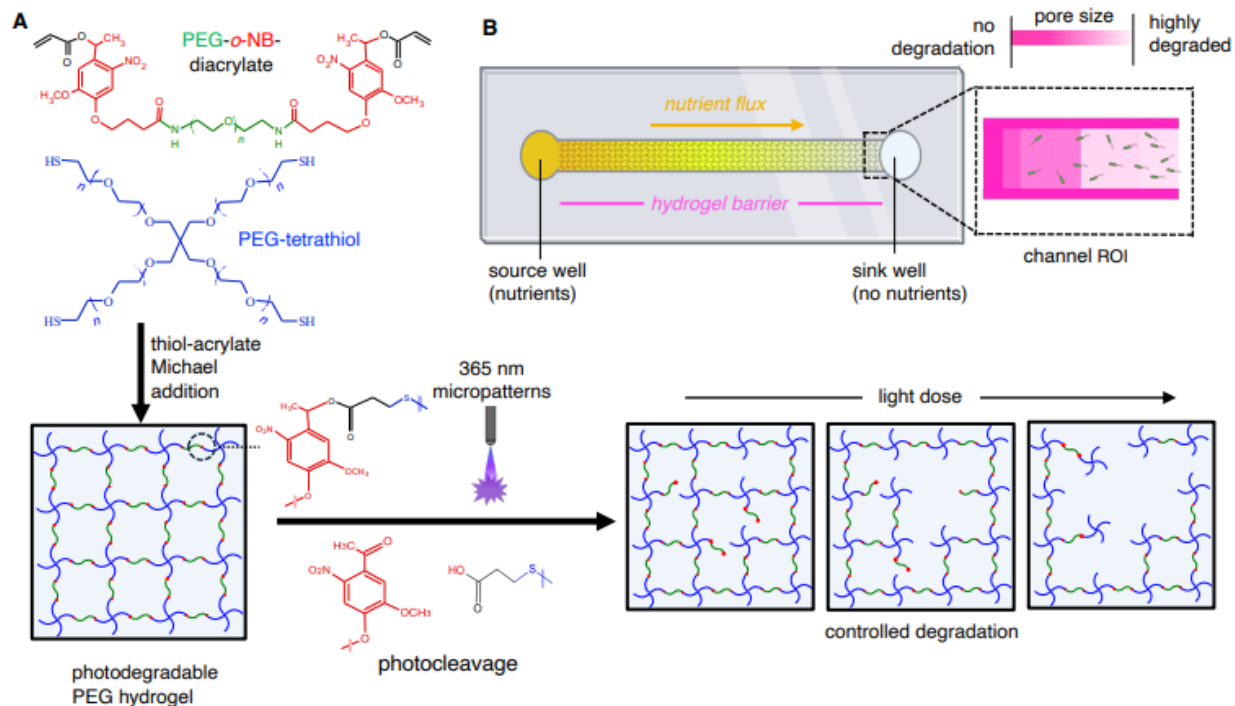


Figure 4.1. (A) Formation of pd-PEG hydrogel using a base-catalyzed Michael-addition reaction between PEG-o-NB-diacrylate and PEG-tetrathiol and photocleaving mechanism using micropatterned 365 nm light. (B) Microfluidic device overview, including chemical gradient generation across a hydrogel barrier and the channel region of interest, where all photodegradation and chemotaxis experiments were performed. Fig. B was completed in Biorender software.

To visualize chemical gradient formation within the microchannel, an Alexa Fluor dye was added to the source well to mimic nutrient diffusion across the hydrogel. 20x fluorescent image montages of the channel from 1 to 48 h show the evolution of the chemical gradient (Fig. 4.2A and 4.2B). It is important to note that camera settings were chosen to quantitatively track chemical gradient formation within the ROI at the end of the channel; thus, the fluorescent signal from the left side of the channel in Fig. 4.2A becomes saturated at later time points when fluorophore concentrations become high. The transient chemical gradient enabled quantification of the effective diffusion coefficient (D_{eff}) through the hydrogel using the early time approximation to Fick's Second Law (Fig. 4.2C). This was calculated to be $D_{eff} = 2.7 \times 10^{-7} \pm 1.5 \times 10^{-7} \text{ cm}^2/\text{s}$, which is a 88% decrease relative to Alexa Fluor 594 diffusivity in water ($2.3 \times 10^{-6} \text{ cm}^2/\text{s}$),¹³³ indicating

hindered diffusion throughout the hydrogel. The decrease is comparable to drops in diffusivities measured for other small molecules in similar step-growth PEG hydrogels of comparable mesh sizes (ex. 87% reduction of acetate diffusivity⁸¹). This indicates that convective flows are minimal or absent across the channel, as convective mass transport in the positive x-direction would lead to an artificially high diffusion coefficient, while convective transport in the negative x-direction would inhibit chemical gradient formation. After 24 h, a linear concentration gradient was noted in the second half of the channel and remained stable through 48 h (Fig. 4.2B), indicating the device reached a pseudo-steady state during this period. To extend these findings to the diffusion of actual nutrients present in tryptic soy broth (TSB) media (glucose, tryptone) for chemotaxis experiments, a mass transfer model that assumes a similar 88% reduction in glucose and tryptone diffusivities was developed (described in Appendix B1). The model predicts steady-state nutrient concentration profiles after 24 h (Fig. B.1-B.3). For this reason, devices were incubated for 24 h prior to introduction of cells to enable formation of a steady nutrient gradient. Subsequent chemotaxis experiments occurred during the 24-48 h period in the channel ROI ($x/L = 0.8-1.0$, Fig. 4.2A and B).

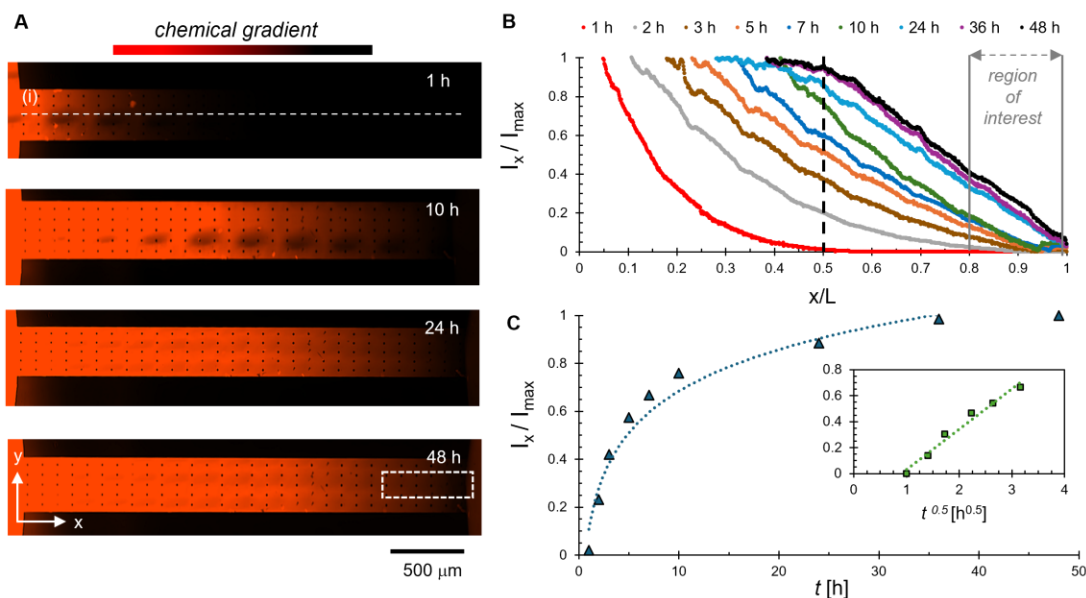


Figure 4.2. (A) 20X image montages of the microfluidic channel at 1, 10, 24, and 48 h after hydrogel formation and loading of Alexa Fluor 594 into source well. Note: Dark marks observed on 1 and 10 h are imaging artifacts. The white rectangle (48 h) corresponds to the region of interest used in all experiments. (B) Fluorescent intensity profiles of the microfluidic channel at 1, 2, 3, 5, 7, 10, 24, 36, and 48 h. Intensities and positions are normalized with respect to maximum intensity (I_{max}) and channel length ($L=3000$ mm), respectively and only quantitative signal data is displayed. (C) Transient fluorescence intensities taken at $x/L = 0.5$ (corresponding to dashed vertical black line in (B)) and (inset) early time approximation ($I_x / I_{\text{max}} < 0.6$) used to determine D_{eff} for Alexa Fluor 594 through the hydrogel.

Next, to confirm the attraction of *B. subtilis*-GFP cells to the nutrient source, devices that contained *pd*-PEG hydrogels were fabricated, and a small square ($140 \times 140 \mu\text{m}$) between the ROI and sink well was formed by exposing the hydrogel to a patterned square exposure with a 168 mJ/mm^2 dose, which was previously characterized to cause reverse gelation in the *pd*-PEG hydrogels.¹¹⁷ This provided an imaging region in the ROI for free swimming bacteria against a fully intact hydrogel barrier. A sharp hydrogel-liquid interface corresponding to the patterned region was observed in brightfield images after degradation (Fig. 4.3A). Next, a nutrient solution (tryptic soy broth (TSB) media) was added into the source well instead of Alexa 594. As a negative control, 1x PBS was loaded into the source well of a separate device. After 24 h, *B. subtilis*-GFP cells were introduced to the sink well and the ROI was monitored. Within 1 h, cells were found

congregating at the hydrogel-liquid interface when the nutrient gradient was present (Fig. 4.3B and C). When the nutrient gradient was absent (Fig. 4.3D and E), cells did not appear at the hydrogel-liquid interface and instead remained dispersed throughout the sink well. Comparing the number of cells present in the 140x140 μm imaging region with and without a nutrient gradient (Fig. 4.3F) confirmed that cellular attraction within the ROI was due to nutrient diffusion across the hydrogel, and not due to passive attachment and aggregation of *B. subtilis* cells at the hydrogel interface. Critically, cell movement was inhibited at the sharp interface between fully degraded and fully intact hydrogel (Fig. 4.3B, Supp. Mov. 1), indicating that bacteria were unable to penetrate the fully intact hydrogel despite the nutrient gradient. These results validated the device design, as both patterned hydrogel photodegradation within the device and a positive responsive from *B. subtilis* to the nutrient gradient generated across the hydrogel were achieved.

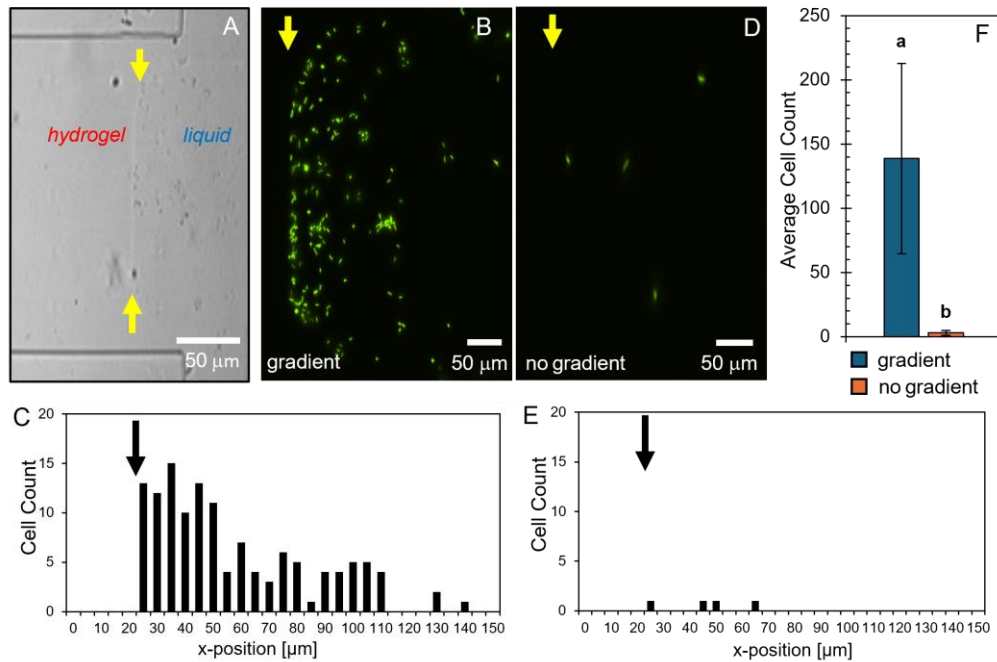


Figure 4.3. (A) Brightfield image of the ROI in the microfluidic device after bacteria loading, showing degraded/undegraded hydrogel. (B and C) Cell count and position of bacteria cells in the ROI region with a nutrient gradient, and (D and E) no nutrient gradient. (F) Average cell count of bacteria in the degraded hydrogel region with and without a chemical gradient (N=3). * = F-value 10.0 and p-value < 0.05.

4.3.2 Controlled Hydrogel Photodegradation within the Channel.

As the crosslinking density of *pd*-PEG hydrogels can be systematically decreased by tuning the dose of 365 nm irradiation, the next step was to use micropatterned light to partially degrade specific areas of the hydrogel within the channel ROI while avoiding reverse gelation. The channel ROI was first exposed to a square UV pattern to reverse gelation ($t_{exp} = 40$ s, dose = 168 mJ/mm²), forming a region for free-swimming bacteria within the channel next to the sink well. Subsequent sections of the hydrogel were then patterned with 110 x 101 μ m rectangles with decreasing exposure times of 10, 3, 1, 0.5 s at constant light intensity, corresponding to light doses of 42, 12.6, 4.2, and 2.1 mJ/mm², forming a degradation ladder (Fig. 4.4A, Figs. B.4A and B). The ladder pattern was designed to provide a comparative analysis of cell movement immediately outside the hydrogel (fully degraded region) with cell movement at specified levels of degradation. As preliminary experiments had shown that *B. subtilis* could not move through hydrogels after a 2.1 mJ/mm² dose, this was chosen as the lowest dose. Importantly, undegraded regions of hydrogel were left between the exposed region and the vertical microfluidic channel wall to enable hydrogel swelling in the y-direction.

It was necessary to characterize the regions of partially degraded hydrogels prior to chemotaxis studies. Because the hydrogel is spatially confined between the glass and the PDMS microfluidic channel, hydrogel swelling for pore size expansion after degradation is restricted. Therefore, *in-situ* characterization of each partially degraded hydrogel region within the device was necessary. With a limited number of experimental methods available for this characterization, a fluorescent staining method utilizing fluorescein-5-maleimide that we previously developed for visualizing PEGDA-PEGTT hydrogels was adapted.¹¹⁶ Covalent coupling of fluorescein-5-maleimide occurs through rapid, specific Michael addition between maleimide groups and pendant

thiol groups within the hydrogel in a manner proportional to PEG chain density, enabling a semi-quantitative analysis of degradation levels within the hydrogel ROI (Fig. 4.4B). After fluorescein-5-maleimide perfusion through the partially degraded hydrogels, fluorescence imaging revealed the degradation pattern (Fig. 4.4C). Some spatial variation in fluorescence intensity appeared within non-degraded portions of the hydrogel in the x-direction, indicating that there was some non-uniformity in the stain, however fluorescence intensity traces in the y-direction were uniform (Fig. B5). Thus, normalized intensity line plots in the y-direction were plotted and compared across each exposure region (Fig. 4.4C and D) and indicate that proportional decreases in PEG chain densities were found in partially degraded hydrogels with increasing UV exposure times, demonstrating patterned and controlled degradation within the channel ROI.

Imaging also revealed structural changes of the hydrogel within the channel. Degradation and lowered crosslinking densities cause an influx of water into the partially degraded hydrogel regions, resulting in swelling that is inversely proportional to crosslinking density.¹³⁴ Within the microfluidic channel, the hydrogel is confined between the glass surface at the device floor and the relatively rigid polydimethylsiloxane (PDMS) elastomer ($E_{PDMS} = 1.9 \times 10^6$ Pa),¹³⁵ constraining swelling in the z-direction. However, because a region of undegraded hydrogel was intentionally left between the patterned region and the channel wall (Fig. 4A), hydrogel swelling occurs in the y-direction to compress the unexposed regions of fully intact hydrogel, which is softer than the PDMS walls ($E_{Hydrogel} \sim 1.0 \times 10^4$ Pa).²⁹ This enabled quantification of a hydrogel swelling ratio, defined here as the width of the swollen hydrogel divided by the width of the exposed region (101 μ m). Consistent with fluorescent intensity plots, increased swelling ratios with higher dose were noted after exposure and equilibrium swelling for 4.2, 12.6, and 42 mJ/mm² (Fig. 4.4E), no differences were found at 2.1 mJ/mm². Taken together, these results indicate that doses between

4.2 and 42 mJ/mm² reduced the crosslinking density without causing reverse gelation, causing hydrogel swelling and increased pore sizes within the channel in a manner proportional to exposure time.

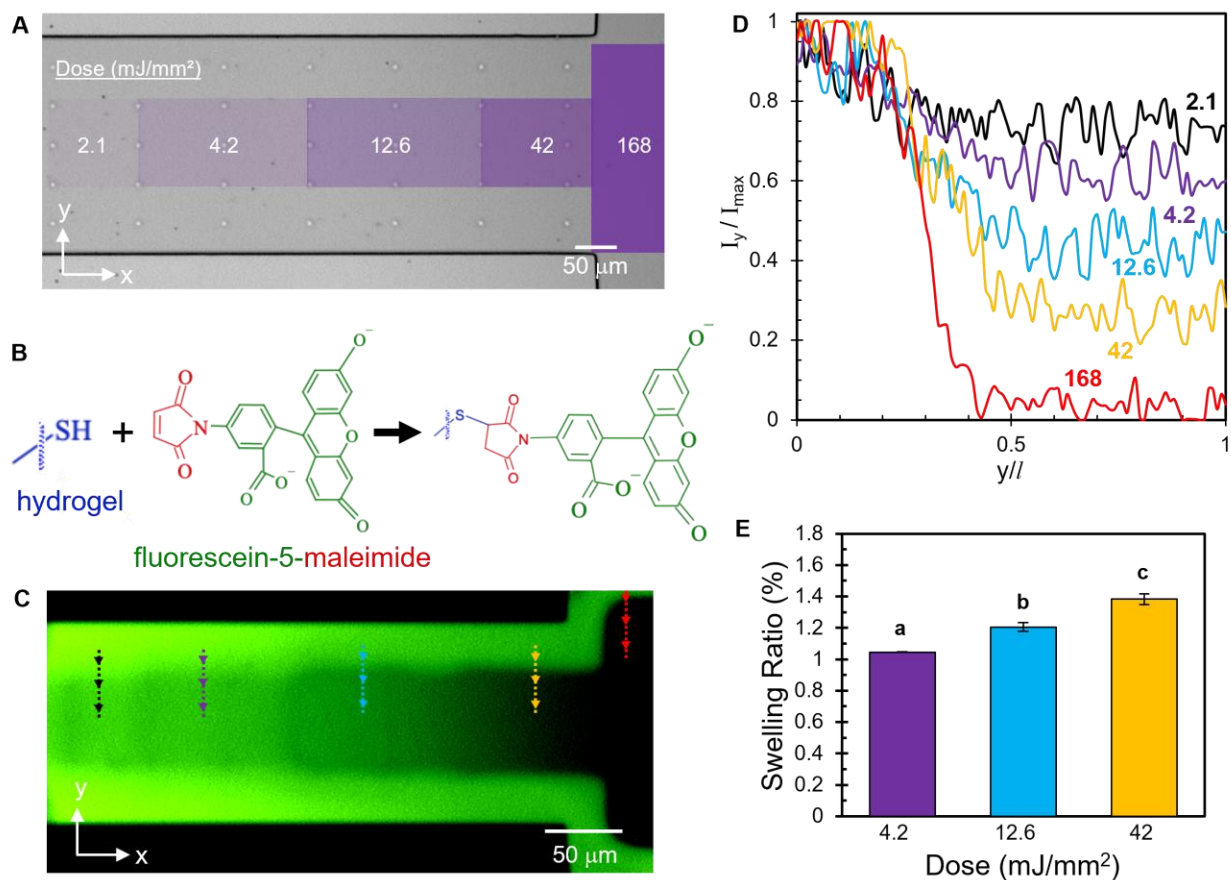


Figure 4.4. (A) Light exposure map for forming degradation ladder. (B) Chemical coupling of fluorescein-5-maleimide to pendant thiols on intact hydrogel. (C) Image of fluorescein stain after exposure according to map in (A). (D) Fluorescent intensity profiles through degraded regions of hydrogel at each specified dose (mJ/mm²). Data correspond to the vertical line scans of length l shown in C. Arrows in line scans denote the direction and start from undegraded hydrogel ($y/l = 0$) and move into a degraded hydrogel region. $I_{\max}$ was taken at the beginning of the line scan ($y/l = 0$). (E) Swelling ratio, taken as the ratio of measured width after swelling to the width of the light exposure pattern (101 µm) for each exposure region ($n = 3$), *p-value<0.01.

4.3.3 Chemotaxis of *B. subtilis* Through Partially Degraded Hydrogels.

To observe bacteria chemotaxis throughout the partially degraded hydrogel regions, *B. subtilis*-GFP cells were introduced into the sink well after nutrient gradient formation and

photodegradation. Bacteria cells moved throughout each rectangular region and up the nutrient gradient, with most cells accumulating at the cell impermeable interface located between the 2.1-4.2 mJ/mm² doses, as cells were unable to penetrate further into the 2.1 mJ/mm² exposed region (Fig. 4.5A, Supp. Mov. 2). Some cells also congregated at the interface of partially degraded and fully intact, impermeable hydrogel, suggesting that the partial degradation pattern also generated a nutrient gradient in the y-direction. Importantly, very few cells remained attached within the central regions of the partially degraded hydrogels, and there was no trend in cell immobilization with PEG density or degradation level across these central regions (Fig. 4.5B). While the few immobilized cells in these regions may also be in contact with the microfluidic device floor (glass) or ceiling (PDMS), this observation indicates that PEG chains do not cause significant bacterial adhesion, as expected. This demonstrates PEG as an ideal hydrogel material for facilitating bacteria transport while minimizing bacteria adhesion.

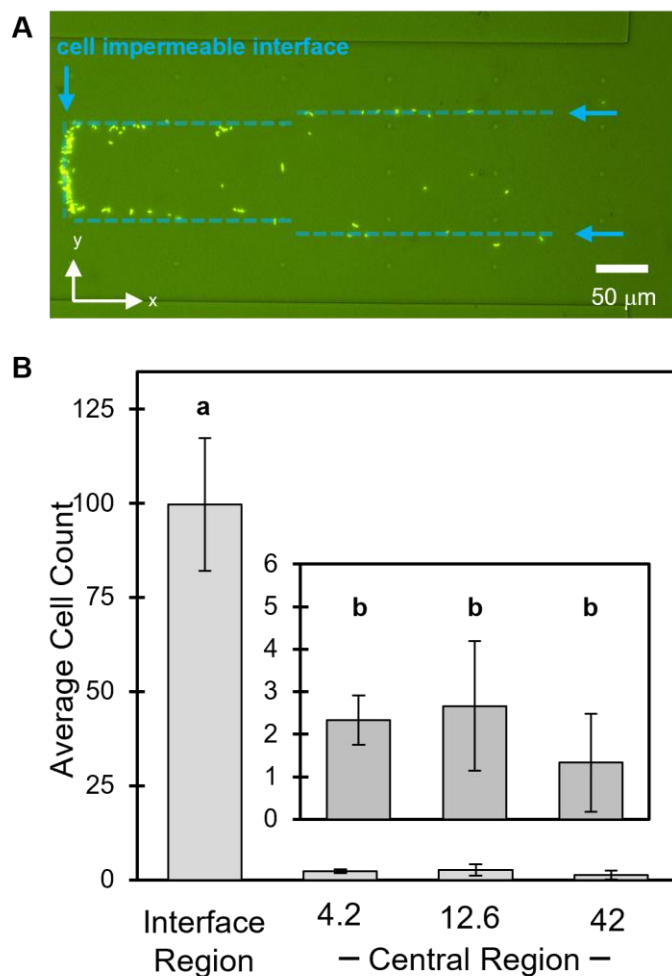


Figure 4.5. (A) Image of *B. subtilis*-GFP cells during chemotaxis through the hydrogel ladder. Blue arrows and dashed lines indicate the interface between cell-permeable and cell-impermeable hydrogels, where cell accumulation was observed. (B) Comparison of the number of cells immobilized at the cell impermeable interface regions with the number of cells immobilized within the central of the 4.2, 12.6, and 42 mJ/mm² dose zones ($n = 3$).

Cell movement in each region of degradation was analyzed using TrackMate software to generate individual two-dimensional cell trajectories and corresponding cell migration maps that show cell densities after 24 h (Fig. 4.6A-D). The trajectories allowed for the mean speed and mean directional change of bacteria populations moving through each region to be quantified in each region of degradation (Fig. 4.7). Without hydrogel confinement, free-swimming cells were observed within the 168 mJ/mm² light dose region, where hydrogel reverse gelation occurred to

generate a liquid phase. Here, *B. subtilis* cells display characteristic run-and-tumble motility patterns and have the highest mean speed and the highest directional change (Fig. 4.6A and Fig. 4.7). The cell migration map indicates net migration towards the nutrient gradient, with significant movement in the y-direction. In partially degraded hydrogels exposed to higher doses (42 and 12.6 mJ/mm² dose), cells experienced proportional decreases in mean speed, and also a significant drop in mean directional change as cells lost their run-and-tumble motility due to confinement within the hydrogel. Trajectories became smoother and more directed towards the nutrient gradient, causing cells to migrate further towards the nutrient source (Fig. 4.6B and 4.6C). Mean cell speed was again reduced proportionally when moving through the region of lowest degradation (4.2 mJ/mm² dose, Fig. 4.7A). Here, cell mean directional change increased significantly compared to the two higher doses that also generated partially degraded hydrogels (Fig. 4.7B), which indicates that cells had to find and follow more tortuous paths to move throughout this region. This caused less net migration of cell populations toward the nutrient gradient (Fig. 4.6D).

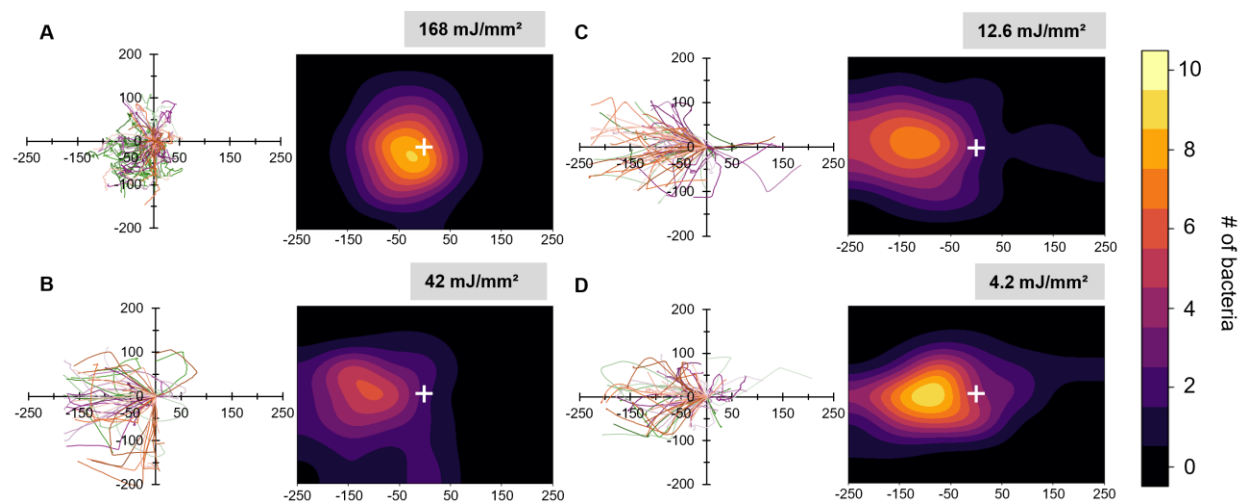


Figure 4.6. Individual two-dimensional cell trajectories tracks (left) and corresponding two-dimensional cell migration maps (right) for (A) 168 mJ/mm², (B) 42 mJ/mm², (C) 12.6 mJ/mm², and (D) 4.2 mJ/mm² exposure regions within the hydrogel. The white cross at the center of each map indicate the initial center of mass for each cell population.

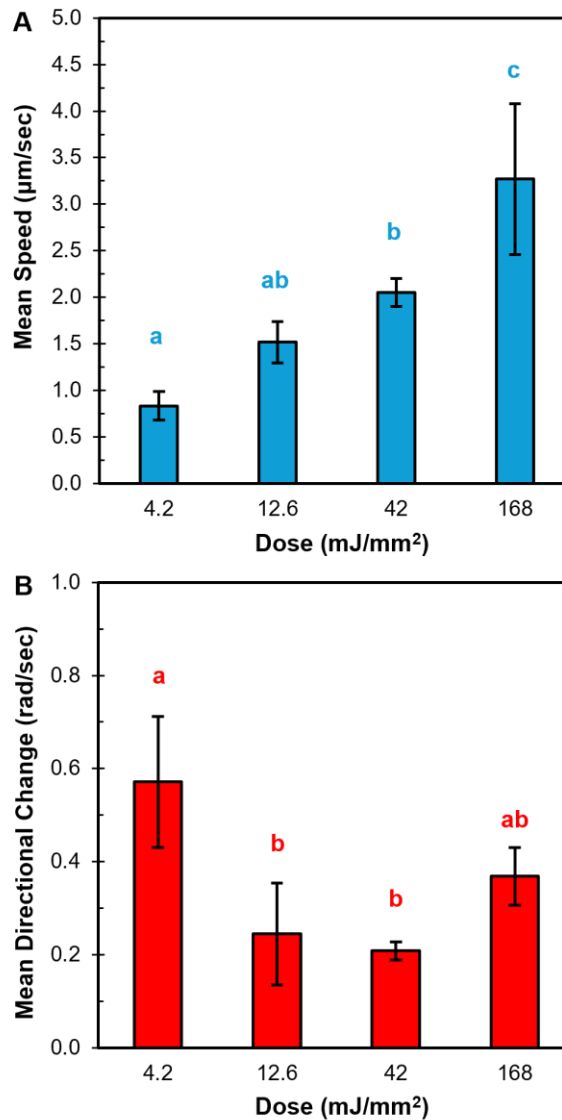


Figure 4.7. Mean speed and mean directional change calculated using TrackMate. An average of 73 ± 4 cells were measured for speed and mean directional change calculations for 4.2, 12.6, and 42 mJ/mm² doses and 261 cells were measured for 168 mJ/mm² dose. Doses of 4.2 to 42 mJ/mm² correspond to partially degraded gels, 168 mJ/mm² correspond to free swimming cells in a fully liquid region. Cells were evaluated over n=3 independent experiments.

Interestingly, during observation of cell movement in real-time, cells in regions of lower degradation could occasionally be found traveling in identical paths towards the chemical gradient at higher speeds (noted example on dashed white line in Supp. Mov. 2). While a qualitative observation only, this led to the hypothesis that cells could find and exploit “paths of least

resistance” within the partially degraded hydrogel to quickly advance toward the nutrient gradient. To further explore this hypothesis, an interface between a fully intact hydrogel and a fully degraded liquid region was again developed. Pathways of compromised hydrogel were then introduced at the liquid-hydrogel interface using three narrow line patterns (9 μm width, 208 μm length of different exposures times (21, 42, 63 mJ/mm^2), offering pathways of compromised hydrogels at varied levels of degradation. After introduction into the device, *B. subtilis* cells were able to find and penetrate through these compromised pathways (Fig. B.6, Supp. Mov. 3) to progress up the nutrient concentration gradient. Here, cell trajectories became increasingly linear with lower dose (Fig. B.6C-E), as cell motion was directed towards two-dimensional paths due to less degradation and swelling. These trends indicate that bacteria find and exploit “paths of least resistance” when penetrating through partially degraded hydrogels.

4.3.4 Selective Delivery of Live Bacteria with Partially Degraded Hydrogels.

A comparison of the averaged cell speeds at different light doses (Fig. 4.7) indicates that controlled degradation can be used to tune *B. subtilis* speed down to nearly 25% of its free-swimming speed. This suggests that this material could be used to control the release and accumulation of bacteria to a targeted location according to its degradation level. To demonstrate this, the degradation pattern within the microfluidic channel was modified to include a fully degraded, liquid region next to the sink well (168 mJ/mm^2 exposure), followed by a partially degraded region (220 μm length x 101 μm width) and finally, a fully degraded “delivery” region (110 μm length by 101 μm width) to collect the bacteria released from the partially degraded hydrogels (Fig. 4.8A, Fig B.4C). To verify that active, motile bacteria were required to reach the delivery region, non-active red fluorescent polystyrene beads of similar size (1 μm diameter) were mixed with *B. subtilis* at a 10:1 bead:cell ratio (Fig. B.7) in the sink well. Degradation levels within

the partially degraded region were tuned using the three different doses (42, 12.6, or 4.2 mJ/mm²), providing increasing resistance to molecular and cellular transport. After loading the bacteria-bead mixture into the well, the hydrogel was monitored (Supp. Mov. 4-6), and the cell and bead counts accumulating in the delivery region were quantified. Over 10 h, cells accumulated in the delivery region at higher numbers when more degradation was provided to the partially degraded region (Fig. 4.8B and C). Statistically significant differences in cell counts were measured between 4.2 and 42 mJ/mm² degradation at later time points ($p \leq 0.05$ at 10 h). The differences in the number of cells released into the delivery region reflect the differences in mean swimming speeds observed at the different exposure doses (Fig. 4.6E). Additionally, despite the 10-fold excess of beads in the sink well, no significant accumulation of beads were measured in the delivery region during the 10 h period (Fig. 4.8C, inset). This indicates that cellular motility, as opposed to passive diffusion or convective transport, was required for cells to reach the delivery region. Overall, the results demonstrate that tuning the hydrogel crosslinking density through controlled degradation can be exploited to control the release profile of motile bacteria, an important need for therapeutic bacteria delivery.¹⁰²

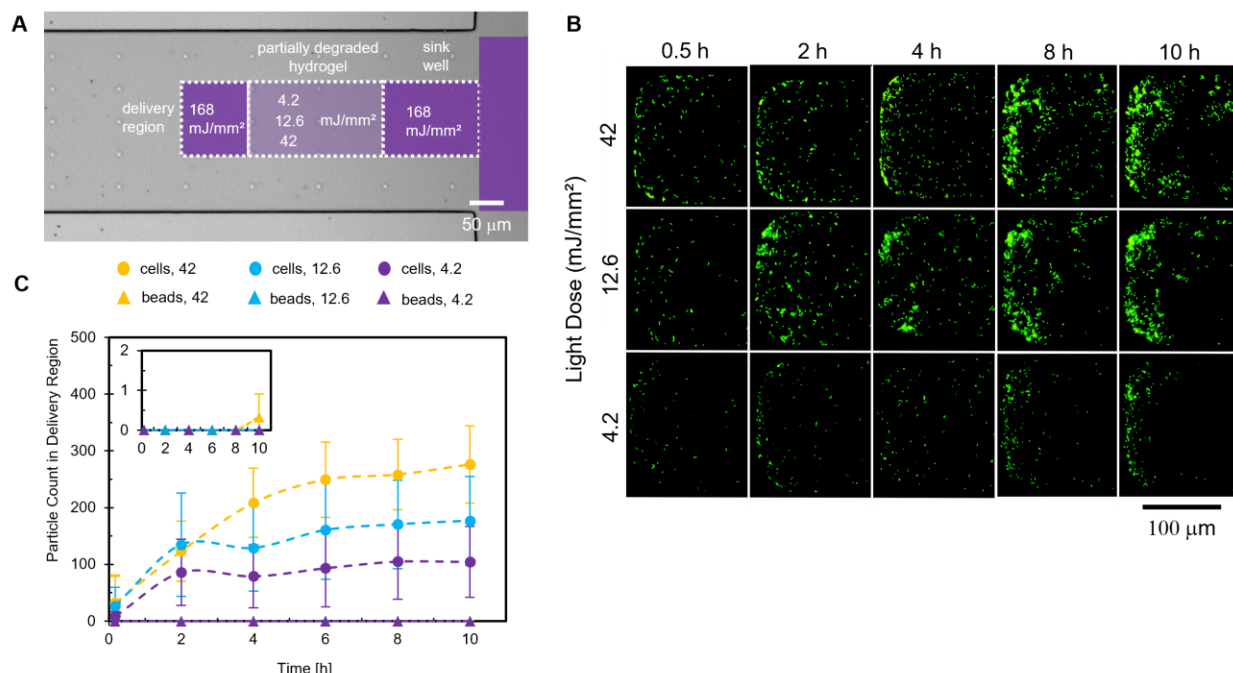


Figure 4.8. (A) Degradation map for selective, controlled release of *B. subtilis* into a fully degraded liquid “delivery” region. (B) Image montage of delivery region with time for each dose. (C) Cell and bead release profiles into the delivery region for 4.2, 12.6, and 42 mJ/mm² dose in the partially degraded region. Inset: Zoomed-in region showing bead accumulation within the liquid delivery region (n=3).

4.4 Conclusions

As PEG is cytocompatible but anti-adhesive to bacteria, PEG-based hydrogels that are designed to provide tunable degradation - whether through light or another stimulus (temperature, pH, enzymatic, mechanical, etc.) – are advantageous for controlled transport of live bacteria. In particular, the ability to guide cell motion, control mean cell speed, and modulate bacteria release profiles by tuning PEG degradation levels, as first demonstrated here, gives these hydrogels potential use as vehicles for controlled, localized *in vivo* delivery of therapeutic bacteria. While UV light is incompatible with bacteria and tissues, photonic nanoparticles can be added to these PEG-based hydrogels to cleave *o*-nitrobenzyl groups using cytocompatible, near-infrared light,¹²² which can provide local release of encapsulated cells within tissues and to a disease site, on-

demand. At a more fundamental level, spatial control of degradation at the microscale combined with a microfluidic device that enables the generation of well-controlled chemical gradients offered insight into the movement of bacteria cells through these materials at varied stages of degradation. Here, we found distinct transitions in cellular motility patterns according to the degradation state of the hydrogel and found that bacteria learn to exploit paths of least resistance within partially degraded hydrogels to advance towards more favorable environments. Beyond bacteria therapeutics, such an experimental system can be applied to probe cell-hydrogel interactions in other applications that require bacteria transport (e.g. porous media analogs, synthetic microbial ecosystems), that require inhibition of bacteria infiltration (e.g. tissue engineering scaffolds, antifouling coatings), or both (microbial separation and isolation).

4.5 Materials and Methods

4.5.1 Design and fabrication of microfluidic devices.

All microfluidic master devices were designed and fabricated at the Center for Nanophase Materials Sciences at Oak Ridge National Laboratory. The microfluidic devices used in all studies were designed in Layout Editor. Devices consisted of two 4-mm circles (source and sink wells) connected by a 250 x 3,000 μm rectangle channel (Fig. B.4A). 10 μm circular support pillars were placed 100 (x-direction) x 45.5 (y-direction) inside the plug region to prevent sagging of the PDMS during soft lithography. Sets of microfluidic masters were created using two processes. Silicon masters were produced using a combination of photolithography and reactive ion etching (RIE) or by photopatterning the negative photoresist SU-8 2015 (Kayakli Advanced Materials) on silicon wafers. Identical mask sets were used for optical contact lithography during both processes. A 5” soda-lime chrome mask, pre-coated with chromium and photoresist (Nanofilm), was exposed with the desired patterns using a Heidelberg DWL 66 mask writer. The mask was developed in MF®

CD-26 developer (Shipley Company) for 1 min, rinsed with DI water, and dried with nitrogen. The mask was then etched in chromium etchant until the patterns were visibly cleared. The photoresist was removed in a heated bath of N-methyl-2-pyrrolidone (Dupont Electronic Materials International). The mask was rinsed in DI water and dried with nitrogen.

Silicon wafers (4-inch diameter <100> P-type, boron-doped to 10-20 Ω resistivity, 500-550 μm thick, single-side polished) were used as the substrate for microfluidic masters in all processes and were patterned on the polished side of the wafer. For silicon master fabrication with RIE, wafers were spin-coated with MicroPrime P20 adhesion promoter (Shin-Etsu MicroSi) to promote photoresist adhesion. NFR 016D2-55cP (JSR Micro, Inc.), a negative photoresist, was spin-coated onto the wafers, soft-baked at 90 $^{\circ}\text{C}$ for 90 s, exposed to 365 nm light (50 mJ/cm^2), and then post-exposure baked at 115 $^{\circ}\text{C}$ for 90 s. Samples were developed using Microposit® MF® CD-26 developer (Shipley Company) for approximately 1 min or until visibly cleared, then rinsed with deionized water and dried with nitrogen. The wafers were etched to a depth of 8.3 μm (~10 cycles) using a modified Bosch process (3 s polymer deposition, 10 s etch). SU-8 masters were created by first baking substrates on a hotplate at 180 $^{\circ}\text{C}$ for 10-30 min to dehydrate the wafers. Wafers were allowed to cool briefly and spin-coated at 3000 RPM with SU-8. Coated samples were soft-baked on a hot plate at 95 $^{\circ}\text{C}$ for 4 min, exposed in a contact aligner (150 mJ/cm^2), and post-exposure baked on a hot plate at 95 $^{\circ}\text{C}$ for an additional 5 min. Wafers were puddle-developed in SU-8 Developer (Kayakli Advanced Materials) for 3 min, rinsed with isopropanol, and dried with nitrogen. Etch depths or SU-8 feature heights were confirmed by measurement of select regions of the sample with a stylus profilometer (KLA Tencor).

4.5.2 Microfluidic device preparation.

All microfluidic devices were made of PDMS. Silicon masters were first treated with trichloro (1H, 1H, 2H, 2H, -perfluorooctyl) silane (PFOS, Sigma-Aldrich) through a vapor deposition process to prevent PDMS adhesion to the microfluidic master during curing. The silicon wafers were placed in a glass 150x50 mm petri dish with an Eppendorf tube cap filled with 20 μ L of PFOS on a hot plate set at 100 °C. After 2 h, the hot plate was turned off, and the master device was left in the petri dish overnight. 100 g total of PDMS was made with a 10:1 DOW SYLGARD 184 (Ellsworth Adhesives) Silicon elastomer base to catalyst ratio. The mixture was then degassed, poured over the silicon master device, degassed again, and cured at 80°C for 2 h. Devices were then cut apart, and 4-mm biopsy punches (Electron Microscopy Sciences) were used to create wells on either side of the plug region. The device and a glass slide (25x75x1mm) were sprayed with 70% isopropanol, dried with nitrogen, and then repeated with deionized water. Both the device and glass slide were then cleaned with oxygen plasma for 30 s using a PDC-001-HGP Plasma Cleaner (Harrick Plasma). The device and glass slide were then contacted together and heat-treated for 10 min at 80°C. A vacuum pump (Pittsburgh Automotive, 3 CFM) and desiccator (SP Bel-Art, 0.20 cu.ft.) were used to purge the air out of the device for 20 min. At this point, devices were ready for perfusion of the *pd*-PEG precursor solution within the channel. A picture of a sample microfluidic device setup is provided (Fig. B.8)

Prior to preparing the *pd*-PEG precursor solution, phosphate-buffered saline (PBS) buffer was made in-house by combining 1,567 mg NaH_2PO_4 , and 187 mg and Na_2EDTA (Sigma-Aldrich) to 90 mL in ultrapure water and adjusting the pH to 8.0. The PBS solution was sterile-filtered and stored at room temperature prior to use. The PEG-*o*-NB-DA macromer was synthesized in-house; a detailed description of its synthesis and an ^1H NMR spectra of the exact

PEG-*o*-NB-DA product used here is available in Fattahi *et al.* (Scheme S1, Fig S1).¹¹⁷ To prepare the precursor solution, 25 μL of PBS was combined with 11.2 μL of PEG-*o*-NB-DA in an Eppendorf tube and mixed thoroughly. 13.8 μL of Pentaerythritol tetra(mercaptoethyl) polyoxyethylene (PEGTT, MW 5.0 kDa, NOF America Corporation) was then added and mixed. Then 10 μL of the solution was quickly pipetted into the 4-mm wells, allowing for perfusion of the precursor throughout the channel. The device was incubated at room temperature for 30 min to allow for hydrogel formation, then 20 μL of TSB (30 g/L, Sigma-Aldrich) was added to one well, and 20 μL of PBS on the other side. This was left covered 24 h before proceeding. After this, the 4-mm biopsy punch was used to clear the hydrogel out of each well and then the wells were washed with 20 μL of PBS.

4.5.3 Chemical gradient characterization and diffusivity calculation.

Fluorescent chemical gradients were produced using a 6.1 mM solution of Alexa Fluor 594 NHS ester (Thermo-Fisher Scientific) in PBS instead of TSB solution. 20 μL of this solution was added to the source well, and 20 μL of PBS only was added to the sink well. Fluorescent images were taken down the channel every hour for 48 h using a Cytation 5 (BioTek) and a 20x objective. Images were montaged together to create a single composite image displaying the entire channel. Intensity profiles down the plug region were then measured using ImageJ software, normalized, and plotted for various time points. To determine the diffusion coefficients (D_{eff}) of this molecule in the hydrogel, transient fluorescent intensity profiles between 0 and 48 h were analyzed at three different positions within the channel ($(x/L) = 0.4, 0.5, \text{ and } 0.6$) using Fick's second law simplified with an early time approximation ($I_x(t)/I_{max} \leq 0.6$):¹³⁶

$$\frac{I_x(t)}{I_{max}} = 4 \left(\frac{D_{eff} t}{\pi x^2} \right)^{0.5}$$

where $I_x(t)$ is the fluorescent intensities in the channel at a given position at time t , I_{max} is the fluorescent intensity at that position at the final time point ($t=48$ h), and x corresponds to the position in the channel. As diffusion is independent of channel position in these regions, the D_{eff} values measured at the different channel positions were averaged together.

4.5.4 Photodegradation of hydrogel in devices using photopatterning.

After deposition in channels, *pd*-PEG hydrogels were exposed to user-defined 365nm light patterns using a Polygon 400 patterned illumination tool (Mightex Systems) attached to an Olympus BX51 upright microscope. This tool allows for control of light intensities ranging from 0.7-7.0 mW/mm² and millisecond control of exposure times at ~9 μ m pattern resolution. Prior to patterning, the Polygon tool was calibrated using a calibration mirror as described in Fattahi *et al.*¹³² All hydrogel degradations were performed using 60% light intensity (4.2 mW/mm²) at a 20x, NA 0.5 objective. For patterning of the degradation ladder, micropatterned rectangles 110 μ m (x-direction) by 101 μ m (y-direction) were used. This pattern corresponds to the distance between one column of support pillars in the x-direction and three rows of support pillars in the y-direction (dashed white line, Fig. B.4A, Fig. B.4B). To ensure that degradation was achieved between different exposure regions, adjacent exposure rectangles overlapped 10 μ m in the x-direction, corresponding to the diameter of a support pillar. Exposure times of 0.5, 1, 3, 10, and 40 s were chosen, generating respective doses of 2.1, 4.2, 12.6, 42, and 168 mJ/mm². The latter was chosen as the upper limit for dose, as this was the dose used for complete hydrogel degradation and reverse gelation in prior studies by Fattahi *et al.*¹¹⁷ The 2.1 mJ/mm² region had one exposure rectangle, and both 4.2 and 12.6 mJ/mm² exposure regions had two exposure rectangles. The 42 mJ/mm² region was exposed from the plug region up to the second set of supporting pillars. To ensure bacteria had access to this region, rectangles (165 μ m x 260 μ m) were exposed where the plug

region met the edge of the well using a 168 mJ/mm^2 dose, which also degraded any residual hydrogel remaining after removing hydrogel from the wells using the 4-mm biopsy punch. After exposing the hydrogel to this degradation pattern, the sink well was briefly rinsed with $20 \text{ }\mu\text{L}$ PBS to remove degraded PEG byproducts. For experiments involving the selective delivery of bacteria, a section of hydrogel $\sim 130 \text{ }\mu\text{m}$ into the channel was fully degraded using 168 mJ/mm^2 doses, up to the second column of supporting pillars. A second $220 \times 101 \text{ }\mu\text{m}$ region of partially degraded hydrogel was exposed to doses of 4.2, 12.6, or 42 mJ/mm^2 . Finally, a $110 \times 101 \text{ }\mu\text{m}$ liquid delivery region was exposed to a 168 mJ/mm^2 dose for reverse gelation (Fig. B.4C).

4.5.5 Hydrogel staining.

To visualize partially degraded hydrogels within the device, a $40 \text{ }\mu\text{M}$ solution of fluorescein-5-maleimide was prepared by adding $4 \text{ }\mu\text{L}$ of 10 mM fluorescein-5-maleimide (Thermo-Fisher Scientific) in DMSO to 1 mL of PBS (pH 8.0). $20 \text{ }\mu\text{L}$ of this solution was then added to each well for 2 h. Each well was then washed with $20 \text{ }\mu\text{L}$ of PBS to remove unbound fluorophore, and the hydrogel was imaged using a Nikon TI-E inverted fluorescent microscope with a FITC filter.

4.5.6 Bacteria chemotaxis through partially degraded hydrogels.

B. subtilis strain 1A1135 (Bacillus Genetic Stock Center, Columbus, OH, United States) modified to express green fluorescent protein (GFP) and for spectinomycin resistance, was used in all chemotaxis experiments. *B. subtilis*-GFP was stored in 50% glycerol stocks at -80°C until use. For chemotaxis experiments, *B. subtilis*-GFP was first cultured in TSB with $100 \text{ }\mu\text{g/mL}$ spectinomycin (Sigma-Aldrich) at 32°C (215 rpm) for 24 h. Optical densities at 600 nm (OD_{600}) were then measured from a $100 \text{ }\mu\text{L}$ sample of a *B. subtilis*-GFP culture solution using an Epoch2 microplate reader (BioTek) in 96-well format. Cultures were diluted with PBS to an OD_{600} of 0.01

for further use. To introduce *B. subtilis*-GFP into the device, 19 μL of PBS and 1 μL of bacteria solution ($\text{OD}_{600} = 0.01$) were added to the sink well, and a 20 μL solution of TSB (16 μL PBS and 4 μL 30 g/L TSB) was added to the nutrient source well. The region of interest containing degraded hydrogel was monitored with time-lapse fluorescence microscopy (TLFM) using a Nikon Ti-E inverted fluorescent microscope with a 20x, NA 0.45 objective, a FITC filter set (gain 25.6x and exposure 100ms), and an X-Cite illumination source immediately after *B. subtilis*-GFP was introduced into the sink well. After this, a glass slide was placed over the top of the device to cover the source and sink wells and prevent evaporation of the liquid and drying of the hydrogel. For experiments involving *B. subtilis*-GFP in red fluorescent polystyrene beads, 18 μL of diluted TSB was added to the source well, and 18 μL of PBS was added to the sink well. 1 μL of *B. subtilis*-GFP at an OD_{600} 0.01 and 1 μL of a 0.025 weight/volume % solution of red fluorescent polystyrene amine-modified latex beads (Sigma Aldrich, 2.5% solids) in PBS were added to the sink well for a final *B. subtilis* OD_{600} of 0.005 and a 0.00125% solid solution, which was approximately equivalent to a 10:1 bead to cell ratio (Fig. B.7). After the introduction of the mixed solution, a glass slide was placed over the top of the device, and the rectangular delivery region was monitored using TLFM. Images of the bacteria and fluorescent beads were taken using the same microscope, objective, and illumination source as stated before for the degradation ladder. A FITC filter set (gain 25.6x and exposure 100 ms) was used for the bacteria and a TRITC filter set (gain 9.3x, exposure 40 ms) was used for the fluorescent beads.

4.5.7 Image analysis.

Images and movies were viewed and edited in FIJI (ImageJ2). Individual cell counts were measured using the FIJI multi-point tool. All mean speeds and mean directional changes, the latter defined as the angle value (in radians) between succeeding links of a track, averaged over the entire

track, were calculated using the TrackMate FIJI plug-in. This plug-in provides x- and y-coordinates for each bacteria detected in each frame. The initial coordinates were set to zero to calculate the individual tracks of bacteria and to provide a comparison of tracks between individual cells. For cell migration maps, the final x- and y-locations for each track after the 24 h chemotaxis experiments from Trackmate were binned within a 7x7 grid. Bin sizes within the grid were created according to the minimum and maximum x and y-locations traveled by cells in each population. The binned bacteria locations created a histogram representing the migration of the entire population of bacteria. Bicubic interpolation of this data was then used to create the cell migration maps. A white cross at the center of the image indicates the initial position of all bacteria.

4.5.8 Statistical Analysis of Data.

All statistical analysis was done using SAS Institute Inc. Statistical significance was determined using student t-tests and Tukey's test; an alpha value less than 0.05 was considered statistically significant. All calculated means and standard deviations are based on a minimum of n=3 independent replicates.

4.6 Acknowledgements

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Chapter 5 - PEG-Based Living Hydrogels Engineered For Tunable Production of Bioactive Lipopeptides

5.1 Overview

Encapsulation of microbial cells within nanoporous hydrogels creates dynamic and responsive living materials well suited for biotherapeutic applications. *Bacillus subtilis* is a promising microbe in this application as it is generally regarded as safe, can be sporulated for long-term stability and resistance to non-ideal environments, and can produce antimicrobial and anticancer molecules such as the cyclic lipopeptide, surfactin. Here, we examine the growth of *B. subtilis* cells and corresponding production of surfactin after encapsulation within poly(ethylene glycol) (PEG) hydrogels at varied levels of nanoconfinement. Encapsulation was achieved through Michael-type addition reactions between PEG diacrylate and PEG tetrathiol macromers, where macromer molecular weight was systematically varied to generate hydrogels across a range of average mesh sizes (9-19 nm). Hydrogels had varied Young's modulus (7.3 ± 1.7 kPa to 16.4 ± 0.7 kPa) and provided a 7-fold range in small molecule diffusivity. In-situ cellular growth monitoring and surfactin quantification revealed that all hydrogels stimulated the production of surfactin with verified antibacterial activity and in a manner tunable with mesh size. Smallest mesh sizes drove highest surfactin production, a ~5-fold increase relative to equivalent cultures of unconfined cells. Cell loading was then varied in 9 nm mesh size hydrogels to reveal that low cell loading (0.1 - 1×10^4 cells per μL hydrogel) promoted sustained growth and surfactin production proportional to the number of cells loaded. Conversely, hydrogels loaded with excessive cells (2.5 - 7.5×10^4 cells per μL hydrogel) resulted in unstained growth and diminished surfactin production. Finally, to develop a more robust material, *B. subtilis* endospores were encapsulated into hydrogels at optimized conditions. Spore-laden hydrogels retained the capability to produce surfactin after

exposure to dehydration and temperature stress. These results indicate that hydrogel encapsulation stimulates *B. subtilis* surfactin production according to the level of nanoconfinement to achieve a tunable engineered living material for production of bioactive molecules.

KEYWORDS: hydrogels, engineered living materials, bacteria, surfactin, poly(ethylene glycol), wound dressings, biotherapeutics

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5.2 Introduction

Engineered living materials (ELMs) are formed by combining live cells with compatible biomaterials to enable a broad range of biomedical applications, including *in situ* production and delivery of biotherapeutic molecules for wound healing and anticancer applications.¹²⁵ Due to their diverse functionality, programmable nature, and relative simplicity, bacteria have received extensive interest as the cellular component in these applications,^{1, 5} while hydrogels are often the biomaterial of choice because they encapsulate cells to control their spatial distribution while providing a microenvironment conducive to their survival, proliferation, and function.^{4, 6} In wound healing applications, bacteria encapsulated within hydrogels can produce therapeutic molecules (e.g. antibiotics) in response to environmental cues such as pH, hypoxia, and reactive oxygen species to treat infection sites.^{137, 138} Similarly, in anticancer applications, hypoxic conditions created by cancer cells can be sensed by bacteria in hydrogels for production of molecules cytotoxic to the tumor environment.¹³⁹ While naturally occurring, polysaccharide-based hydrogels (alginate, chitosan, hyaluronic acid) are most commonly used,¹⁴⁰ synthetic hydrogels such as poly(ethylene glycol) (PEG), poly(acrylic acid) (PAA), and poly(vinyl alcohol) (PVA) offer distinct design advantages, including precise control of physicochemical hydrogel properties,¹⁴¹ long-term stability,¹⁴² or tunable hydrogel degradation.⁷¹

Use of PEG in ELM applications is particularly advantageous because PEG is chemically and biologically inert and resistant to cellular adhesion and biomolecule adsorption.¹⁴³⁻¹⁴⁵ These features yield PEG-based hydrogels that resist external bacterial colonization and biofilm formation while promoting the function of encapsulated bacteria, a critical feature for wound healing applications.¹⁴⁶ Further, PEG-based macromers used in hydrogel precursor solutions are commercially available across a wide variety of molecular weights and end-group chemistries and

can be modified to incorporate stimuli-responsive moieties,¹⁴⁷ enabling flexible and creative hydrogel design options tailored towards specific applications.¹⁴⁸ In particular, the molecular weight of the PEG precursors can be varied to tune hydrogel average mesh size (ξ) for control of mass transport, particularly in step-growth hydrogels that yield high mesh size uniformity.²⁹ Mesh size also determines hydrogel mechanical properties, equilibrium swelling, and the degree of physical confinement imposed on encapsulated cells, with smaller mesh sizes corresponding to lower swelling, stiffer gels, and greater diffusion limitations.¹⁴⁹ These factors influence how cells regulate gene expression, growth, and morphology. While such behaviors are well studied with encapsulated eukaryotic cells for tissue engineering applications,¹⁵⁰ similar studies with microbial cells for emerging ELM applications are currently underexplored.⁶

The primary goals of this study are to understand the effects that hydrogel nanoconfinement, as determined by hydrogel mesh size, and *Bacillus subtilis* loading play in cellular growth and corresponding production of the secondary metabolite, surfactin. *B. subtilis* has generally recognized as safe (GRAS) designation and the unique ability to form endospores, decisive advantages for real-world ELM applications. *B. subtilis* strain 21332 was chosen here, as it produces bioactive lipopeptide molecules (e.g. surfactin, fengycin) with potent antibacterial, antifungal, and anticancer properties,^{151, 152} and is the “gold-standard” surfactin-producing strain amongst non-genetically modified *B. subtilis* organisms.¹⁵³ Surfactin has been demonstrated to prevent the growth of pathogenic biofilms and decrease wound closure times,¹⁵⁴ while also inducing apoptosis in human breast cancer cells.¹⁵⁵ For example, previous work by David et al. demonstrated the encapsulation of *B. subtilis* within PVA microparticles for producing surfactin at 350 $\mu\text{g/mL}$ concentrations to treat both *Staphylococcus aureus* and methicillin-resistant *S. aureus*.²⁰ Here, we encapsulate *B. subtilis* in hydrogels of varied mesh sizes using Micheal-type

addition reactions between PEG diacrylate (PEGDA) and PEG tetrathiol (PEGTT) macromers across a range of macromer molecular weights (Fig. 5.1). This encapsulation chemistry was previously demonstrated to immobilize bacteria cells at high viability,⁸³ generating hydrogels that facilitate bacteria growth,^{81, 117, 156} and that also provide controlled transport and release of bacteria on degradation.^{82, 118, 157}

The results reveal that hydrogel mesh size ($\xi = 9\text{-}19\text{ nm}$) can be used to provide *B. subtilis* with varied levels of nanoscale physical confinement to enhance the quorum-mediated production of surfactin. Production was tunable with mesh size, with the lowest mesh size and highest nanoconfinement generating the highest surfactin production. Variation of *B. subtilis* cell loading levels demonstrated that low cell loading provides surfactin levels proportional to the number of cells added, while overloading hydrogels with cells diminishes growth and surfactin production. Finally, using hydrogels designed for maximum surfactin production, we encapsulate *B. subtilis* endospores and demonstrate the ability to retain surfactin production after exposing hydrogels to dehydration and temperature stress. These combined results will inform the future design of PEG hydrogels in ELM applications that include living bandages, wound dressings, and anticancer therapies.

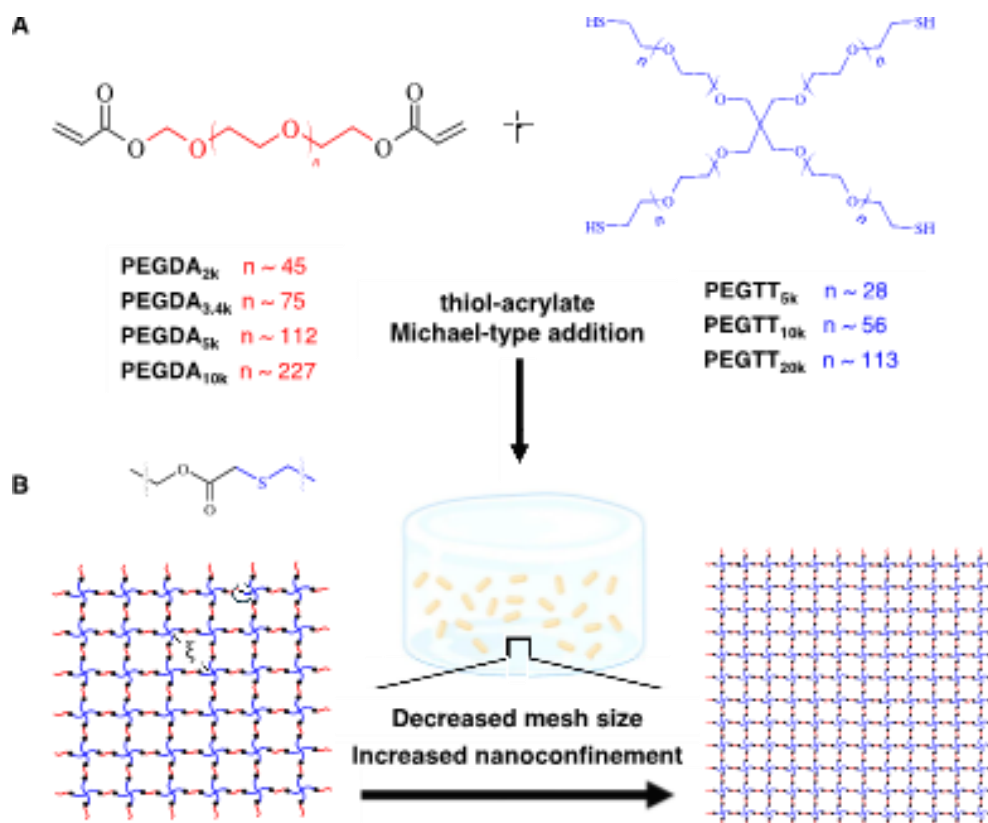


Figure 5.1. (A) Formation of PEG hydrogels through Michael-type addition reaction between PEGDA and PEGTT macromers of varied molecular weights. (B) Decreasing mesh sizes (ξ) increase nanoconfinement on encapsulated bacteria. Panel (B) was partially completed in Biorender software.

5.3 Materials and Methods

5.3.1 Material preparations.

Four separate PEGDA macromers of different molecular weights ($M_n = 2, 3.4, 5,$ and 10 kDa) were purchased from Creative PEGWorks, and three separate PEGTT macromers of different molecular weights ($M_n = 5, 10,$ and 20 kDa) were purchased from NOF America. *B. subtilis* strain 21332 was obtained from the American Type Culture Collection (ATCC), and *B. subtilis* strain 168 was obtained from the Bacillus Genetic Stock Center (Strain 1A1135). *Escherichia coli* strain K12 was gifted from Oak Ridge National Laboratory. Stocks of each strain were maintained in a

50% glycol solution and stored at -80°C . Nutrient broth, buffer salts, and other reagents were purchased from Sigma-Aldrich unless otherwise noted. Phosphate-buffered saline (PBS) buffer at 0.1 M was made in-house by dissolving 1,567 mg NaH_2PO_4 and 187 mg Na_2EDTA in 90 mL ultrapure water and adjusting the pH to 8.0 with aqueous 5 M NaOH. The PBS buffer was sterile-filtered and stored at room temperature prior to use. ATGN-phosphate buffer was prepared by combining 1,506 mg of sodium phosphate monobasic dihydrate with 95 mL of 2 $\times$ AT minimal medium, supplemented with glucose (Fisher Scientific) and ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$, Fisher Scientific), as described by Morton and Fuqua.¹⁵⁸ The pH of the solution was then adjusted to 8.0 using an aqueous 5 M sodium hydroxide solution (Fisher Scientific), sterile filtered, and stored at -20°C until further use. 2 $\times$ Schaffers glucose (2 $\times$ SG) medium from Leighton and Doi¹⁵⁹ was used for *B. subtilis* endospore formation. 2 $\times$ SG media was made by combining 16 g per liter nutrient both No. 4 with 0.5 g per liter $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ (Macron Fine Chemicals), 2.0 g per liter KCl (Fisher Scientific), and autoclaved. After autoclaving, the medium was supplemented with 1 mM $\text{Ca}(\text{NO}_3)_2$ (Fisher Scientific), 0.01 mM MnCl_2 , 0.001 mM FeSO_4 (added as $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$, Wards Science), and 0.1% (w/v) glucose. All growth experiments with bacteria encapsulated within a hydrogel used M9 minimal media unless otherwise noted. M9 minimal media was made according to Miller,¹⁶⁰ combining 6 g/L Na_2HPO_4 , 3.0 g/L KH_2PO_4 , 0.5 g/L NaCl (Fisher Scientific), 1.0 g/L NH_4Cl (Fisher Scientific), and autoclaving. Post autoclave additions included 10 mL of 100 mM MgSO_4 , 20% glucose, 10 mM CaCl_2 (Fisher Scientific), and 100 mM thiamine HCl. Tryptic soy broth (TSB) was made according to the manufacturer's instructions.

5.3.2 Hydrogel preparation and mesh size determination.

Hydrogels of various mesh sizes were formed via Michael-type addition reaction on mixing of an aqueous solution of PEGDA macromer with an aqueous solution of PEGTT

macromer. PEGDA macromers were reacted pairwise with the PEGTT macromers in varied molecular weight combinations to create a total of 11 different macromer combinations, generating hydrogels of 11 different mesh sizes. Hydrogels were formed at either 25, 50, 100, or 200 μL volumes. For 100 μL hydrogel volumes, 50 μL PBS was mixed with 22.4 μL of PEGDA (49 mM) dissolved in MilliQ water. After thorough mixing, 27.6 μL of PEGTT (20 mM) dissolved in MilliQ water was then added, achieving an equimolar ratio of acrylate and thiol groups. Volumes were scaled accordingly for 25, 50, or 200 μL hydrogels. Each mixture was reacted at room temperature for 30 min for hydrogel formation.

For hydrogel swelling experiments, hydrogels were prepared at 200 μL , then fully swollen or dried, weighed, and Equilibrium Swelling Theory as described by Canal and Peppas was used to calculate average mesh size for each hydrogel.⁹⁷ For equilibrium swelling, 500 μL of water was pipetted on top of a hydrogel in a 2 mL vial and incubated for 24 h. For drying, 200 μL hydrogels were placed in a 2 mL vial in an oven at 80°C for 24 h. After swelling or drying, hydrogel samples were removed from their respective vials and weighed with a digital analytical balance (U.S. Solid, ± 0.1 mg precision). A summary of the equations and constants used for equilibrium swelling calculations is provided in Appendix C (C1). All swollen and dry hydrogel masses were measured on $n=3$ independent hydrogels for each of the 12 macromer combinations.

5.3.3 Diffusional and mechanical hydrogel characterizations.

Effective diffusion coefficients of Alexa Fluor 488 (D_{eff}) through PEG hydrogels of varied mesh size were experimentally determined using a small molecule desorption approach adapted from previous hydrogel diffusion studies.⁸¹ Cylindrical 150 μL hydrogels were formed inside 2 mL glass vials and swollen in 1 mL ultrapure water for 24 h. The ultrapure water was removed, and the hydrogels were subsequently equilibrated with a 282 μM Alexa Fluor 488 solution for 24

h. Following loading, the Alexa Fluor solution was removed and replaced with 1.0 mL ultrapure water to initiate Alexa fluor diffusion out of the hydrogel and into solution. At selected time points, 2 μ L aliquots of the release solution were collected at and analyzed using a Nanodrop One UV–Vis spectrophotometer at 494 nm. Alexa fluor concentrations were calculated using Beer’s law from standards prepared by diluting the original 282 μ M Alexa fluor to varied concentrations. Released tracer concentrations were normalized to the equilibrium release concentration at the final time point (C_t/C_∞). Effective diffusion coefficients were then estimated using the early-time approximation of Fick’s second law for slab geometries when $\frac{C_t}{C_\infty} \leq 0.5\text{--}0.6$.¹³⁶

$$\frac{C_t}{C_\infty} = 4 \left(\frac{D_{eff} t}{\pi L^2} \right)^{1/2}$$

where C_t and C_∞ are the Alexa Fluor 488 concentrations measured at time t and at equilibrium, respectively, L is the swollen hydrogel thickness, and D_{eff} is the effective diffusion coefficient of Alexa Fluor 488 through the hydrogel. Swollen hydrogel thicknesses used for diffusion calculations were estimated from equilibrium swelling measurements and experimentally estimated via cross-sectional areas (C.2, Table C.1).

Hydrogel mechanical properties were evaluated by compression using an Instron universal testing system equipped with a 5 kN load cell and 3D printed flat plate cylinders (diameter = 16 mm, height = 25 mm) designed in AutoCAD. Hydrogels were formed as nominal $4 \times 4 \times 4$ mm cubes by mold casting, pipetting 64 μ L hydrogel precursor solutions into cubic molds of the same dimension (Fig. C.1). Following gelation, hydrogels were removed from the molds and immediately subjected to compression testing to minimize dehydration effects. Individual hydrogel cubes were positioned between the compression plates and compressed at a constant crosshead speed of 0.5 mm/min at room temperature. Force and displacement data were recorded

every 0.01 s throughout testing. Compression was continued until the stress–strain response became nonlinear. Engineering stress was calculated using the nominal hydrogel cross-sectional area of 16 mm², and engineering strain was calculated by normalizing plate displacement to the nominal initial hydrogel height of 4 mm. The apparent compressive Young’s modulus (herein referred to only as Young’s modulus) was determined from the slope of the linear region of the engineering stress–strain curve between 5–15% strain, excluding the initial toe region associated with plate seating/contact artifacts. Samples exhibiting visible tearing, slipping, or nonuniform deformation during compression were excluded from analysis. Mechanical properties were evaluated using n=3–5 independent hydrogel replicates per formulation.

5.3.4 Cell encapsulation and growth tracking in hydrogels.

For bacterial growth tracking, hydrogels encapsulating *B. subtilis* were formed at 100 μ L volumes in 48-well plates and optical density at 600 nm (OD₆₀₀) was monitored during culture using a microplate reader (Epoch2, Biotek). This experimental setup was designed to probe bacteria while confined in hydrogels with diffusive exchange of nutrients and metabolic products to and from the bulk fluid. These confinement and transport principles are shared across several biotherapeutic formats, including implantable or injectable bacteria-loaded hydrogels, skin and wound facing hydrogels.¹⁶¹⁻¹⁶³ The 100 μ L volumes were necessary to align with our 48-well plate reader and were sufficient for probing bacteria growth dynamics governed by the varied nanoscale environments created across the different hydrogel samples.

Prior to encapsulation, *B. subtilis* cells were cultured in TSB for 24 h (32°C, 215 rpm). Cells were then spun in a centrifuge (20 min, 4400 RPM), spent media was removed, then the cells were resuspended in PBS (pH 8.0). To account for a 50% dilution when combined with the PEG precursors during encapsulation, cells were diluted to twice the desired concentration in ATGN-

phosphate buffer. For example, for a hydrogel encapsulating cells at $OD_{600} = 0.1$, cells were first diluted in ATGN-phosphate buffer to $OD_{600} = 0.2$ before adding the PEGDA and PEGTT solutions. After encapsulation, hydrogels were gently washed with 1 mL of ultrapure water for 30 seconds to remove any planktonic or loosely attached cells. Hydrogels were deposited in 48-well plates by adding 50 μ L of the *B. subtilis* cell suspension to the wells, then 22.4 μ L of PEGDA (49 mM) followed by 27.6 μ L of PEGTT (20 mM) solutions were added into the wells to initiate the crosslinking reaction. Solutions were incubated at room temperature for 30 min, a time sufficient for complete hydrogel formation.¹⁵⁶ After hydrogel formation, 1 mL of M9 minimal media was dispensed on top of each hydrogel for culture, then bacterial growth was tracked over 72 h at 32°C (unless otherwise stated), with OD_{600} measurements recorded every 10 min in all growth experiments. A 1 s linear shake at 567 cycles/min occurred before each measurement. M9 minimal media was used to create a well-controlled growth environment that enabled systematic investigation of bacteria growth behavior under different levels of hydrogel confinement. Growth kinetics were fitted to the Gompertz equation using Mathematica (Wolfram, Inc.), which models the bacterial growth curve as an asymmetrical sigmoidal function allowing for precise estimation of lag phase duration and growth rate.¹⁶⁴ While the present studies focus on bacteria growth and behavior over shorter periods, these living hydrogel systems can be transitioned to function over longer periods through additional media supplementation or perfusion to sustain metabolically active bacteria.^{17, 161, 165}

5.3.5 Surfactin quantification in cell free culture fluid by CPC-BTB assay.

All surfactin quantification experiments were done using the cetylpyridinium chloride (CPC)-bromothymol blue (BTB) colorimetric assay from Yang *et al.*, which was specifically developed to quantify surfactin concentrations in the cell free culture fluid (CFCF) of *B. subtilis*

cultures and was validated against reverse-phase high-performance liquid chromatography (RP-HPLC).¹⁶⁶ The CPC-BTB assay leverages the affinity of surfactin with CPC, dissociating the CPC-BTB complex to cause a color shift and a corresponding increase in absorbance at 600 nm. A 0.20 mM CPC solution was prepared by dissolving CPC in 0.1 M PBS (pH 8.0). Separately, a 0.20 mM BTB solution was prepared in the same buffer. Equal volumes of the CPC and BTB solutions were mixed in a glass tube and vortexed thoroughly to yield a uniform dark-green CPC–BTB indicator solution. This mixture was freshly prepared immediately prior to each assay. Culture fluid above each hydrogel after 48 or 72 h culture was then collected and centrifuged (4,400×g, 20 min) to obtain CFCF, as any free cells can interfere with the CPC-BTB complex. For unconfined culture controls, CFCF was collected in the same manner after culture in identical medium containing the equivalent amount of nutrients and inoculated with the same initial number of bacteria. Then, 100 µL of CPC–BTB indicator solution was combined with 100 µL of CFCF in a 96-well microplate. The plate was agitated for 5 min in a microplate reader at 567 cycles·min⁻¹ and orbital movement of 3 mm diameter to allow for CPC-surfactin complexation. Absorbance at 600 nm was then measured (Epoch2, Biotek). A schematic describing the surfactin quantification experiment can be found in Appendix C (Fig. C.2). Surfactin concentration was quantified using standard calibration curves relating absolute surfactin concentrations (µg/mL) to absorbance at 600 nm. Surfactin standards were prepared using purified surfactin diluted in both M9 (Fig. C.3) and TSB (Fig. C.4) culture media to normalize for any background signal caused by the media components.

5.3.6 Fluorescent imaging of cells following encapsulation and culture.

An inverted fluorescence microscope (Nikon Ti-E, NIS-Element software) was used to image bacteria within hydrogels, both after encapsulation and at different stages of culture. For imaging, *B. subtilis* cells were encapsulated within a ~25 µm thick hydrogel layer chemically

attached onto 18×18 mm glass coverslips. Prior to hydrogel attachment, inert (fluorinated) glass slides (25×75×1 mm) and glass coverslips functionalized with a chemically reactive (thiolated) attachment layer were fabricated using a chemical vapor deposition method. For slide fabrication, each slide or coverslip was first cleaned by rinsing with 70% isopropanol in deionized water, rinsing with deionized water, and then drying with nitrogen. Cleaned slides or coverslips were then placed in a glass petri dish (150×50 mm) next to an Eppendorf tube cap filled with 20 μ L of either trichloro (1H, 1H, 2H, 2H-perfluorooctyl) silane for inert slides or (3-mercaptopropyl) trimethoxysilane for thiolated coverslips. The glass petri dish was set on a hot plate at 100 °C for 2 h. The hot plate was then turned off, and the slides/coverslips were left in the petri dish overnight. Next, hydrogel precursor solutions containing *B. subtilis* 21332 cells were created as described in Section 2.4 at a 25 μ L volume. Immediately after addition of the PEGTT, 7 μ L of the precursor solution was pipetted onto a hydrophobic glass slide and placed onto a thiolated coverslip with 25 μ m spacers separating the pieces of glass (Fig. C.5). This was incubated for 30 min to allow for hydrogel formation and chemical attachment to the thiolated coverslip. After gelation, the inert slide was peeled off, leaving the hydrogel attached to the thiolated coverslip. Before staining or culturing, each coverslip was washed 3× with ultrapure water. A detailed demonstration of hydrogel functionalization over glass coverslips is available in video format in Fattahi et al.¹⁵⁶ The coverslip was placed in a 3D-printed coverslip holder (Fig. C.6A). The coverslip holder was designed in house; the STL file is available upon request.

To determine initial cell viability after hydrogel encapsulation, a Live/Dead Bacterial Viability Kit (Thermo-Fisher Scientific)¹⁶⁷ was used. Here, hydrogels were stained according to the manufacturer's instructions by adding 9 μ L of the reagent mixture per 100 μ L of hydrogel, followed by a 15 min incubation at room temperature in the dark. Excess dye was then removed

by washing the samples with a 0.85 wt% NaCl solution. Fluorescence intensity was then recorded using FITC/TRITC filters at 10× magnification. The intensity ratio of green/red cells gives the overall initial cell viability using a calibration curve available in the supplementary information file (Fig. S1) in Gutierrez et al.⁸³

For imaging cell growth within hydrogels during culture, hydrogel-functionalized coverslips (7 µL) were added to the coverslip holder which was then placed in a live cell incubation chamber (Tokai Hit, Fig. C.6B). 140 µL of a 50:50 mixture of M9 and ultrapure water was then pipetted on top of each hydrogel. To account for evaporation during the culture period, 67 µL of ultrapure water was added to the culture media every 24 h, which was equivalent to the amount of water evaporated. After the desired culture time, hydrogels were stained by pipetting 100 µL of a 10 µM SYTO 9 solution over the hydrogel and incubating for 15 min at room temperature in the dark. Hydrogels were finally washed three times with ultrapure water to remove excess or unbound dye and imaged (Fig. C.6C). All images were taken with a 10×, NA 0.3 objective and a FITC filter set.

5.3.7 Antibacterial activity of *B. subtilis* cell free culture fluid.

To assess the activity of surfactin produced by *B. subtilis*, 100 µL *H_{9.5}* hydrogels inoculated at 1×10^4 cells per µL hydrogel were cultured with 1 mL of TSB at 37°C for 48 h. The CFCF supernatant was then collected, centrifuged for 15 min at 4,400 rpm, and sterile filtered using a 0.2 µm filter to remove any residual cells. Surfactin levels in the CFCF were then quantified using the CPC-BTB assay calibration curve (Fig. C.4), then diluted to a final concentration of 1,000 µg/mL in fresh TSB media. This concentration corresponds to the reported minimum inhibitory concentration (MIC) for purified *B. subtilis* lipopeptide biosurfactant against *Escherichia coli*.¹⁶⁸ 100 µL of the CFCF supernatant was then inoculated with *E. coli* at an initial OD₆₀₀ of 0.1 in 96

well plates and cultured at 37°C overnight. As a control, *E. coli* growth in supernatant from empty hydrogels without encapsulated *B. subtilis* cells was used under identical experimental conditions.

5.3.8 Preparation and Encapsulation of *B. subtilis* spores.

For experiments involving endospores, *B. subtilis* 21332 was first cultured overnight in TSB media (32°C, 215 rpm), then centrifuged at 4,400 g for 20 min, washed twice with MilliQ water, resuspended to OD₆₀₀ = 0.1 in 2×SG sporulation media and incubated in a shaker for 48 h (50°C, 215 RPM). To purify endospores from any remaining vegetative cells, the culture tube was then placed in a 75°C water bath for 20 min.¹⁶⁹ To confirm the formation of endospores, the LIVE/DEAD BacLight Bacterial Viability Kit was applied according to the manufacture's protocol, which stains spores or live cells green and dead cells red.¹⁷⁰ 3 µL of each stain was added per 1 mL of bacterial suspension and incubated for 15 min in the dark at room temperature. A sporulation of ~96% cells was quantified using image analysis. Spores were encapsulated within the hydrogel at an initial concentration of OD₆₀₀ = 0.1 following the procedure described in Section 2.4. Following encapsulation, heat stress was applied to the hydrogels within the 48-well plate by placing it in a convection oven at 80°C for 20 min. After heat treatment, the plate was cooled for 5 min then 1 mL of TSB was dispensed on top of each gel. Bacterial growth was then tracked for 48 h at 37°C, with OD₆₀₀ measurements recorded every 10 min. A 1 s linear shake at 567 cycles/min occurred before each measurement.

5.3.9 Statistical Analysis.

SAS software (SAS Institute Inc.) was used for statistical analysis. Statistical significance was determined using a Student's t-test and Tukey's test, with an $\alpha < 0.05$ considered statistically significant. All calculated means and standard deviations are based on a minimum of n=3 independent replicates.

5.4 Results and Discussion

5.4.1 Generation and Characterization of Hydrogels of Varied Mesh Size.

Molecular weights (MW) of both two-arm PEGDA and four-arm PEGTT macromers were first varied in precursor solutions to generate hydrogels with a range of average mesh sizes (ζ) (Fig. 5.1). Equilibrium swelling theory⁹⁷ calculations indicated that mesh sizes ranged from 9-19 nm with larger MW macromers generating larger mesh sizes, as expected. Statistical comparisons across all macromer combinations demonstrated that mesh size was tunable with PEG macromers MW (Fig. 5.2A). The smallest mesh size ($\zeta_1 = 9.5 \pm 0.7$ nm) was generated by PEGDA_{2k}–PEGTT_{5k} macromers, the middle mesh size ($\zeta_2 = 14.7 \pm 0.8$ nm) was generated by PEGDA_{10k}–PEGTT_{10k} macromers, and the largest mesh size ($\zeta_3 = 19.0 \pm 0.6$ nm) was generated by PEGDA_{10k}–PEGTT_{20k} macromers. This mesh size range provides a distinct set of PEG hydrogels that can be fabricated reproducibly from commercially available PEG macromers and that are potentially suitable for ELM applications. The mesh size range is comparable to other step growth PEG hydrogels used in related investigations, including a 5-15 nm mesh size range created with PEG thiol-norbornene macromers for tunable protein release,⁵⁸ and a 10-13 nm mesh size range created with PEG thiol-acrylate hydrogels for tunable hydrolytic degradation.¹⁷¹ While a ~ 10 nm range in mesh size may seem small, when propagated throughout the entire hydrogel network, this range results in significantly different hydrogel characteristics, including notable macroscopic differences in swelling (Fig. 5.2B). In fact, assuming a homogeneous network and cubic pores (as depicted in Fig. 5.1), the difference from $\zeta = 9.5$ to 19 nm results in a near order-of-magnitude difference in the number of pores ($8\times$) in hydrogels of equivalent volumes. Hydrogels spanning this range of mesh sizes, herein referred to as $H_{9.5}$, H_{15} , and H_{19} to correspond with $\zeta = 9.5$ nm, $\zeta = 15$ nm and $\zeta = 19$ nm respectively, were selected to enable systematic studies of bacteria function at varied

levels of nanoconfinement. For clarity, Table 5.1 displays the notation for these hydrogels, as they are used in further studies.

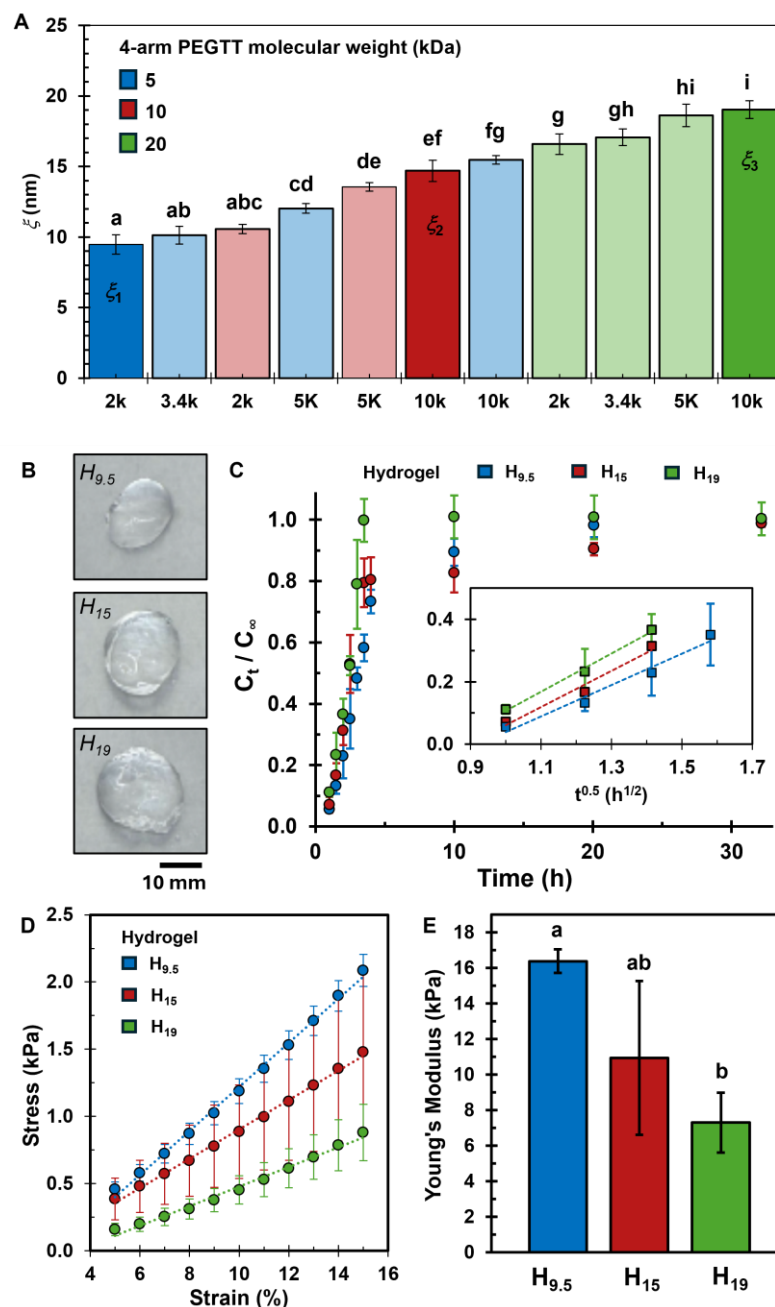


Figure 5.2. (A) Average mesh size (ξ) for each PEGDA and PEGTT macromer molecular weight combination. Error bars indicate standard deviation and differing letters indicate statistical significance ($p < 0.05$, $n=3$). (B) Images of hydrogels of each mesh size chosen for further experiments after 24 h of swelling. All hydrogels were of equivalent 50 mL volumes prior to swelling. (C) Transient release of Alexa Fluor 488 from $H_{9.5}$ (blue), H_{15} (red), and H_{19} (green) hydrogels. (Inset) Early time ($C_t/C_\infty < 0.6$) data used to determine De_{eff} for Alexa Fluor 488 through the hydrogel. (D) Stress-strain curve for uniaxial compression tests and (E) Young's modulus from the slope of the engineering stress-strain curve for $H_{9.5}$, H_{15} , and H_{19} hydrogels. Error bars indicate standard deviation ($n=3-5$ replicates) and differing letters indicate significance ($p < 0.05$) by Tukey's test.

Table 5.1. PEG hydrogel notation for cell encapsulation at varied nanoconfinement levels^a

notation	PEGDA molecular weight (Da)	PEGTT molecular weight (Da)	ξ (nm)
$H_{9.5}$	2,000	5,000	9.5 ± 0.7
H_{15}	10,000	10,000	14.7 ± 0.8
H_{19}	10,000	20,000	19.0 ± 0.6

^aAll hydrogels were prepared at 49 mM PEGDA and 20 mM PEGTT concentrations

Following the quantification of average mesh sizes, mass transport through each of the three selected hydrogels was characterized. Here, diffusion of Alexa Fluor 488 through the hydrogel was measured, creating release profiles for each hydrogel (Fig. 5.2C), Fick's Law for early release (Fig. 5.2C, inset) was then used to quantify diffusivity values of $5.17 \pm 1.36 \times 10^{-7}$ cm²/s, $1.04 \pm 0.40 \times 10^{-6}$ cm²/s, and $3.48 \pm 0.47 \times 10^{-6}$ cm²/s for $H_{9.5}$, H_{15} , and H_{19} hydrogels, respectively. For comparison, the diffusivity of Alexa Fluor 488 in water is 4.47×10^{-6} cm²/s when adjusted to room temperature,¹⁷² indicating that each hydrogel created diffusion limitations, and that diffusion became more hindered as mesh size decreased. Importantly, the 7-fold difference in diffusivity indicates these hydrogels created a wide range of nutrient environments for cell growth. Finally, hydrogel stiffness was quantified, as this is an additional factor that can influence bacteria growth in hydrogels.⁵¹ Stress-strain curves of $H_{9.5}$, H_{15} , and H_{19} hydrogels (Fig. 5.2D) provide Young's moduli values ranging from 16.4 ± 0.7 kPa to 7.3 ± 1.7 kPa (Fig. 5.2E), falling in the expected range for PEG hydrogels with this mesh size range,⁵⁸ and decreasing with increasing mesh size, as expected.

5.4.2 Cellular Growth in Hydrogels at Varied Levels of Nanoconfinement.

After hydrogel characterization, a systematic study of *B. subtilis* growth and surfactin production was performed. Here, *B. subtilis* was cultured in M9 minimal media after encapsulation in hydrogels $H_{9.5}$, H_{15} , and H_{19} at a constant cell loading level (1×10^4 cells per μL hydrogel). Images of *B. subtilis* cells throughout hydrogels immediately after encapsulation revealed differences in the initial distribution of cells, where the highest frequency of single cells and most uniform cell distributions were noted in $H_{9.5}$ hydrogels (Fig. C.7A). As mesh size increased, larger cellular aggregates appeared more frequently, driving non-uniform cell distributions (Figs. C.7B and C.7C). This may reflect the fact that higher molecular weight PEG precursors form hydrogels with more network heterogeneity,⁵⁸ which could create larger local pockets that favor multicellular encapsulation and aggregation during hydrogel formation.

Cell microscopy at different growth stages (Fig. 5.3A) and corresponding kinetic growth curves (Fig. 5.3B) were used for comparative analysis across hydrogels. Growth within each hydrogel was marked by the disappearance of individual cells and formation of spherical aggregates (24 and 72 h, Fig. 5.3A), and culture within each hydrogel displayed characteristic lag, exponential growth, and stationary phases (Fig. 5.3B). Importantly, OD₆₀₀ measurements were also taken from supernatant above each hydrogel (triangles, Fig. 5.3B). These values remained consistently low during the culture period and did not increase over time, indicating that all hydrogel formulations retained encapsulated cells throughout the growth period. If cells had escaped from hydrogels and into bulk culture fluid, OD₆₀₀ readings would have increased in the supernatant as it would become an unconfined culture.⁸³

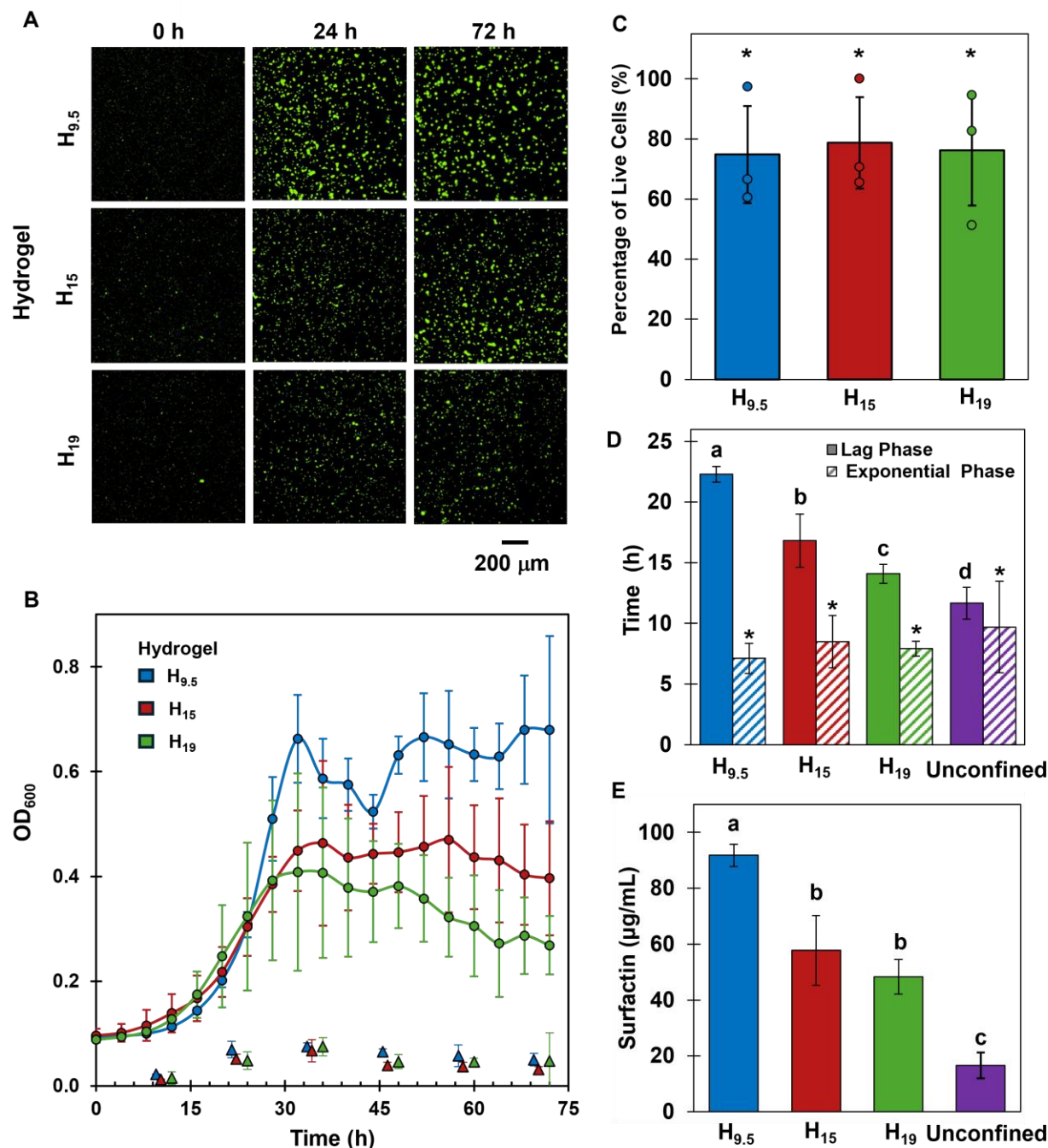


Figure 5.3. (A) 10× objective image montage of encapsulated *B. subtilis* 21332 at different culture stages for hydrogels of varied mesh size. (B) Growth kinetics of *B. subtilis* encapsulated within H_{9.5} (blue), H₁₅ (red), and H₁₉ (green) hydrogels. Triangles: OD₆₀₀ measurement of supernatant above encapsulated bacteria. (C) *B. subtilis* cell viability after encapsulation in selected hydrogels of varied mesh size. (D) Effect of hydrogel mesh size on lag (solid) and exponential (striped) growth phase duration. (E) Surfactin production from each hydrogel, measured using the CPC-BTB colorimetric assay. Error bars indicate standard deviation and differing letters indicate statistical significance ($p < 0.05$, $n=3$). A single asterisk (*) is not significant, $p > 0.05$.

Noting that physicochemical hydrogel properties can impact bacteria viability,⁶ live-dead staining of *B. subtilis* immediately after encapsulation in each selected hydrogel was checked. Here, *B. subtilis* cells in $H_{9.5}$, H_{15} , and H_{19} had equivalent cell viability levels, indicating that mesh size did not significantly impact viability and that all formulations supported bacterial survival (Fig. 5.3C). The averaged viability across all three hydrogels was $76.62 \pm 16.6\%$, which is consistent with Gutierrez *et al.*,⁸³ where PEGDA_{3.4k}-PEGTT_{10k} ($\zeta = 12.3 \pm 0.3$ nm) hydrogels yielded a statistically equivalent level of cell viability ($82.49 \pm 12.36\%$).⁸³ These findings are also consistent with analogous studies with mammalian cells by Zustiak *et al.*,¹⁴¹ where the viability of mouse fibroblast (Balb/3T3) cells after encapsulation in step growth PEG hydrogels of varied mesh sizes was characterized, and no correlation was found. This indicates that differences in initial cell viability after encapsulation was not a cause of growth differences between hydrogels.

Comparisons of growth curves (Fig. 5.3B) within each hydrogel revealed significant differences. In particular, cells in $H_{9.5}$ had delayed but more sustained growth to higher levels compared to H_{15} and H_{19} , as indicated by OD₆₀₀ values after 72 h. This agreed with corresponding microscopy images, where largest aggregates were observed in $H_{9.5}$ hydrogels (20.5 ± 2.2 μm) at 72 h compared to 13.8 ± 1.4 μm and 11.6 ± 0.9 μm for H_{15} and H_{19} , respectively (Fig 5.3A). To decipher differences in growth kinetics, the lag and exponential growth phases in each hydrogel were quantified (Fig. 5.3D). For further comparison of growth to an unconfined culture, *B. subtilis* was also inoculated in bulk culture media under identical inoculum, growth media, and culture conditions, and its growth kinetics were also measured (Fig. C.8). A significant trend in the lag phase was found, as its duration increased as average mesh size decreased. No differences were found in the exponential phase. The lag phase in bulk culture was also significantly lower than the lag phases of all hydrogel cultures.

Taken together, these growth trends reflect the increasing diffusion limitations created with smaller mesh sizes, which when combined with the more uniform initial distribution of single cells, promoted more sustainable growth and larger aggregate formation. By comparison, more frequent aggregates and heterogeneous cell distributions created by larger pore sizes, accompanied with lower diffusion limitations and higher nutrient flux, led to faster local nutrient consumption around cell clusters to limit growth and eventually cause cellular decay.

5.4.3 Surfactin Production in Hydrogels at Varied Nanoconfinement Levels.

Spatially confined environments can cause bacteria to coordinate gene expression and display cell density-dependent (i.e. quorum-mediated) behaviors, such as biofilm formation, chemotaxis, and secretion of metabolites.¹⁷³ Further, artificial systems designed to confine cells within controlled micro and nanoscale environments, including microfluidic droplets¹⁷⁴ and microwells,¹⁷⁵ have been used to study quorum-sensing processes in a variety of bacteria. With respect to hydrogels, Gao *et al.* used *Vibrio harveyi* cells within alginate-based hydrogels to demonstrate that both the spatial distribution of cells and the mass transfer environment are critical components for bacterial quorum sensing.¹⁷⁶ Their results indicate that both diffusion-limited environments and cellular aggregation within hydrogels promotes quorum sensing, and that larger aggregates produced higher levels of autoinducer molecules than smaller aggregates. Given this premise, and the fact that surfactin biosynthesis in *B. subtilis* is quorum-mediated,¹⁷⁷ we hypothesized that $H_{9.5}$, H_{15} , and H_{19} would stimulate surfactin production according to the level of nanoconfinement.

For surfactin quantification, the CPC-BTB assay was used.¹⁶⁶ The specificity of the CPC-BTB colorimetric assay to surfactin produced by *B. subtilis* CFCF was first validated by comparing the colorimetric response of surfactin-positive *B. subtilis* 21332 CFCF to surfactin-

negative *B. subtilis* 168 ($\Delta srfa$ operon¹⁷⁸) CFCF. The CFCF from *B. subtilis* 21332 caused a significant colorimetric shift and increase in absorbance at 600 nm, while *B. subtilis* 168 CFCF had no significant change over background levels (Fig. C.9). Combined with RP-HPLC validation of surfactin specificity provided in Yang *et al.*,¹⁶⁶ it was concluded that CPC-BTB assay could be used reliably for surfactin quantification from *B. subtilis* 21332 CFCF. Surfactin concentrations at 72 h for $H_{9.5}$, H_{15} , and H_{19} were next measured and compared to unconfined free-media culture prepared with identical inoculum, growth media, and culture conditions. The results revealed that hydrogel confinement was a strong promoter of surfactin production (Fig. 5.3E), as a surfactin concentration of 91.8 ± 3.9 $\mu\text{g/mL}$ was produced from $H_{9.5}$ hydrogels, representing greater than a 5-fold increase relative to the unconfined culture, with a surfactin concentration of 16.6 ± 4.6 $\mu\text{g/mL}$. H_{15} and H_{19} hydrogels, with successively higher mesh sizes, showed corresponding decreases in surfactin levels, but were still significantly higher than the unconfined culture. Taken together with the growth measurements (Fig. 5.3A-D), the results indicate the formation of larger aggregates and higher diffusion limitations created by smaller mesh sizes increase the quorum-mediated production of surfactin. As larger mesh sizes transition the hydrogel towards lower confinement and a more viscous liquid state, surfactin production approaches that of the unconfined culture.

In light of wound-healing and anticancer applications, it is notable that the surfactin concentrations measured from $H_{9.5}$ to H_{15} in these experiments (48-92 $\mu\text{g/mL}$, Fig. 5.3E) falls below the minimum inhibitory concentration (MIC) of most bacteria (ex. *Staphylococcus aureus*, 200 $\mu\text{g/mL}$ ²⁰; *E. coli*, 1,000 $\mu\text{g/mL}$ ¹⁶⁸) and below the levels reported to exhibit antitumor activity against cancer cell lines (100 $\mu\text{g/mL}$).¹⁷⁹ However, this is due to the limited nutrients in the minimal M9 media used, and changing nutrient environment can increase surfactin production.¹⁸⁰

To verify that similar gains in surfactin production could be obtained from hydrogel confinement in a more nutrient-rich environment, *B. subtilis* growth was again tracked in $H_{9.5}$ hydrogels and unconfined cultures (Fig. 5.4A and B) in TSB media, and corresponding surfactin levels after growth were again measured. A ~4-fold gain in surfactin production was achieved with hydrogel confinement, where $H_{9.5}$ hydrogels produced surfactin at a concentration of $1,841 \pm 54 \mu\text{g/mL}$ (Fig. 5.4C), which well exceeds concentrations necessary for antitumor activity and inhibitory concentrations of key pathogens in wound infections. To test the antibacterial activity of the solution released from the *B. subtilis*-PEG hydrogel, supernatant from these hydrogels was collected, centrifuged to obtain CFCF, then diluted to $1,000 \mu\text{g/mL}$ in TSB media to match the MIC for *E. coli* (Fig. 5.4D).¹⁶⁸ The growth of *E. coli* was then monitored during culture in 96-well plate format. As a control, supernatant from empty hydrogels with no *B. subtilis* was collected in the same manner and diluted in TSB media by the equivalent amount. Over a 20 h period, media supplemented with CFCF from the *B. subtilis*-PEG hydrogel diminished and eventually completely suppressed *E. coli* growth (Fig. 5.4E). In stark contrast, *E. coli* proceeded to growth to high levels in the control media over the same period. This demonstrates the antibacterial activity of these hydrogels and advances its use towards biotherapeutic ELM applications that leverage bioactive secondary metabolites produced from hydrogels loaded with *B. subtilis*.

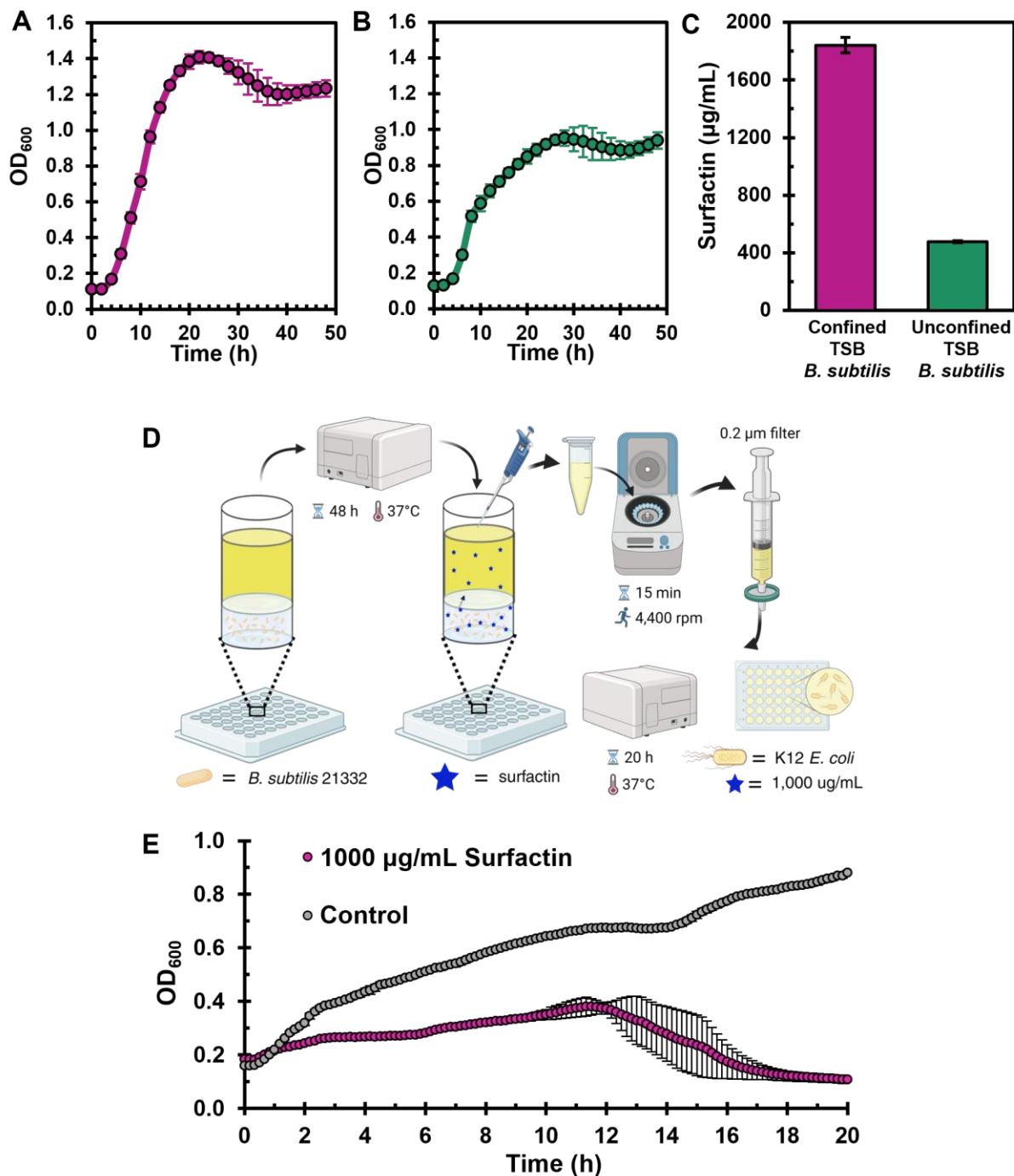


Figure 5.4. (A) Growth kinetics of *B. subtilis* during confinement within a H9.5 hydrogel and (B) in unconfined culture. Both cultures used nutrient-rich TSB media. (C) Corresponding surfactin production in confined (magenta) or unconfined (teal) cultures. (D) Biorender sketch of experimental workflow for assessing bioactivity of hydrogel supernatant against *E. coli*. (E) Growth kinetics of *E. coli* inoculated with a fresh media-CFCF at 1,000 µg/mL surfactin concentration (magenta) and fresh media-supernatant from a gel with no encapsulated *B. subtilis* (control, grey). Error bars indicate standard deviation (n=3)

5.4.4. Impact of Cell Loading on Surfactin Production.

As $H_{9.5}$ hydrogels produced the highest surfactin concentrations, these hydrogels were selected to next investigate the impact of bacteria cell loading, defined as the initial number of cells per volume of hydrogel precursor solution, on surfactin production. Cell loading was varied from 1.0×10^3 to 7.5×10^4 cells per μL hydrogel. These cell loading levels fall within a commonly reported range for other ELM bacteria encapsulation systems,⁵¹ including those used in wound-facing and injectable living hydrogels.¹⁶² After encapsulation at each cell loading level, equilibrium swelling experiments were again applied to determine if the presence of bacteria altered the average pore size. No significant difference in equilibrium swelling was found between the control set of hydrogels containing no bacteria and bacteria at any loading level (Fig. C.10), indicating that the presence of cells did not significantly alter the hydrogel network structure. Growth kinetics and surfactin concentrations in CFCF after 72 h culture were then measured in the same manner as previously described (Fig. C.2). Microscopic images of each hydrogel after encapsulation but before culture display a wide range of encapsulated cell densities with increased cell loading levels (Fig. 5.5A), as expected. Prior reports from Rivera-Tarazona et al. have indicated that loading covalently crosslinked hydrogels with increasingly high cell loading levels can lead to the escape of cells from the hydrogel matrix.¹⁸¹ To check for cell release from the hydrogel during culture, the supernatant fluid was sampled after 72 h culture in M9 media for OD_{600} measurements, as done in the previous experiment. Low OD_{600} values were measured, indicating that cells were not released from the hydrogel at any cell loading level (Fig. C.11). At low cell loading levels (1.0×10^3 to 1.0×10^4 cells per μL hydrogel), cells within the hydrogels displayed growth trends according to the number of cells initially present (Fig. 5.5B). Similar lag phases were measured, and OD_{600} values at 72 h increased with the initial number of cells loaded,

indicating increased biomass accumulation within hydrogels as cell loading levels increased. Corresponding surfactin levels generated from this range of cell loading also increased with the number of cells loaded, specifically between 2.5×10^3 to 1.0×10^4 cells per μL hydrogel (Fig. 5.5C). These trends indicate that at lower cell loading levels, cells initially encapsulated within the hydrogel were able to develop without interference from surrounding aggregates within the hydrogel.

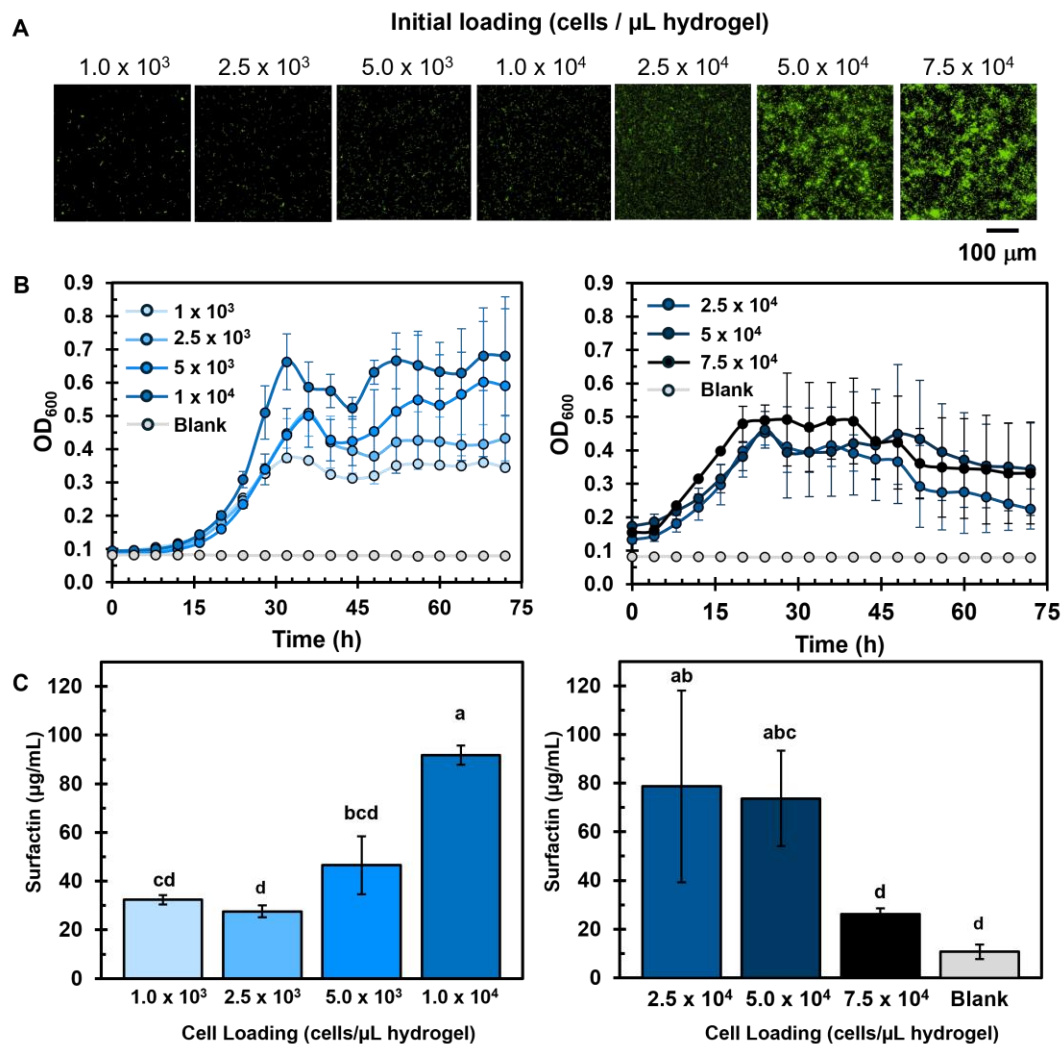


Figure 5.5. (A) 10 $\times$ objective image montage of H9.5 hydrogels with encapsulated *B. subtilis* 21332 at different initial cell loading densities (cells/ μL hydrogel). (B) Growth kinetics of *B. subtilis* encapsulated within H9.5 hydrogels at varied cell densities. (C) Corresponding surfactin production at 72 h. Error bars indicate standard deviation and differing letters indicate statistical significance ($p < 0.05$, $n=3$).

Distinctly different behavior was observed when hydrogels were encapsulated with cells at higher loading levels (2.5 to 7.5×10^4 cells per μL hydrogel). Here, growth kinetics were marked by a minimal lag phase and rapid growth phase to reach peak cell levels and the stationary phase after 24 h culture, followed by slow decay between 24 and 72 h (Fig. 5.5B). Unlike the lower cell loading range, surfactin levels did not increase with cell densities at these higher loading levels. In fact, at the highest loading level (7.5×10^4 cells per μL hydrogel), surfactin levels showed a significant decrease (Fig. 5.5C). These trends indicate that loading hydrogels at high cell densities can cause interference between neighboring cell aggregates during culture. It was likely that nutrients within the culture media were rapidly consumed, leading to unsustainable growth and limited surfactin production. Similar observations have been made in other studies that investigate bacteria growth within spatially confined systems. For example, Hansen *et al.* seeded *P. aeruginosa* cells at different inoculum levels into microwells of sub-picoliter volumes and monitored resulting cell growth during culture to determine that growth becomes inhibited as cells are seeded at excessively high inoculum levels.¹⁸² Taken together, these results demonstrate the importance of carefully determining optimal cell loading levels in accordance to the nutrient and physicochemical hydrogel environments; just as low cell densities can limit bioproduction, overloading cells within the hydrogel matrix can lead to interference between developing cellular aggregates to also limit or even inhibit bioproduction.

5.4.5. Encapsulated *B. subtilis* Endospores Bioproduction After Heat Stress.

In nutrient-poor environments, *B. subtilis* can form dormant endospores with a multilayered architecture that preserves genomic integrity and enables long-term survival. By withstanding harsh environments common during ELM fabrication or storage, endospores hold decisive advantages for real world application. Previous work by Gonzalez *et al.* demonstrated

that *B. subtilis* endospores embedded within 3-D printed agarose hydrogels could be germinated to retain cellular function after exposure to desiccation, organic solvents, high acidity (pH=1), ultraviolet and γ -irradiation, and high (75-100°C) temperatures.¹⁸³ However, prior studies have not investigated surfactin production from spore-laden hydrogels. This motivated the investigation of *B. subtilis* spores within $H_{9.5}$ hydrogels, optimized from the prior studies, to achieve surfactin production after dehydration and exposure to elevated temperatures, conditions relevant for ELM storage.

Prior to hydrogel encapsulation, a live-dead assay was used to characterize the treatment of vegetative *B. subtilis* cells with 2×SG sporulation media to obtain endospores. The live-dead stain was first performed on vegetative *B. subtilis* populations (no sporulation media) before and after treatment with a heating bath (75°C, 20 min). Entire *B. subtilis* cell populations appeared live before the heat treatment and dead afterwards (Fig. 5.6A(i) and (ii)), validating both the live-dead stain and the heat treatment protocol for removing live, vegetative cells. Next, populations of *B. subtilis* treated with 2×SG sporulation media were also exposed to the heat bath. Live-dead staining indicated that $96 \pm 1\%$ of cells remained alive, with the remaining fraction of intact dead cells often appearing elongated (Fig. 5.6A(iii)). This indicates the successful formation of *B. subtilis* endospores using the 2×SG sporulation media.

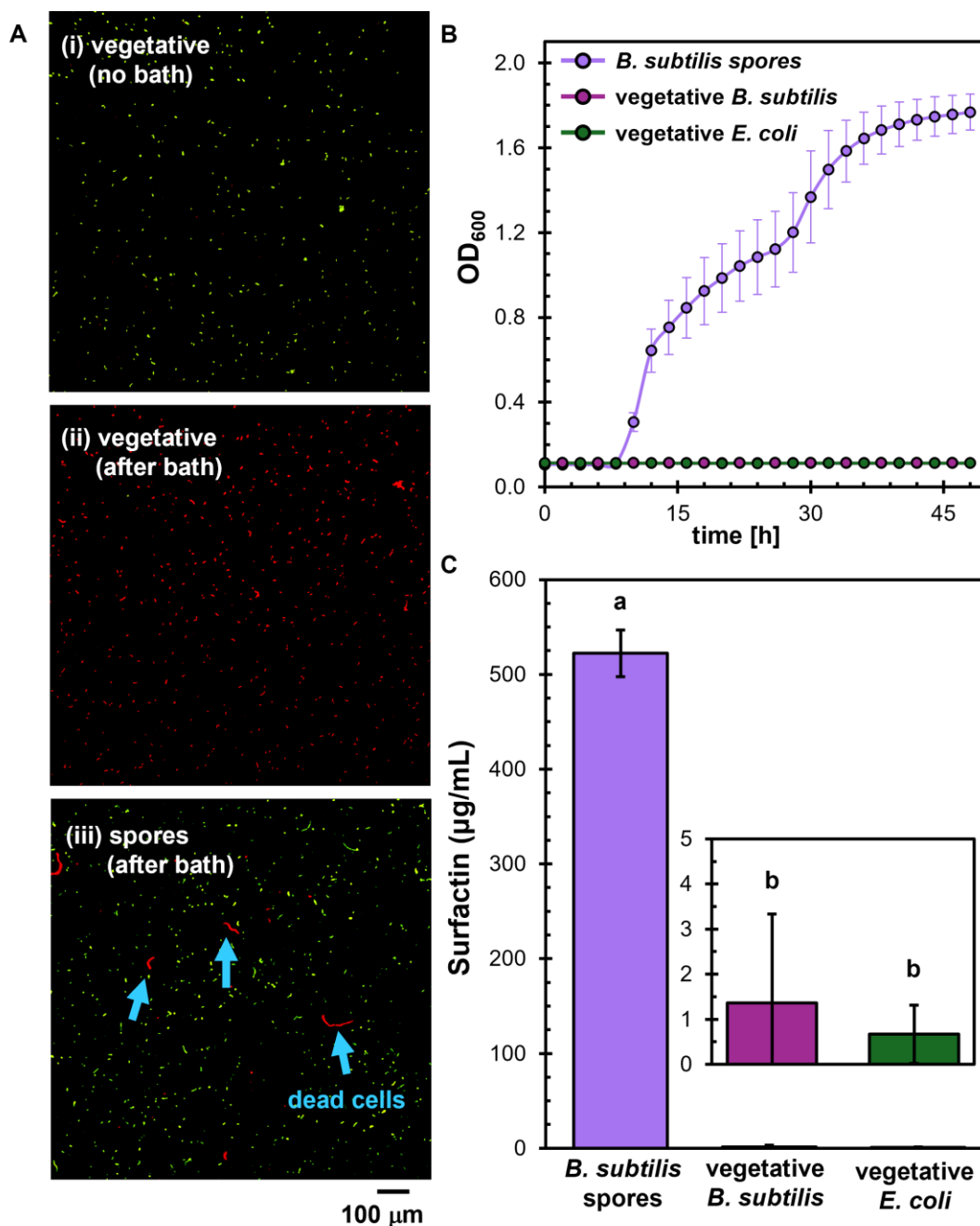


Figure 5.6. (A) 10 $\times$ green-red fluorescent images of *B. subtilis* after live-dead staining of (i) vegetative cells before and (ii) after exposure to the water bath (75 $^{\circ}\text{C}$, 20 min) and (iii) endospores after exposure to the same water bath. Green denotes live cells and red denotes dead cells, images were adjusted to maximize contrast in the figure. (B) Growth kinetics of encapsulated *B. subtilis* spores and *B. subtilis* / *E. coli* vegetative cells (negative controls) within $H_{9.5}$ hydrogels after double heat-treatment. (C) Surfactin quantification of *B. subtilis* endospores and *B. subtilis* / *E. coli* vegetative cells in $H_{9.5}$ hydrogels. Error bars indicate standard deviations and differing letters indicate statistical significance ($p < 0.05$, $n=3$).

Following validation of the sporulation protocol, *B. subtilis* endospores were placed in a 75 °C water bath for purification, then encapsulated within *H9.5* hydrogels at 1×10^4 cells per μL hydrogel. To impose dehydration and temperature stress on the material, hydrogels were then exposed to a high temperature of 80 °C in an oven for 25 min, conditions that fully deactivate vegetative *B. subtilis*.¹⁸⁴ Two negative control hydrogels were also performed. The first control used *H9.5* hydrogels loaded with vegetative *B. subtilis* cells not treated with 2×SG sporulation media, testing the ability of *B. subtilis* to adapt and survive the dehydration and temperature stress within the material without prior sporulation. The second control used *H9.5* hydrogels loaded with *E. coli*, a Gram-negative, non-sporulating bacteria which grew as expected within hydrogels that were not heat-treated (Fig. C.12). All hydrogels were baked (80 °C, 25 min) then placed in TSB media, and growth kinetics and 48 h surfactin production of each were measured (Fig. 5.6B and C), as in the previous studies. After an 8 h lag period, growth commenced only in hydrogels containing *B. subtilis* endospores, with a 48 h surfactin concentration of $522 \pm 25 \mu\text{g/mL}$ (Fig. 5.6C). The extended lag phase noted here is comparable to *B. subtilis* LMG16 spores exposed to temperatures ranging from 60-100 °C (Supp. Fig. 28 in Gonzalez et al.)¹⁸³, and can be attributed to the time necessary to re-activate the spores. A second growth phase was observed after 28 h and could be due to cells being partially released into the growth media above the hydrogel, likely due to mechanical hydrogel fracturing of the hydrogel due to high bacteria proliferation in a nutrient rich media, a result also seen by Kalairaj et al.⁵⁹ In contrast, no growth was measured in vegetative *B. subtilis* or *E. coli* cells after heat treatment (Fig. 5.6B), and no significant amount of surfactin was measured (Fig. 5.6C). The combined results indicate that the spore-laden hydrogel materials can produce surfactin after exposure to dehydration and heat stress, creating a more robust ELM material. While only short periods of environmental stress were investigated here, it is expected

that these materials can survive similar stress for longer periods of time, as *B. subtilis* endospores can remain dormant indefinitely (i.e. millions of years¹⁸⁵).

5.5 Conclusions

These studies provide three major findings that advance *B. subtilis*-PEG hydrogel systems towards ELM applications. First, hydrogel mesh size was found to be a regulator of microbial function, both with respect to growth and the amount of surfactin produced. Across all average mesh sizes examined ($\xi = 9\text{-}19\text{ nm}$), confinement within the hydrogel network boosted the production of surfactin compared to equivalent, unconfined cultures in bulk media. As low product yield and corresponding high production costs have traditionally limited surfactin production in *B. subtilis* bulk cultures,¹⁸⁶ this finding uncovers a clear advantage of cellular nanoconfinement within hydrogels. The increased levels of surfactin production noted with decreasing mesh sizes was likely due to the more uniform initial distribution of single cells after encapsulation to drive the formation of larger cell aggregates, combined with the effects of increased nanoconfinement. The latter includes a stiffer hydrogel matrix and higher diffusion limitations – evidenced by the 7-fold drop in diffusivities measured across these hydrogels - to activate the quorum-sensing system in *B. subtilis* necessary for surfactin production. Second, cell loading studies demonstrated that while low cell densities result in limited surfactin production as expected, excessive cell loading also diminishes surfactin production due to unsustainable growth and reduced nutrient availability. This emphasizes the need for careful selection of cell loading levels in accordance with the nutrient and physicochemical environment to optimize material performance. Finally, *B. subtilis* endospores were loaded into hydrogels at optimized conditions to demonstrate production of surfactin after exposure to dehydration and heat stress. These fundamental insights lay the foundation for future design of PEG hydrogels in ELM applications that require stable

encapsulation of bacteria, protection of bacteria from chemical and mechanical stresses, and optimized production of antimicrobial molecules or other secondary metabolites.

5.6 Acknowledgments

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Chapter 6 - Biofunctional PEG-Hydrogel Coatings for Protection of Bacteria from Reactive Oxygen Species

6.1 Overview

The loss of cell function due to oxidation is relevant to many systems across biomedical, environmental, and bioelectrochemical applications, creating a need to develop materials capable of protecting encapsulated cells while maintaining viability and function. Here we examine the response of encapsulated *Bacillus subtilis* cells, a model Gram-positive bacterium that is generally regarded as safe and found in the gut, soil, and plant microbiomes, when exposed to varying challenge concentrations of hydrogen peroxide (H_2O_2). Hydrogels formed through a base-catalyzed Michael-type addition reaction between two-arm PEG vinyl sulfone (PEGVS) and four-arm PEG tetrathiol (PEGTT) remained stable for long periods of time (>60 days) when exposed to varying H_2O_2 concentrations. Following measurement of the H_2O_2 effective diffusion coefficient and the apparent Michaelis-Menten kinetics of encapsulated catalase, a one-dimensional reaction-diffusion model was developed to guide subsequent enzyme loading using an empirical exposure threshold established from hydrogels with no encapsulated catalase. The results show that co-immobilization of enzymes and microbial cells within PEG hydrogels creates protective materials that reduce exposure to environmental stressors while providing a physicochemical environment that supports bacterial proliferation and bioproduction. Following a two-h challenge with 0.5% w/v H_2O_2 , hydrogels containing 20–200 U of catalase supported bacterial recovery with no significant difference in production of surfactin, a cyclic lipopeptide with antibacterial and anticancer properties, across recovered hydrogels. Agreement between experimental results and the predicted protected hydrogel volume indicates that this model can be

used to optimize hydrogel geometry, thickness, and enzyme loading based on predicted chemical exposure and guide the rational design of future engineered living hydrogel (ELH) applications.

KEYWORDS: Engineered living materials, hydrogels, bacteria, hydrogen peroxide, enzymes, protection

6.2 Introduction

Engineered living hydrogels (ELHs) integrate live microorganisms into hydrogel scaffolds to create biomaterials with dynamic function adaptable to the external environment.⁶ This enables a range of novel applications, from cancer and antimicrobial therapies to environmental sensors to wastewater treatment.¹⁸⁷⁻¹⁸⁹ Due to their metabolic diversity, rapid growth rates, and genetically-programmability, bacteria are often used as the living component in ELHs.^{190, 191} Hydrogels, with high water content and flexible chemical design features, enable stable encapsulation and diffusive exchange of nutrients and small molecules to support bacteria viability and proliferation.^{2, 192} ELHs commonly use hydrogels from naturally occurring materials (e.g. gelatin, alginate) or synthetic polymers (e.g. poly(ethylene glycol) (PEG), polyacrylamide, or elastomer-hydrogel hybrids), with the latter offering distinct advantages that include highly tunable mechanical properties, homogenous gel networks, control of network stability and degradation, and incorporation of stimulus-responsive moieties into the hydrogel structure to enable SMART materials.^{6, 192}

Even with careful selection of the hydrogel material, ELH applications often become limited when exposed to non-ideal chemical environments which can disrupt cellular metabolism and destroy cells to reduce and eventually eliminate ELH functionality.^{14, 81, 193} This includes extreme pH, toxic chemical shocks, and exposure to reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl ($\bullet OH$) radicals. ROS are commonly encountered in ELH therapeutic applications that aim to treat infection sites, tumors, and

gastrointestinal inflammatory disorders, where activated immune cells and stressed host tissues produce locally high ROS molecules (ex. H_2O_2 , in the range of 50-100 μM)¹⁹⁴ as part of the inflammatory and defense response.¹⁹⁵ Beyond biomedical applications, biological wastewater treatment systems can utilize ELHs to stabilize microbial communities,⁶ however these materials encounter ROS in both activated sludge wastewater treatment processes (H_2O_2 , 0.8-290 μM) and anaerobic treatment processes (H_2O_2 , 0.27-0.29 mM per cycle).¹⁹⁶ Similarly, in bioelectrochemical systems, the activity of biofilms established at an anode interface can become limited due to H_2O_2 generation at the cathode (147-882 mM).¹⁹⁷ While hydrogels have been widely used as coatings over anodic biofilms to improve their activity,¹⁹⁸ cells within hydrogel-anode interfaces remain susceptible to ROS. These examples, which span a diverse range of applications, highlight the broad need to advance the capabilities of ELHs towards detoxification of ROS to protect microbial viability and activity.

As ROS is ubiquitous in natural ecosystems, microbes have adapted both intercellular and extracellular strategies to withstand oxidative stress. A striking example comes from *Salmonella* biofilms, which manages H_2O_2 from immune cells through the combined action of extracellular polymeric substances (EPS) and antioxidant enzymes.¹⁹⁹ The EPS matrix creates a diffusional barrier to slow the transport of H_2O_2 to embedded cells, while catalase enzymes secreted throughout the EPS layer decompose infiltrating H_2O_2 into water and oxygen before it reaches cells, providing community-level protection against ROS. Inspired by this coupled reaction-diffusion protection strategy, here we develop synthetic hydrogel materials that mimic this approach. PEG-based hydrogels are particularly well-suited for this task, as PEG is chemically and biologically inert, non-adhesive to bacteria, and can encapsulate bacteria uniformly and at high viability.⁸³ Another enabling feature is that PEG macromers are commercially available across a

range of molecular weights, allowing for the design of hydrogels that provide varied resistances to mass transfer.^{141, 200} For example, we recently used step-growth, thiol-acrylate PEG systems from PEG macromer molecular weights to generate hydrogels with a range of nanoscale mesh sizes (9-19 nm), providing a 7-fold range of hindered diffusivities that was used to manipulate bacterial growth and function.⁸⁴

Here we couple catalase enzymes into diffusion-limited PEG-based hydrogels to demonstrate protection of bacteria in high ROS environments where bacteria are typically not viable (Fig. 6.1).²⁰¹ While various enzymes have been encapsulated within hydrogels for mammalian, therapeutic, and biocatalytic applications, use of enzymes to protect encapsulated bacteria in ELH systems is comparatively less explored.²⁰²⁻²⁰⁴ Hydrogels are polymerized with 2-arm PEG-vinyl sulfone (PEGVS) and 4-arm PEG tetrathiol (PEGTT) to offer gel stability in the presence of H₂O₂. These hydrogels contain pendant thiol throughout, which are then targeted for covalent immobilization of bovine liver catalase (BLC) using acrylamide-PEG-NHS molecules.^{202, 205} The activity of encapsulated and covalently immobilized BLC was then measured to inform a one-dimensional, transient diffusion model that predicts H₂O₂ concentrations throughout the hydrogel. The viability and growth of *Bacillus subtilis*, a model organism for ELM applications due to its generally regarded as safe designation and production of beneficial molecules,^{206, 207} was then investigated after exposure to varied concentrations of H₂O₂. The results reveal that varying the concentration of encapsulated catalase modulates growth and metabolic productivity of *B. subtilis* and can be used for complete protection of cells in high ROS environments. These findings demonstrate the design of PEG-based hydrogels to reduce diffusive mass transfer while employing enzymatic catalysis to detoxify the cellular microenvironment, a concept that can be extended towards the design of ELHs for function in other toxic environments.

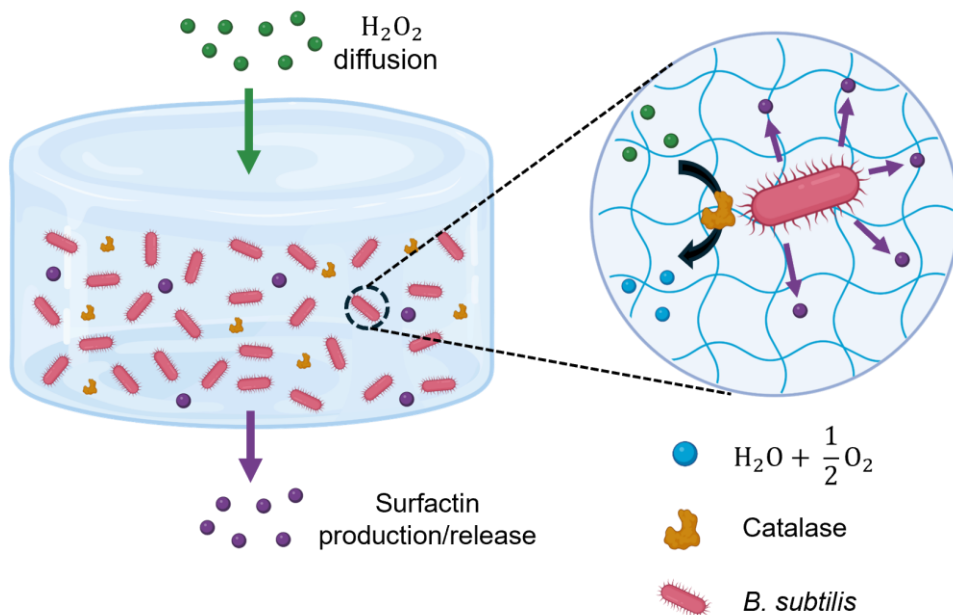


Figure 6.1. Catalase-functionalized engineered living hydrogel for reducing H₂O₂ cell exposure while maintaining bacterial surfactin production

6.3 Materials and Methods

6.3.1 Material Preparations.

Poly(ethylene glycol) divinyl sulfone (PEGVS, 3.4 kDa), poly(ethylene glycol) diacrylate (PEGDA, 3.4 kDa), and acrylamide-poly(ethylene glycol)-succinimide ester (ACA-PEG-SCM, 3.4 kDa) were purchased from Creative PEGWorks. Poly(ethylene glycol) tetrathiol (PEGTT, 5 kDa) was purchased from NOF America. Bovine liver catalase (herein referred to as catalase) was purchased from Millipore Sigma (C3155). *Bacillus subtilis* strain 21332 was obtained from the American Type Culture Collection (ATCC) and maintained as 50% glycerol stocks stored at -80°C . Alexa fluor 488 NHS ester was purchased from Thermo-Fisher Scientific. Unless otherwise specified, all other chemicals, media components, and reagents were obtained from Sigma-Aldrich. For preparation of PBS (0.1 M), NaH_2PO_4 (1,567 mg) and Na_2EDTA (187 mg) were dissolved in 90 mL ultrapure water and the pH was adjusted to 8.0 using 5 M NaOH (Fisher

Scientific). The resulting solution was sterile filtered and stored at room temperature until use. For bacteria encapsulated hydrogels, ATGN-phosphate buffer was used. 1,506 mg of sodium phosphate monobasic dihydrate was dissolved in 95 mL of 2× AT minimal medium, supplemented with glucose (Fisher Scientific) and ammonium sulfate ((NH₄)₂SO₄, Fisher Scientific), as described by Morton and Fuqua.¹⁵⁸ The pH was adjusted to 8.0 using aqueous 5 M NaOH, and stored at -20°C after sterile-filtering. Bacterial growth experiments involving bacteria-encapsulated hydrogels used M9 minimal medium prepared according to Miller.¹⁶⁰ The medium consisted of 6 g/L Na₂HPO₄, 3.0 g/L KH₂PO₄, 0.5 g/L NaCl (Fisher Scientific), 1.0 g/L NH₄Cl (Fisher Scientific), sterilized by autoclaving prior to supplementing with 10 mL of 100 mM MgSO₄, 20% glucose, 10 mM CaCl₂ (Fisher Scientific), and 100 mM thiamine HCl. Various H₂O₂ solutions were prepared by diluting a 50 wt/v% stock solution with ultrapure water to the desired concentration prior to use.

6.3.2 Hydrogel Fabrication and Estimation of Average Mesh Size.

Hydrogels were synthesized through Michael-type addition reactions between vinyl sulfone and thiol groups under basic conditions (pH 8). For a 100 µL PEGVS-PEGTT hydrogel volume, 50 µL PBS was first mixed with 22.4 µL of PEGVS (49 mM) dissolved in ultrapure water, then 27.6 µL of PEGTT (20 mM) dissolved in ultrapure water was added to achieve an equimolar ratio of vinyl sulfone to thiol groups. Solutions were incubated at room temperature for 30 min to allow for gelation. Hydrogel volumes of 36, 50, 100, 150, or 200 µL were prepared by scaling the volumes of PEG macromers accordingly. The same procedure was followed to fabricate PEGDA-PEGTT, with a PEGDA precursor solution replacing the PEGVS precursor solution.

Average hydrogel mesh size was determined using Equilibrium Swelling Theory as described by Canal and Peppas.⁹⁷ Hydrogels at 50 µL volumes were prepared and placed in 10

mL of ultrapure water (for swollen weight measurement) or 2 mL glass jars placed in an oven at 60°C for 24 h (dry weight measurement). The swollen and dry hydrogels were then weighed using a digital analytical balance (U.S. Solid, ± 0.1 mg precision). Excess water was removed from swollen hydrogel by dabbing the hydrogel on a KimWipe prior to weighing. A minimum of $n=3$ independent hydrogel replicates were used for swollen or dry weight measurements. An example calculation including the equations and PEG-specific constants can be found in Reed et al. (S1.0).⁸⁴

6.3.3 Hydrogel Characterization with H₂O₂ Exposure.

Long-term stability of PEGVS-PEGTT hydrogels against H₂O₂ was analyzed using gravimetric analysis as described by Paez et al.²⁰⁸ For comparison, PEGDA-PEGTT hydrogels containing a hydrolytically labile ester groups were also used as a chemically distinct comparison network.²⁷ All experiments used 200 μ L hydrogels in 2 mL glass jars. After gelation, 1 mL of ultrapure water was pipetted onto each hydrogel and incubated for 24 h to allow for equilibrium swelling of the hydrogel. Water was then removed and replaced with 1 mL of H₂O₂ solutions at varied concentrations (0.01, 0.1, and 1.0% w/v or 2.94, 29.4, and 294 mM, respectively), and hydrogels were incubated for 65 days. Hydrogel mass was recorded at varied time points (M_t) and normalized to the initial mass recorded (M_0). An increase in the M_t/M_0 ratio from 1.0 indicates increased swelling in the hydrogel due to degradation of covalent crosslinks and subsequent water influx to the hydrogel.²⁰⁸ A minimum of $n=3$ hydrogels was used for each condition.

Fourier transform infrared (FTIR) analysis was performed with a Cary 630 FTIR spectrometer to investigate chemical changes within PEGVS-PEGTT hydrogels due to H₂O₂ exposure. A full spectral range (4,000- 650 cm^{-1}) was collected for each sample with background and sample scans set to 128 with 4 cm^{-1} resolution using air as a background. Prior to FTIR measurement, 50 μ L hydrogels were formed on parafilm squares and placed in 60 mm plastic petri

dishes with 10 mL of varied H₂O₂ concentrations (1, 2, 5, and 10% w/v) and exposed for 72 h and washed before measuring. One set of control hydrogels was exposed to PBS only. SpectraGryph V1.2(ACD Labs) was used to analyze/smooth all spectra taken.

6.3.4 Experimental Determination of Diffusive and Kinetic Model Parameters.

The effective diffusion coefficient of H₂O₂ through PEGVS-PEGTT hydrogels ($D_{H_2O_2,eff}$) was experimentally determined using a small molecule desorption approach adapted from previous hydrogel diffusion studies.^{81, 84, 209} Cylindrical 150 μ L hydrogels were first formed inside 2 mL glass vials and incubated for 24 h in 1 mL ultrapure water to allow for swelling. The ultrapure water was then removed, and hydrogels were subsequently equilibrated with 1 mL of 1% w/v H₂O₂ for 24 h for H₂O₂ loading. The H₂O₂ solution was then replaced with 1.0 mL ultrapure water to initiate H₂O₂ release from the hydrogel. 2 μ L aliquots of the supernatant were collected at selected time points and analyzed using a Nanodrop One UV–Vis spectrophotometer at 240 nm (Fig. D.1A). H₂O₂ concentrations were calculated using a calibration curve constructed over a range of H₂O₂ standards (0.01-2.5% w/v, Fig. D.1B). H₂O₂ release was normalized to the equilibrium concentration at the final time point, (C_t/C_∞), where C_t is the concentration measured at time t and C_∞ is the equilibrium concentration. For $\frac{C_t}{C_\infty} \leq 0.5-0.6$, the early-time approximation of Fick's second law for one-dimensional diffusion was used to estimate $D_{H_2O_2,eff}$ at each time point, and the values within this range were averaged.¹³⁶

$$\frac{C_t}{C_\infty} = 4 \left(\frac{D_{H_2O_2,eff} t}{\pi L^2} \right)^{1/2} \quad (1)$$

where L is the swollen hydrogel thickness. Swollen hydrogel thicknesses used for diffusion calculations were measured using FIJI (ImageJ V2) to analyze images of the hydrogel.

To quantify kinetic parameters, catalase enzymes were added to PEG hydrogels to determine the apparent H_2O_2 degradation kinetics when immobilized within the hydrogels. Hydrogel formulations contained 3,520 U catalase and were prepared in 200 μL gel volumes inside 2 mL plastic cuvettes. This level of catalase loading was selected to ensure measurable H_2O_2 depletion over the 5 min assay period, enabling quantification of the apparent Michaelis Menton kinetic parameters. Following the same experimental setup as diffusivity measurements, catalase-loaded hydrogels were submerged in 2 mL H_2O_2 solutions ranging from 0.1–1.0% (w/v) and the solutions were sampled over 5 min time intervals. H_2O_2 concentrations were quantified using 240 nm absorbance measurements and the calibration curve generated from H_2O_2 standards (Fig. D.1B). H_2O_2 depletion data was analyzed using an the apparent Michaelis-Menten model. Because enzyme immobilization within the hydrogel can alter the observed kinetics relative to free catalase, the fitted parameters were interpreted as apparent rather than intrinsic catalytic constants. Simultaneous fitting of H_2O_2 depletion curves yielded the apparent kinetic parameters $V_{max, app}$ and $K_{m, app}$ and represent the effective H_2O_2 consumption from encapsulated catalase inside the hydrogel. The apparent Michaelis-Menten parameters determined from these conditions served as the reference kinetic parameters for all reaction-diffusion simulations. To apply the apparent catalytic activity to hydrogels loaded with different levels catalase , an effective activity coefficient ($\eta_{\text{cat,hydrogel}}$) was calculated from $V_{max, app}$ and the theoretical V_{max} ($V_{max, theoretical}$) which taken according to the manufacturer's reaction rate of 1 U degrades 1 μmol of H_2O_2 per minute.

$$\eta_{\text{cat,hydrogel}} = \frac{V_{max, app}}{V_{max, theoretical}} (2)$$

This coefficient was used to scale the apparent catalytic activity proportionally with catalase loading in the reaction-diffusion model. To simulate hydrogels of varied volume and varied units of encapsulated catalase, $V_{max, model}$ was calculated according to:

$$V_{max,model} = \eta_{cat,hydrogel} * \frac{U_{gel}}{Vol_{gel}} \quad (3)$$

Where U_{gel} and Vol_{gel} are the catalase units loaded and hydrogel volume, respectively.

6.3.5 Reaction-Diffusion Modeling of H₂O₂ Transport Through Hydrogels.

Using the experimentally quantified values for $D_{H_2O_2,eff}$, $V_{max,app}$, and $K_{m,app}$, a one-dimensional reaction-diffusion model was developed in Python to estimate the concentration profile of H₂O₂ throughout PEG hydrogels ($C_{H_2O_2}(z,t)$), both with and without catalase. The model geometry represented a cylindrical 100 μ L hydrogel within a 48-well plate exposed to a finite H₂O₂ solution, which corresponds to the experimental setup used. The model uses a numerical solution Fick's second law coupled with the apparent Michaelis-Menten kinetics:^{210, 211}

$$\frac{\partial(C_{H_2O_2}(z,t))}{\partial t} = D_{H_2O_2,eff} \frac{\partial^2 C_{H_2O_2}(z,t)}{\partial z^2} - \frac{V_{max,model} * C_{H_2O_2}(z,t)}{K_{m,app} + C_{H_2O_2}(z,t)} \quad (4)$$

Boundary conditions included a finite, time-dependent H₂O₂ concentration at the hydrogel-reservoir interface ($z=0$), represented by:

$$C_{H_2O_2}(0,t) = C_{H_2O_2,soln}(t) \quad (5)$$

and a no-flux boundary condition at $z=z_L$ which represents the hydrogel-well interface that is impermeable to H₂O₂, represented by:

$$\frac{\partial C_{H_2O_2}(z_L,t)}{\partial z} = 0, t > 0 \quad (6)$$

Finally, the initial condition assumes that no H₂O₂ is initially present throughout the hydrogel:

$$C_{H_2O_2}(z,0) = 0, 0 \leq z \leq z_L \quad (7)$$

The initial time point ($t=0$) corresponds to the time at which the H₂O₂ solution is placed over the hydrogel. Hydrogel thickness (z_L) was calculated according to the volume of a cylinder with the cross-sectional area calculated from the standard diameter of a 48-well plate. The

governing reaction-diffusion equation was solved numerically using an explicit forward-time, centered-space finite difference method in Python. H₂O₂ concentrations were updated at each time step using the diffusion and Michaelis-Menten reaction terms.

6.3.6 Covalent attachment of catalase to the hydrogel network.

Catalase was functionalized with AF488 and ACA-PEG-SCM (3.4 kDa, CreativePEGWorks) to generate fluorescently labeled, hydrogel-reactive catalase following the Molecular Probes Handbook protocol for amine-reactive labeling.²¹² 10 mg catalase was first dissolved in 1 mL of 0.1 M sodium bicarbonate buffer (pH 8.3). 1 mg of AF488 was dissolved in 100 μ L anhydrous DMSO and 50 μ L of the resulting stock solution was added to the catalase solution under gentle stirring. ACA-PEG-SCM was dissolved in ultrapure water and added at an equivalent molar concentration to the AF488. The reaction mixture was protected from light and incubated for 1 h at room temperature with continuous stirring. Following conjugation, unreacted AF488 and ACA-PEG-SCM were removed by centrifugal ultrafiltration using 10 kDa molecular weight cutoff filters (Amicon Ultra-4, Millipore Sigma) with repeated PBS (pH 7.4) buffer exchange. Purified conjugates were recovered from the retentate, protected from light, and stored at 4 °C until use.

A confocal laser scanning microscope (Zeiss LSM PASCAL) was used to image bacteria and fluorescently labeled catalase within hydrogels following encapsulation and throughout culture. For imaging, green fluorescent protein (GFP)-expressing *B. subtilis* strain 168 cells were encapsulated within approximately 25 μ m thick PEG hydrogels chemically attached to 18 $\times$ 18 mm glass coverslips prepared as previously described. Z-series were acquired using a Plan-Neofluar 20 $\times$ /0.5 Ph2 objective at a pixel resolution of 1024 $\times$ 1024 with GFP fluorescence, Alexa Fluor 633 fluorescence, and brightfield channels collected over 10 optical sections. The voxel

dimensions were $0.45 \times 0.45 \times 2.48 \mu\text{m}$ (x, y, z). Maximum-intensity projections and orthogonal (x–z) reconstructions were generated in Fiji (ImageJ) to visualize the spatial distribution of encapsulated bacteria and catalase throughout the hydrogel thickness.

6.3.7 Bacterial encapsulation, dosing, culture, and surfactin quantification.

For all encapsulation experiments, *B. subtilis* was cultured overnight in tryptic soy broth (TSB) media at 32 °C and 215 rpm. After culturing, cells were centrifuged (4,400 rpm, 20 min), the supernatant was removed, and cells were resuspended in an equivalent volume of PBS (pH 8.0). A detailed protocol for cell encapsulation in a similar PEG-based hydrogel is previously described in Reed et al.⁸⁴ Immediately following the addition of PEGTT, the precursor mixtures were transferred to individual wells in a 48-well plate and were incubated for 30 min at room temperature, which was sufficient for gelation.²¹³

After hydrogel formation, 1.0 mL of H₂O₂ at concentrations between 0.1 and 1% w/v was pipetted onto the hydrogel and incubated for 2 h. After this, the H₂O₂ solution was removed and 1 mL of ultrapure water was added on top of each hydrogel to remove residual H₂O₂ diffused into the hydrogel. After 1 h, the water was removed and replaced with 1 mL of M9 minimal medium and bacterial growth was measuring by monitoring the optical density of each well at 600 nm (OD₆₀₀) using a microplate reader (Epoch2, BioTek). Cultures were maintained at 32 °C and OD₆₀₀ measurements were collected every 10 min with a 1 s linear shake at 567 cycles min⁻¹ prior to each measurement. Growth curves were fitted to the Gompertz equation, an asymmetrical sigmoidal function, using Python, and the maximum and minimum points of the fitted curve's second derivative were used to determine the endpoints and durations of the lag and exponential growth phases according to Buchanan and Cygnarowicz.¹⁶⁴ To compare culture

progression across conditions, the observed OD₆₀₀ value at different stages of culture (24, 48, and 72 h) was also recorded for each replicate and used for subsequent analysis.

It is important to note that the H₂O₂ concentrations used here are well above concentrations characteristic of many applications, but they were required to generate the necessary range of H₂O₂ exposures throughout the relatively thick (~1 mm) hydrogels for real-time growth track experiments using a 96-well plate reader. Consequently, these H₂O₂ concentrations should be interpreted as geometry-specific experimental conditions used to validate the approach, rather than an application-specific oxidative environment.

For surfactin production quantification, the fluid above the hydrogels was collected at the end of the 72 h culture and centrifuged (4,400 ×g, 20 min) to obtain cell-free culture fluid (CFCF). Surfactin concentrations were quantified using the cetylpyridinium chloride–bromothymol blue (CPC–BTB) colorimetric assay described previously.^{84, 166} A 0.20 mM CPC solution and a separate 0.20 mM BTB solution were prepared separately in 0.1 M PBS (pH 8.0). Equal volumes of the CPC and BTB solutions were then mixed in a glass tube and vortexed thoroughly to yield a uniform dark-green CPC–BTB indicator solution. This solution was freshly prepared immediately prior to each assay. Equal volumes of CPC–BTB reagent and CFCF were combined in a 96-well plate, agitated for 5 min in a microplate reader at 567 cycles·min⁻¹, and absorbance was measured at 600 nm. Surfactin concentrations were determined from calibration curves prepared using purified surfactin standards diluted in M9 minimal medium, and are available in Appendix C (Fig. C.3) of Reed et al.⁸⁴

6.3.8 Statistical Analysis.

Statistical analyses were performed using SAS software (SAS Institute Inc.). Differences between groups were evaluated using Student's *t*-tests and Tukey's multiple-comparison tests, with

statistical significance defined as $\alpha < 0.05$. Reported means and standard deviations were calculated from a minimum of three independent replicates ($n \geq 3$).

6.4 Results

6.4.1 PEGVS-PEGTT hydrogel chemical stability during H₂O₂ exposure.

Step growth PEG hydrogels formed by base-catalyzed Michael addition reactions can utilize a variety crosslinking chemistries, including thiol-acrylate, thiol-vinyl sulfone, and thiol-norbornene coupling, generating hydrogels that contain crosslinks with varied levels of chemical stability.²¹⁴ With the goal of stability in the presence of H₂O₂, vinyl sulfone-thiol crosslinking was investigated. The PEGVS-PEGTT hydrogels form spontaneously by mixing PEGVS and PEGTT macromers together at an equimolar VS:thiol ratio in basic conditions, forming thioether-sulfone bonds at each crosslink (Fig. 6.2A). As thiol-vinyl sulfone hydrogels are generally hydrolytically stable in aqueous environments, it was hypothesized that PEGVS-PEGTT hydrogels would also remain intact in the presence of the H₂O₂ oxidizing agent.^{27, 213} For side-by-side comparison, PEGDA-PEGTT hydrogels were also included, as these hydrogels contain thioether ester crosslinks that are readily susceptible to acid or base hydrolysis (Fig. 6.2B), and are also likely less stable in the presence of H₂O₂.²⁷

PEGVS-PEGTT and PEGDA-PEGTT hydrogels were challenged with increasing concentrations of H₂O₂ (0.01, 0.1, 1.0% w/v, pH 6.9, 6.0, and 5.2 respectively) during long term exposures (65 days), and the stability of each hydrogel was characterized by measuring the swelling ratio each day (Fig. 6.2C and 6.2D). Degradation is indicated by an increase in hydrogel mass at each time point relative to the initial mass (M_t/M_o), and is typically due to reduced crosslinking density which causes an influx of water into the hydrogel up to the point of reverse gelation and dissolution.¹³⁴ PEGDA-PEGTT hydrogels displayed rapid swelling in the presence of

all concentrations of H₂O₂. In fact, PEGDA-PEGTT hydrogels were unable to withstand 1.0% H₂O₂ for any amount of time and showed complete degradation and dissolution at 7 and 15 days for H₂O₂ concentrations of 0.1 and 0.01%, respectively. By contrast, PEGVS-PEGTT hydrogels remained stable across the entire 65-day period under 0.1 and 0.01% H₂O₂, where the M_t/M_0 showed no significant deviation. At the highest H₂O₂ concentration of 1.0%, slower swelling was observed during the first 20 days, however no significant change occurred during the remaining 45 days of the experiment, indicating that the crosslinked network structure remained intact. The PEGVS-PEGTT hydrogels had no noticeable change in stiffnesses after the 65-day exposure, regardless of H₂O₂ concentration.

The temporary swelling to a stable hydrogel observed at 1% H₂O₂, suggests that other chemical changes that did not result in bond cleavage or network degradation had occurred. To further investigate chemical changes occurring within PEGVS-PEGTT hydrogels on high H₂O₂ exposure, FTIR spectra was collected after 72 h incubation in H₂O₂ solutions ranging from 1-10% (Fig. 6.2D). The higher H₂O₂ concentrations were used here to provide hydrogels with exposures comparable to that from the previous swelling studies at lower incubation times. After exposure, a broad increase in peak intensity was observed across the fingerprint regions (1200-1650 cm⁻¹) with increasing H₂O₂ concentrations, which may reflect changes in network hydrophilicity and the local PEG-water environment.²¹⁵ Other notable changes were a shoulder that emerged in the C-H stretch region near 2,800 cm⁻¹ with increasing H₂O₂ concentrations, and the broad O-H stretching region near 3,300 cm⁻¹. These peaks are commonly attributed to changes in water content and hydrogen-bonding within the PEG network, and serve as indicators of hydrogel swelling.^{216, 217}

²¹⁵ We hypothesized that swelling was induced by oxidation of thioether-containing polymers, which increase hydrophilicity to promote water uptake within the hydrogel network.²¹⁸⁻²²⁰ Prior

work by Tan et al. and others has shown that thioethers can undergo a two-step oxidation process when exposed to H_2O_2 to yield sulfoxide and sulfone groups, with peaks near $1,030\text{-}1,055\text{ cm}^{-1}$ and $1,250\text{-}1,380\text{ cm}^{-1}$, respectively.^{221 222, 223} The PEGVS-PEGTT hydrogels did not display peaks at these wavenumbers after H_2O_2 exposure, however this does not indicate the absence of sulfur oxidation, as sulfur-related absorbances in poly(ether-thioether) materials are weak and often difficult to isolate,²²² and in PEGVS-PEGTT hydrogels, resolving these peaks can be complicated by overlapping vibrations from the PEG-rich network. Therefore, the concentration-dependent increase in absorbance across the fingerprint region may instead reflect changes in the hydrogel network due to swelling.²¹⁵ Thus, the FTIR spectra serve as a secondary indication of H_2O_2 - induced swelling, likely through sulfur oxidation. Taken together, the data shows that PEGVS-PEGTT remain chemically stable in H_2O_2 , even under high H_2O_2 exposures where mild swelling is observed.²²⁴

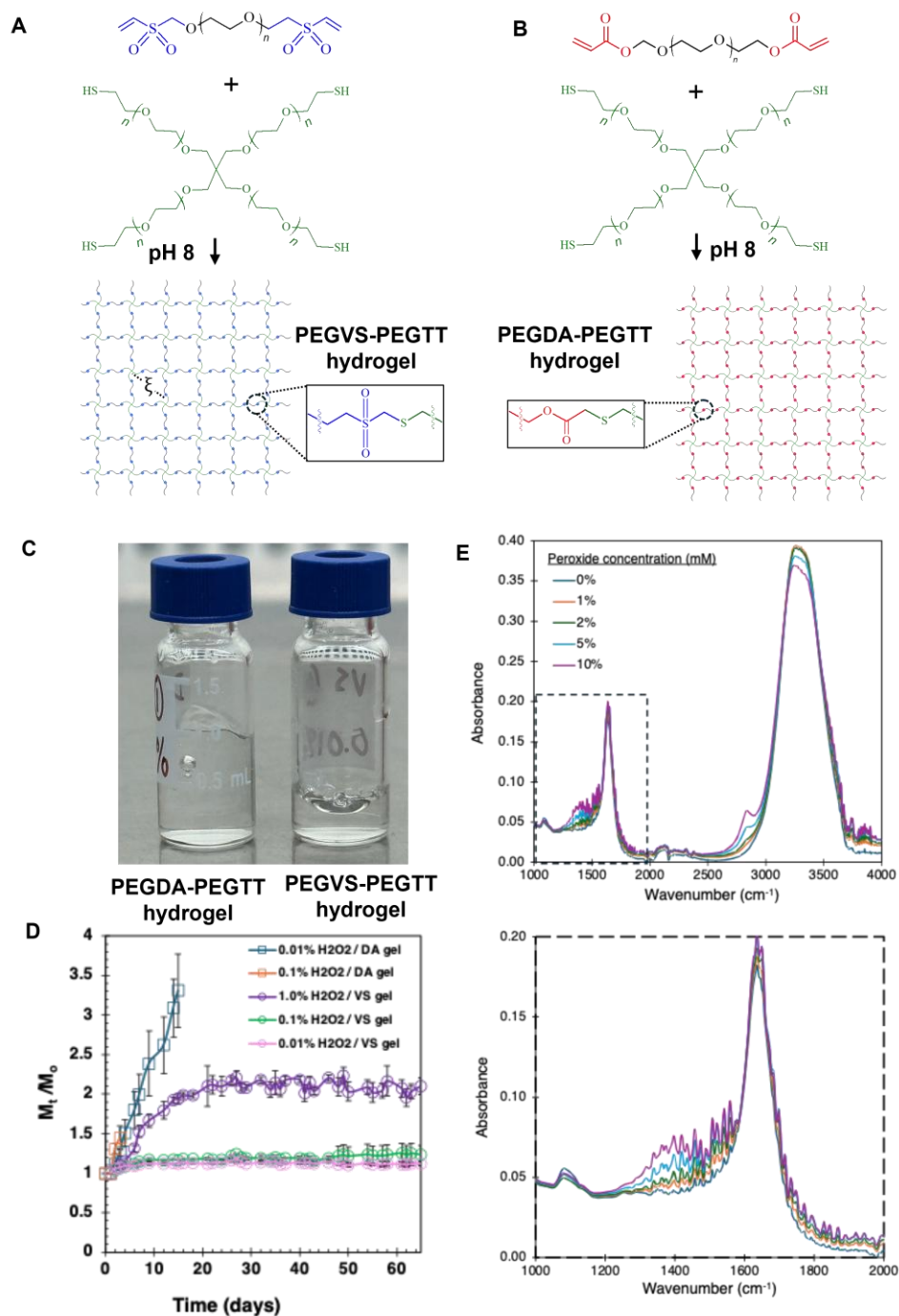


Figure 6.2. Formation of PEG hydrogels through Michael-type addition reaction between PEGTT and (A) PEGVS or (B) PEGDA macromers. (C) Image of PEGDA-PEGTT (left) and PEGVS-PEGTT (right) hydrogels at day 14 (0.01% H_2O_2), before complete dissolution of PEGDA-PEGTT. (D) Swollen PEGVS-PEGTT and PEGDA-PEGTT hydrogel mass (M_t) relative to initial hydrogel mass (M_0) during incubation in 0.01, 0.1, or 1.0% H_2O_2 . (E) FTIR spectra of PEGVS-PEGTT hydrogels exposed to 0-10% H_2O_2 solutions. (F) Zoomed-in FTIR spectra highlighting changes of C-H bending vibrations in fingerprint region.

6.4.2 H₂O₂ transport in catalase-free PEGVS-PEGTT hydrogels.

With the goal of modeling spatiotemporal H₂O₂ concentrations throughout PEGVS-PEGTT hydrogels, the effective H₂O₂ diffusivity ($D_{H_2O_2,eff}$) in the hydrogels was quantified. Fully swollen hydrogels were first characterized gravimetrically to calculate an average pore size of 11 ± 0.3 nm according to equilibrium swelling theory.⁹⁷ Swollen hydrogels were then saturated with a 1% (294 mM) aqueous solution of H₂O₂. The supernatant was then replaced with pure water, providing a concentration gradient to drive the diffusive release of H₂O₂ from the hydrogel into solution over time (Fig. D.1), generating a H₂O₂ release profile (Fig. 6.3A). This revealed hindered diffusion, with $D_{H_2O_2,eff} = 1.04\times10^{-6}$ cm²/s, a 90.5% reduction relative to the temperature-adjusted diffusion coefficient of H₂O₂ in water.²²⁵ This reduction in diffusivity is comparable to other small molecules whose diffusivities have been quantified in similar PEGVS-PEGTT hydrogels, including acetate (87% reduction) and ammonium (90% reduction),⁸¹ indicating that hydrogels impose similar diffusional resistance across various low molecular weight solutes with size well below the hydrogel average mesh size. This suggests that small molecule diffusion is governed by polymer volume fraction and the structural characteristics of the PEGVS-PEGTT hydrogel network, which reduce the size and connectivity of water-rich pathways to cause diffusion hinderance.^{53, 56, 226} This is likely due to the homogeneous, nanoscale network structure characteristic of step-growth hydrogels formed with Michael-type “click” reactions.²²⁷

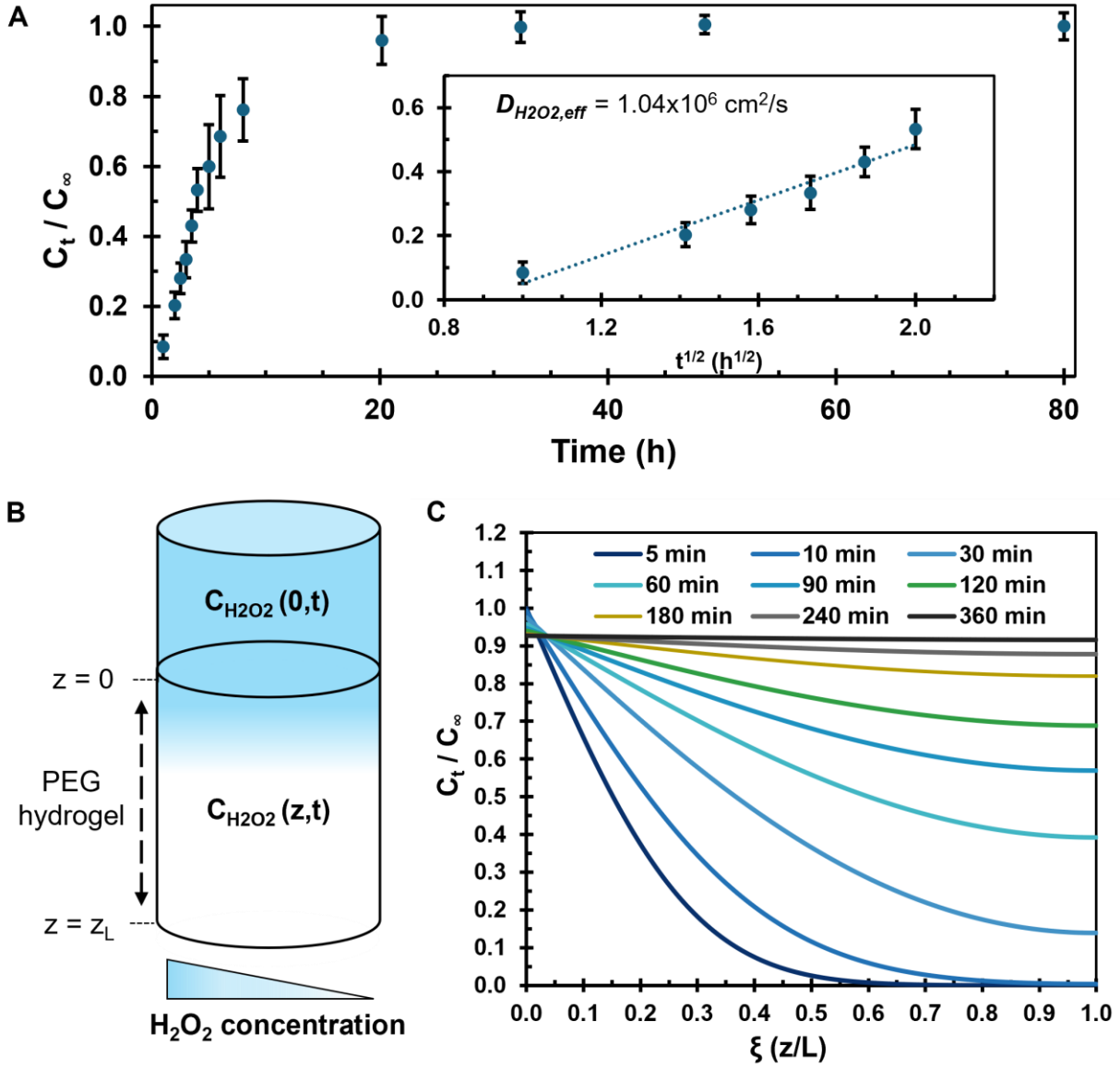


Figure 6.3. (A) Transient release of hydrogen peroxide from PEGVS hydrogels. (Inset) Early-time ($C_t/C_\infty < 0.6$) data used to calculate the diffusion coefficient (D_{eff}) through the hydrogel. (B) Schematic of 1-D transient model of H_2O_2 diffusion through cylindrical hydrogel geometry. (C) H_2O_2 concentration profiles throughout hydrogel at increasing time points.

Using $D_{H_2O_2,eff}$, one-dimensional transient diffusion of H_2O_2 through the hydrogel was modeled according to Fick's second law to predict the H_2O_2 concentration profile. The model geometry was based on a 100 μL cylindrical hydrogel geometry that fits within a standard 48-well plate and assumes one-dimensional diffusion in the $+z$ -direction (Fig. 6.3B). This geometry

matches the hydrogel dimensions used in subsequent bacterial growth experiments. Boundary conditions consisted of a known H_2O_2 concentration in the solution above the hydrogel ($z = 0$) and a no-flux condition at the bottom hydrogel surface (hydrogel-well plate interface at $z = z_L$). The resulting concentration profiles (Fig. 6.3C) demonstrate temporary protection from H_2O_2 offered by the hydrogel due to hindered diffusion. For example, cells positioned at $z/z_L > 0.75$ from the hydrogel-solution interface would be exposed to $< 1\%$ of the solution H_2O_2 concentration at a 5 min exposure time. Previously, we leveraged this effect to design of PEG coatings over electroactive bacteria on anode interfaces in a MEC system.⁸¹ The coatings provided a diffusive barrier that was proven effective for protecting encapsulated bacteria from temporary chemical (ammonium) shocks.⁸¹ However, as evident in Fig. 6.3C, without enzymatic H_2O_2 degradation capability, protection breaks down at increasing exposure times as the system reaches equilibrium and the concentration gradient approaches zero, generating high levels of H_2O_2 throughout the hydrogel. Knowledge of $C_{\text{H}_2\text{O}_2}(z, t)$ informs the design of subsequent H_2O_2 dosing experiments using encapsulated bacteria.

6.4.3 Quantification of H_2O_2 exposure thresholds due to hindered diffusion.

To access the benefits of the hydrogel for protection of bacteria from H_2O_2 toxins exclusively through hindered diffusion, bacteria were next encapsulated in hydrogels and then exposed to H_2O_2 solutions for 120 min, when H_2O_2 penetrates the entire hydrogel at high levels (Fig. 6.3C). Varying the H_2O_2 solution concentration (0-1% w/v) provided a range of H_2O_2 exposures to access bacteria survival. These conditions are representative of transient oxidative stress environments where one-dimensional diffusion dominates transport, such as MECs or peroxide-producing cells. Integration of $C_{\text{H}_2\text{O}_2}(z, t)$ with respect to time provides the local H_2O_2 exposure $E(z)$ according to hydrogel

depth ($E(z) = \int_0^t C_{H_2O_2}(z, t) dt$) and shows that cells positioned at greater depth experienced lower exposures (Fig. 6.4A). Because our cellular growth tracking technique measures population-level changes across cells within the hydrogel, a cumulative exposure ($\overline{E}_h = \frac{1}{L} \int_0^L \int_0^t C_{H_2O_2}(z, t) dt dz$) was also calculated for each H_2O_2 concentration (Fig. 6.4A).

With knowledge of H_2O_2 exposure profiles, the next step was to investigate the ability of the hydrogel to protect bacteria through hindered diffusion and to quantify the H_2O_2 exposure threshold, defined as the highest H_2O_2 exposure that bacteria can survive and recover after a 72 h culture period. *B. subtilis* cells were first encapsulated within PEGVS-PEGTT hydrogels at a cell loading of $OD_{600} = 0.1$, then 1 mL of H_2O_2 solutions with varied concentrations of 0.1-1% (0-294 mM) were added on top of each hydrogel and incubated for 120 min. After exposure, the H_2O_2 solutions were removed and replaced with ultrapure water to reduce any residual H_2O_2 within the hydrogel before 1 mL of M9 minimal medium was placed on top of each hydrogel to commence the growth of any surviving bacteria. A minimum H_2O_2 concentration of 0.5% (147 mM) was required to inhibit bacterial growth, while bacteria exposed to 0.25% and 0.1% H_2O_2 concentrations were eventually able to grow to similar levels as the unexposed (0% H_2O_2) control (Fig. 4B and C). Growth curves from these two intermediate concentrations showed significantly longer lag times (lag time = X h and Y h for 0.1 and 0.25 % H_2O_2 , respectively) compared to the unexposed control (lag time = Z h), and lag time increased as H_2O_2 exposure increased. This indicates that only a fraction of bacteria within these hydrogels were able to survive, those cells were likely present within deeper regions of the hydrogel where there was less H_2O_2 exposure (Fig. 6.3A). Because 0.25% H_2O_2 was the highest measured concentration with bacteria growth after exposure ($\overline{E}_h = 4,800 \text{ mM} \cdot \text{min}^{-1}$), the resulting H_2O_2 profile was taken as the empirical exposure threshold for post- H_2O_2 exposure bacterial recovery in this system. Hydrogels

that experience this or lower H_2O_2 exposures are designated as viable, in these hydrogels at least a fraction of the encapsulated bacterial cells survived after H_2O_2 exposure. Critically, this provided an experimentally established a no-catalase reference for benchmarking the impact of adding catalase enzymes to the hydrogel matrix and decoupling the effects of hindered diffusion and H_2O_2 degradation.

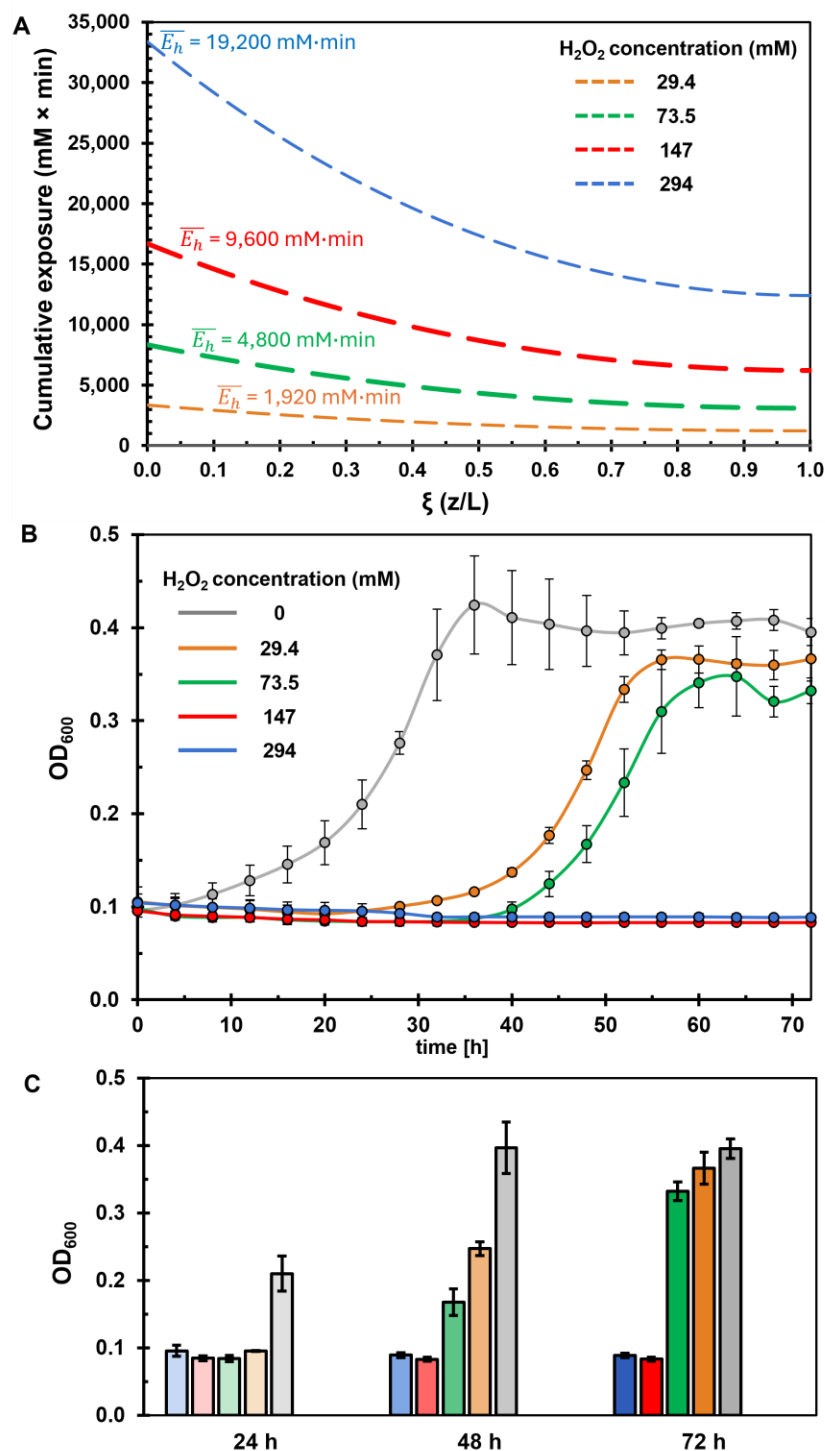


Figure 6.4. (A) Integrated dose profile for a $t = 120$ min dose, highlighting protected regions (growth observed, green), transition regions (grey striped), and unprotected regions (no growth, red) within the hydrogel. (B) Growth kinetics of encapsulated *B. subtilis* cells exposed to 0 (grey), 0.1 (orange), 0.25 (green), 0.5 (red), and 1.0% (blue) H₂O₂ solutions for 120 min. (C) Maximum OD₆₀₀ of each growth profile up to 24, 48, or 72 h. (C)

6.4.4 Reaction-diffusion modeling in catalase-functionalized hydrogels.

Addition of catalase enzymes within the PEGVS-PEGTT hydrogels enables decomposition of H_2O_2 to O_2 and H_2O , adding a new mechanism of protection to the hydrogels. Prior to experimentation with catalase-functional hydrogels, it was necessary to update the H_2O_2 – hydrogel transport model to include catalase-mediated H_2O_2 decomposition .

Michaelis-Menten kinetics are commonly used to describe catalase-mediated H_2O_2 consumption.^{211, 228} As enzymatic immobilization can impact kinetic parameters,²²⁹ it was necessary to experimentally quantify the apparent Michaelis-Menten constant ($K_{m,app}$) and the apparent maximum H_2O_2 consumption rate ($V_{max,app}$) for catalase within PEGVS-PEGTT hydrogels. For this, hydrogels were loaded with 3,520 U catalase then exposed to H_2O_2 concentrations ranging from 0.1 to 1.0% w/v. H_2O_2 concentrations in the well-mixed bulk solution above the hydrogel were then measured over a 5 min period. H_2O_2 becomes depleted due to diffusion into the hydrogel and catalase-mediated decomposition (Fig. 6.5A) and was consumed rapidly and in a concentration-dependent manner (Fig 6.5B) to confirm that catalase retained strong activity within the hydrogel. The depletion rate ($r_{\text{H}_2\text{O}_2}$) increased with increasing H_2O_2 concentration and then plateaued at higher concentrations, consistent with Michaelis-Menten saturation behavior. Nonlinear regression of the initial-rate data (Fig. 6.5C) yielded apparent kinetic parameters of K_m , $app = 57.9 \text{ mM}$ and $V_{max, app} = 160.6 \text{ mM min}^{-1}$. These values are best interpreted as effective hydrogel-scale kinetic parameters rather than intrinsic free-enzyme constants because they reflect the combined effects of catalase activity, H_2O_2 diffusion through the swollen network, and the spatial distribution of catalase throughout the gel. While system-dependent, apparent K_m values reported for immobilized catalase range from approximately 24 to 211 mM, corresponding to a 1.2- to 2.5-fold increase relative to the free enzyme.^{228, 230, 231} $K_{m,app}$ measured here falls within this

reported range for immobilized and encapsulated catalase systems. Although differences in $V_{\max}$ are also expected after immobilization, direct literature comparisons are less informative because $V_{\max}$ is highly dependent on catalase loading and system geometry.^{211, 229}

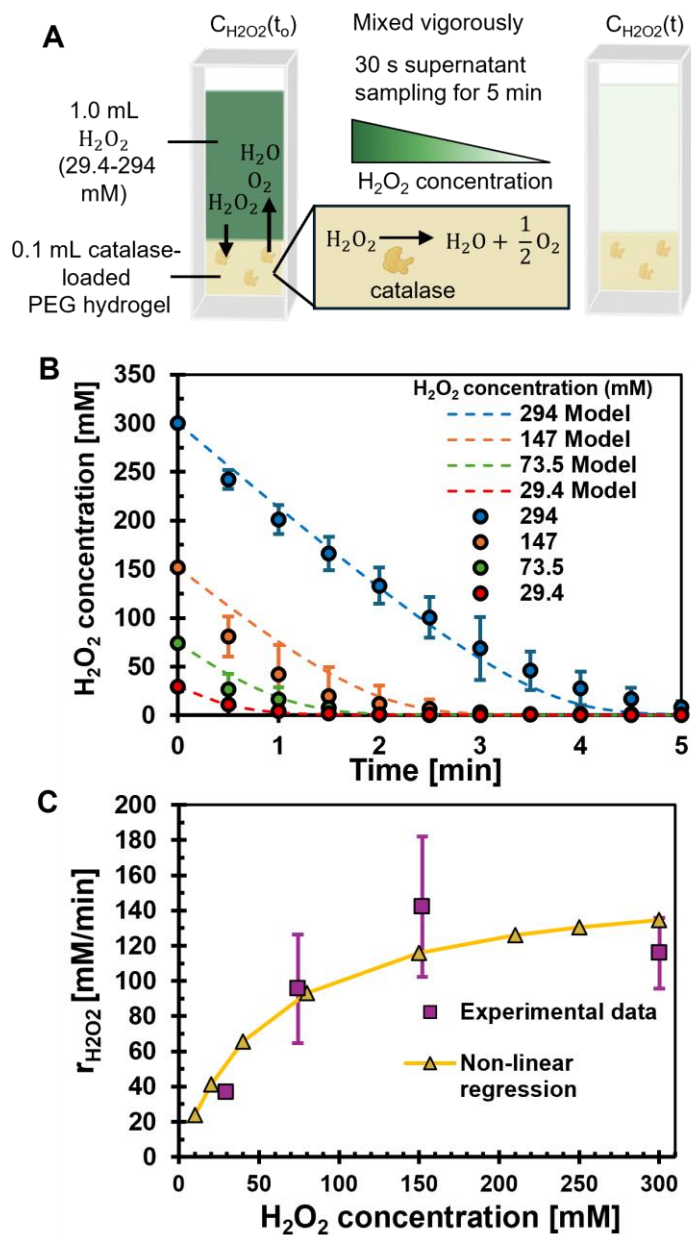


Figure 6.5. (A) Hydrogen peroxide degradation at four concentrations (0.1, 0.25, 0.5, and 1.0%) at a fixed catalase concentration of 3,520 units per hydrogel. (B) Experimental (purple) and theoretical (yellow) maximum reaction rates determined from non-linear regression of experimental data.

Using the apparent kinetics parameters quantified within the hydrogel, the one-dimensional H_2O_2 transport model (Eqn. 4) was solved to predict the H_2O_2 concentration profiles throughout hydrogels with the addition of various levels of catalase. A 0.5% H_2O_2 concentration and 20 min exposure time were used, as these were conditions where no bacteria survived the resulting exposure without H_2O_2 decomposition (Fig. 6.4). Comparison of H_2O_2 exposure profiles from this condition (also shown as red curve in Fig. 6.6A) to H_2O_2 profiles with catalase addition unveils the protective benefits of catalase, as higher catalase loading progressively lowers exposures throughout the hydrogel (Fig. 6.6A). For further comparison, the H_2O_2 threshold exposure determined from in the prior experiment ($\overline{E_h} = 4,800 \text{ mM min}^{-1}$, Fig. 6.4A) is also plotted again here (shaded green region, Fig. 6.6A); we denote the area under this as a viable region. This is significant because a portion of bacteria within this hydrogel were able to survive and grow after being subjected to this exposure profile (Fig. 6.4B and 6.4C). Portions of catalase-loaded hydrogel that fall below this viable region would therefore be expected to also harbor cells that survive H_2O_2 exposure. As catalase loading increases, higher portions of the hydrogel fall within this viable region (Fig. 6.6B). The model predicts that a minimum catalase loading of 20 U is necessary for some cells to remain viable within the hydrogel.

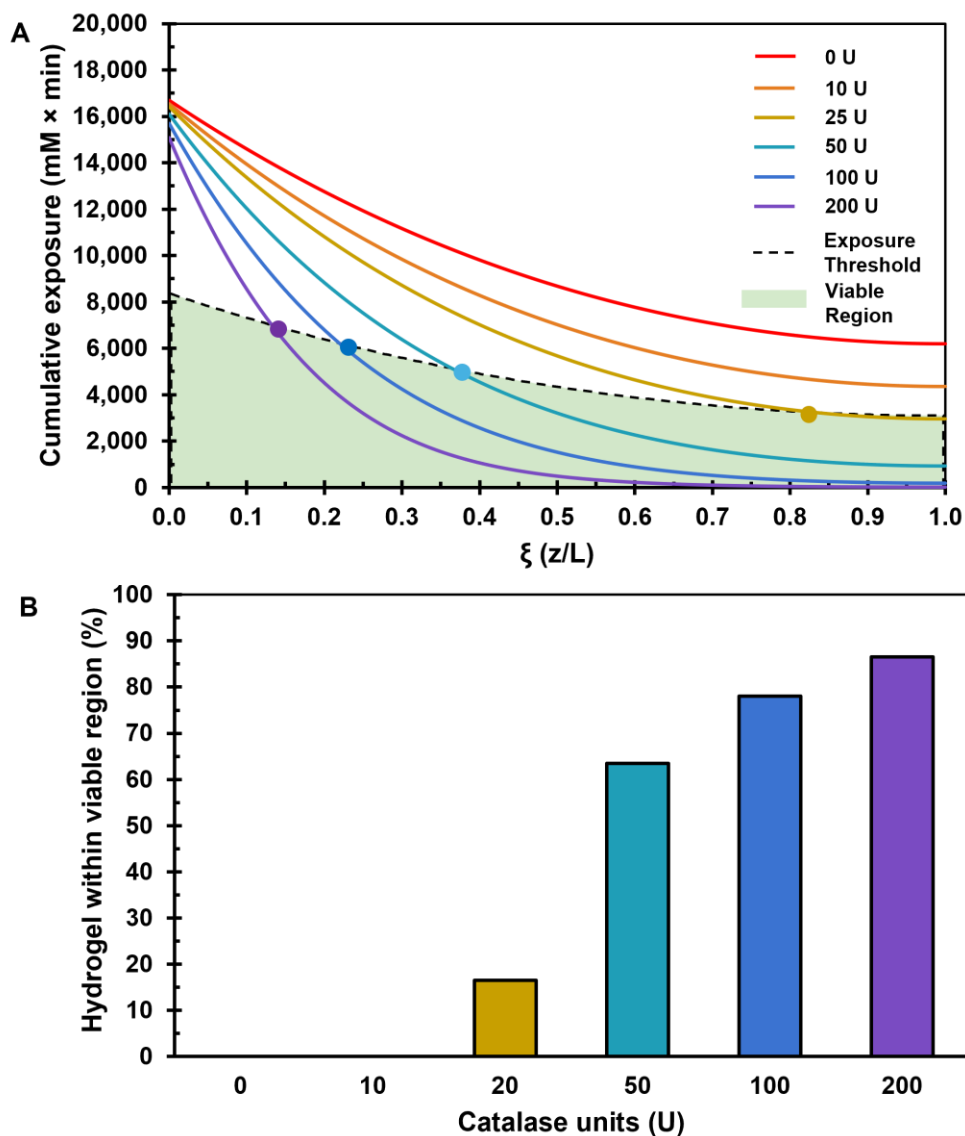


Figure 6.6. (A) H₂O₂ exposure profiles across normalized hydrogel depth as a function of catalase loading, adding the Michaelis-Menten reaction term integrated within the model. (B) Percentage of the hydrogel with cumulative H₂O₂ exposure below the empirically defined exposure threshold, where bacterial recovery was previously observed.

6.4.5 Bacteria and catalase distribution throughout hydrogels.

Prior to evaluation of the performance of catalase-loaded hydrogels, it was important to understand the spatial distribution of both bacteria and catalase throughout the hydrogel immediately after encapsulation. Two color confocal microscopy images of catalase-AF633 and

B. subtilis-GFP reveal that both catalase and cells were randomly distributed throughout the hydrogel. Cells appeared primarily as single cells, while some cellular aggregates were also present (Fig. 6.7A), consistent with prior studies that have investigated bacteria morphology after encapsulation in PEGVS-PEGTT hydrogels.⁸³ Importantly, z-stack images reveal that bacteria are randomly dispersed throughout the hydrogel depth (Fig. 6.7B and 6.7C). Cells within the encapsulated population will therefore be exposed to varied levels of H₂O₂ according to z-position, with cells at the interface hydrogel-liquid interface receiving the highest exposures, as previously depicted (Fig. 6.6A). At 3,520 U concentrations, catalase enzymes also appear uniformly distributed throughout the hydrogel at a micron scale. These findings support the assumptions in the transport model and provide insight into the cellular response curves after H₂O₂ exposures.

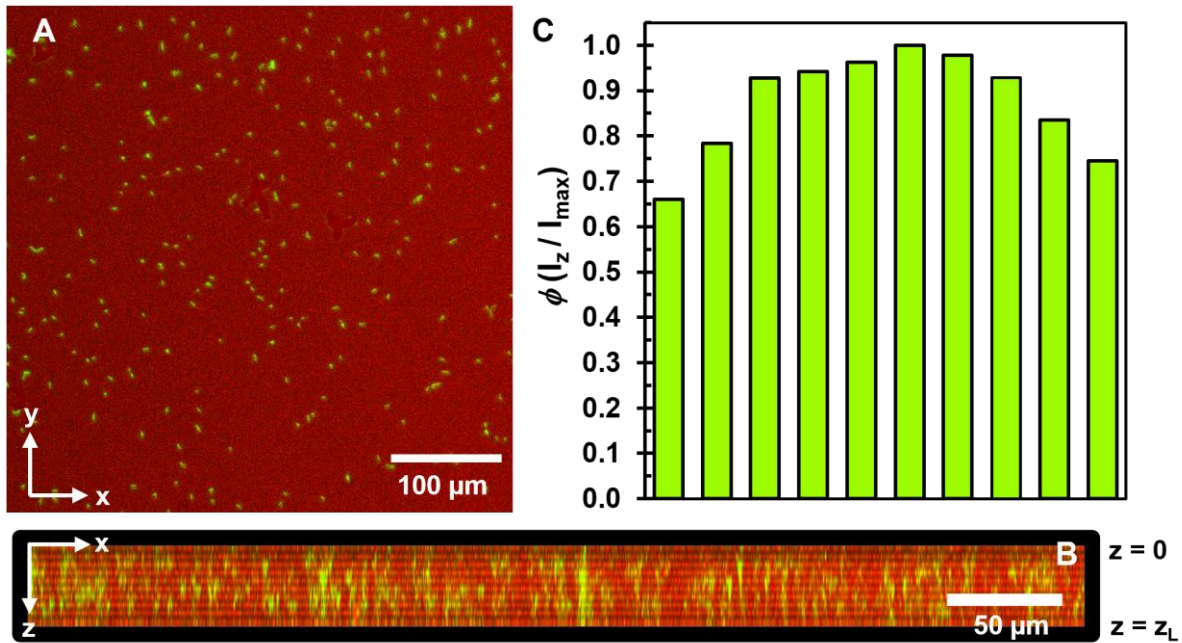


Figure 6.7. (A) Representative 20x green-red confocal image of a hydrogel with encapsulated green fluorescent protein-expressing *B. subtilis* and catalase (3,520 U) labeled with Alexa Fluor 633. (B) Representative X-Z cross-sectional projection of the confocal fluorescence z-stack containing GFP-*B. subtilis* and AF633-labeled catalase. (C) Normalized mean GFP fluorescence intensity for each optical slice within a 20x objective z-stack.

6.4.6 Enzymatic protection of bacteria from H₂O₂ exposure.

The impact of catalase loading on protection of *B. subtilis* cells from H₂O₂ within the hydrogels was finally evaluated. *B. subtilis* cells were encapsulated at an initial cell density of OD₆₀₀ = 0.1 then exposed to 0.5% H₂O₂ for 2 h, conditions which resulted in no survival in the prior system, when no catalase was present (Fig. 6.4B and 6.4C). Following H₂O₂ exposure and washing, bacteria growth kinetics within the hydrogel were again monitored by measuring the OD₆₀₀ for 72 h during incubation in culture media. Two controls were included, the first was no exposure to H₂O₂ to quantify the growth of fully viable, unexposed cells within the hydrogel. The second control hydrogels without catalase (0 U) to verify that cells within the hydrogels could not survive the prescribed H₂O₂ exposure without decomposition.

During incubation in culture media, H₂O₂-exposed hydrogels without catalase and with 10 U catalase showed no bacteria growth, indicating that the cells within these hydrogels did not survive the H₂O₂ exposure (Fig. 6.8A). In contrast, bacteria growth was observed when H₂O₂-exposed hydrogels contained a minimum of 20 U catalase. At 200 U catalase, growth trajectories of encapsulated bacteria nearly matched the no H₂O₂ exposure control curve, indicating that the majority of cells remained viable. To better visualize differences in recovery kinetics across catalase loadings, the OD₆₀₀ observed at 24, 48, and 72 h after exposure was plotted from each growth curve (Fig. 6.8B). While longer delays in growth were observed with lower catalase loading, all hydrogels loaded with ≥ 20 U catalase grew to equivalent levels by the end of the culture (72 h), suggesting that each of the surviving cell populations were sufficient to repopulate the hydrogel. A key metric is the duration of the lag phase, which inversely correlates with catalase loading (Fig. 6.8C) to indicate that higher numbers of cells in the hydrogels survived with higher catalase loading. Finally, surfactin released from cells within the hydrogel into the supernatant was

also quantified using a CPC-BTB colorimetric assay.¹⁶⁶ Surfactin is a bioactive lipopeptide produced as a secondary metabolite by *B. subtilis* and has notable anticancer and antimicrobial activity.^{124, 232} Our prior work investigated the use of PEG hydrogels containing *B. subtilis* to stimulate surfactin production for ELH biotherapeutic applications,⁸⁴ it was therefore quantified as an additional measure of cell function here. Consistent with growth recovery, surfactin measurements after 72 h of growth showed that all recovered hydrogels produced equivalent surfactin concentrations relative to the 0 and 10 U hydrogels that did not survive (Fig. 6.8D). The concentrations (42-55 μ M) were comparable to those quantified in the previous study,⁸⁴ indicating that the surviving cells within the hydrogel remained viable and metabolically active, and that this beneficial output was not compromised after H₂O₂ exposure.

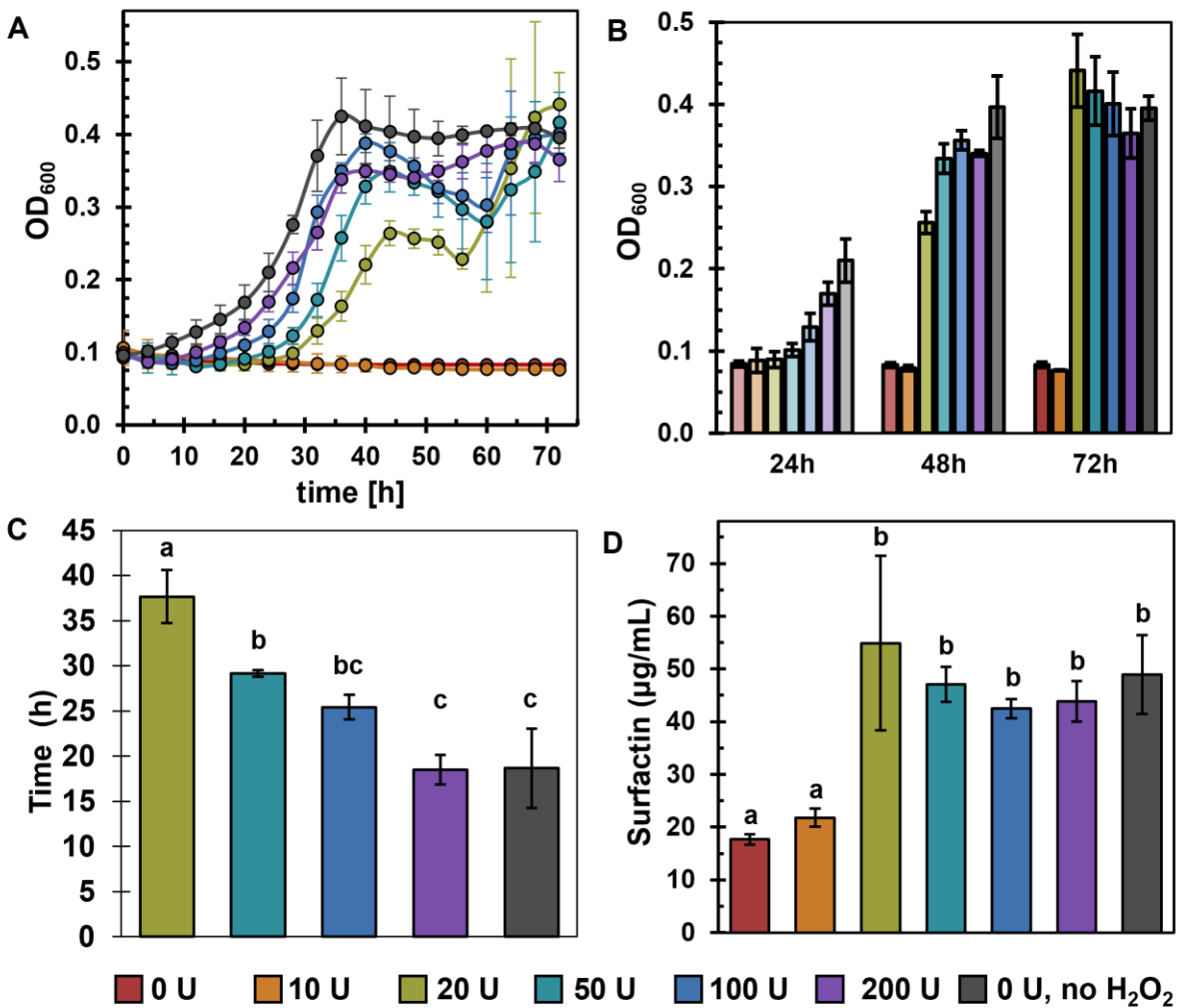


Figure 6.8. (A) Growth trajectories of encapsulated *B. subtilis* cells after H₂O₂ exposure (0.5%, 2h) with varying catalase loading (U) or without catalase or H₂O₂ (control) (B) Averaged OD₆₀₀ readings for each condition at 24, 48, or 72 h. (C) Effect of catalase loading (U) on lag phase duration (D) Surfactin production levels after 72 h culture at each condition. Error bars indicate standard deviation (n=3 replicates), differing letters indicate statistical significance ($p < 0.05$).

6.5 Discussion

The experimental trends observed agree with the exposure profiles generated by the H₂O₂ hydrogel reaction-diffusion model and the resulting cellular behavior that was predicted. Under the H₂O₂ exposure used (0.5%, 2h), no growth was predicted to occur at 10 U, as this was higher

than the H_2O_2 exposure threshold, thus no portion of hydrogel fell within the viable region. The onset of bacteria growth was predicted to occur as catalase loading approached 20 U, where enough H_2O_2 decomposition lowered the exposure enough for a small fraction of the hydrogel (16.5%) to fall below the H_2O_2 exposure threshold and into the viable region (Fig. 6.6A and B). Experimental measurements of cell growth after H_2O_2 exposure matched these predictions (Fig. 6.8A-C). Addition of higher amounts of catalase (50-200 U) further lowered H_2O_2 exposures to move successively larger fractions of the hydrogel into the viable region. The larger fractions of surviving cell populations with each incremental increase in catalase was experimentally verified by a corresponding decrease in lag time (Fig. 6.8A-C).

With demonstrated ability to match cell viability observations, the reaction-diffusion model coupled with the experimentally determined H_2O_2 exposure threshold can now be used to reduce the need for extensive empirical testing. For example, the bacterial survival at a given catalase loading for other H_2O_2 concentrations not directly investigated here can be estimated (Fig. D.2). Further, the approach can be used to guide the design of these hydrogels by estimating catalase loading levels to ensure bacteria protection in other H_2O_2 environments, and to predict the necessary catalase concentrations required to accommodate increasing H_2O_2 exposures that accompany lower diffusion lengths (Fig. D.3), as many applications require thinner protective coatings (5-100 μm).^{233, 234} The reaction-diffusion model can also accommodate changes in diffusivity, which can be modulated by controlling hydrogel mesh size.²³⁵ Finally, while the reaction-diffusion model describes one-dimensional axial transport through a cylindrical hydrogel, the same framework also applies to other systems where one-dimensional mass transport limitations govern chemical penetration to establish concentration gradients normal to the material surface. This includes protective hydrogel coatings on planar substrates (i.e. one-dimensional

transport in cartesian coordinates) and the design of protective microspheres (i.e. one-dimensional radial transport in spherical coordinates).

There are several considerations that should be made when applying this approach to new systems. The current reaction-diffusion model assumes a uniform reaction field throughout the hydrogel from a homogenous catalase distribution, which is reasonable at moderate to high functional catalase loading levels (Fig. 6.7B). With lower catalase loading however, H_2O_2 decomposition may become spatially heterogenous, and bacterial protection would be expected to become localized around regions of high catalase activity. Also, it is important to note that cellular response is inferred from empirical H_2O_2 exposure thresholds (Fig. 6.4). As both anerobic and aerobic bacteria can have widely varied tolerance to ROS, even at the genus, species, strain, and growth phase level,²³⁶⁻²³⁸ this experimental characterization must be repeated for other bacteria. Finally, as bacteria in this study were distributed throughout the hydrogel depth (Fig. 6.7A), the measured recovery reflects a collective response from a population of cells experiencing different H_2O_2 exposures. This matches some applications, such as suspended hydrogel encapsulants for water treatment,²³⁹ while other applications present differing cellular orientations. For example, protection of functional biofilms, such as electroactive biofilms in MxC systems,⁸¹ would require deposition over the top of the biofilm. Here it would be expected that cells across the biofilm-hydrogel interface would instead experience more uniform H_2O_2 exposure, given a coating of uniform thickness.

6.6 Conclusions

To our knowledge, this is among the first examples of an enzyme-functionalized ELH to protect bacteria from a targeted environmental toxin and create a protective microenvironment for encapsulated bacteria. The hydrogels remained chemically stable in the presence of H_2O_2 over

extended time periods (>60 days), enabled viable cell encapsulation and growth, and lowered the cellular exposure to H_2O_2 through hindered diffusion combined with enzymatic decomposition to O_2 and H_2O . A one-dimensional reaction-diffusion model was developed based on experimentally measured transport and kinetic parameters ($D_{\text{H}_2\text{O}_2, \text{eff}}$, $K_{m, \text{app}}$, $V_{\text{max}, \text{app}}$) to model spatiotemporal H_2O_2 concentrations throughout the hydrogels. The model was used to relate catalase enzyme loading to H_2O_2 transport. When combined with an experimentally determined H_2O_2 exposure threshold for *B. subtilis*, catalase loading levels that enabled cell survival from a H_2O_2 exposure were correctly predicted. The established framework provides a quantitative link between experimental and application-relevant hydrogel geometries, allowing H_2O_2 exposure profiles to be translated across different oxidative environments to optimize hydrogel geometry, thickness, and enzyme loading. Relevant ELH systems include biomedical applications (implant coatings, wound dressings, and oral microbial delivery), environmental applications (hydrogel biofilm coatings, wastewater-treatment reactors, biosensing, and bioremediation), and bioelectrochemical applications (microbial electrolysis cells and microbial fuel cells), as these systems may involve chemical environments where ROS is present to compromise cellular function within ELH materials.

6.7 Acknowledgments

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Chapter 7 - Summary and Future Work

7.1 Summary

As engineered living hydrogels continue to expand across a wide range of biomedical, environmental, industrial, and other emerging applications, advancing the potential of these materials requires a fundamental understanding of how molecular transport, cellular confinement, and the local biochemical environment influence the viability and function of encapsulated microorganisms. Rather than serving solely as passive encapsulation matrices, ELHs are increasingly being designed as active materials capable of regulating transport, cellular behavior, and the surrounding biochemical microenvironment. This dissertation addressed these needs through the development of experimental platforms and PEG-based hydrogel systems that progressively advanced from the characterization of molecular transport to the regulation of bacterial behavior through hydrogel architecture and ultimately to the engineering of the hydrogel microenvironment through enzymatic functionality.

The development of a photodegradable PEG hydrogel platform integrated within a microfluidic device established a foundation for understanding and controlling bacterial transport within ELHs. Spatially controlled hydrogel degradation enabled systematic study of *B. subtilis* chemotaxis through partially degraded hydrogels while maintaining precise control over the surrounding chemical environment. Controlled photodegradation produced up to a 2.6-fold difference in bacterial migration speed while regulating migration directionality and enabling controlled bacterial release, demonstrating that hydrogel degradation can be used to precisely modulate microbial transport. Collectively, these findings demonstrate that degradable PEG hydrogels can serve as both experimental platforms for investigating bacterial transport and as functional materials for controlling microbial movement and release.

Building upon this understanding of cellular transport, the next study investigated how hydrogel network architecture influences bacterial behavior and function. Systematic variation of PEG macromer molecular weight produced hydrogels with mesh sizes ranging from approximately 9 to 19 nm, resulting in nearly a 7-fold difference in small molecule diffusivity and providing a well-defined platform to investigate the effects of nanoscale confinement on encapsulated bacteria. The results demonstrated that hydrogel mesh size serves as a critical design parameter governing microbial activity, with nanoscale confinement significantly influencing bacterial growth, surfactin production, and biomolecule release. These findings established fundamental relationships between hydrogel structure, molecular transport, and microbial function, demonstrating that hydrogel architecture can be rationally designed to regulate cellular function and the performance of ELHs.

The final study extended these design principles beyond passive regulation of transport and confinement by engineering the hydrogel microenvironment through the incorporation of immobilized catalase enzymes within PEG-based engineered living hydrogels. To our knowledge, this work represents one of the first enzyme-functionalized engineered living hydrogels designed to protect encapsulated bacteria from a targeted environmental toxin through the creation of a localized protective biochemical microenvironment. Experimentally determined transport and kinetic parameters were integrated into a one-dimensional reaction-diffusion model that quantitatively linked enzyme loading, hydrogel geometry, and hydrogen peroxide transport, to accurately predict bacterial survival under a given hydrogen peroxide exposure. Together, these findings establish a predictive framework for the rational design of enzyme-functionalized engineered living hydrogels and demonstrate how enzymatic functionality can be integrated within

synthetic hydrogel matrices to actively regulate the microenvironment surrounding encapsulated microorganisms in order to preserve cellular function.

7.2 Future Work

The studies presented in this dissertation establish fundamental design principles for ELHs that extend beyond the individual systems investigated here. Collectively, these findings provide a starting point for the continued development of ELHs through advances in material design, biochemical functionality, and transport modeling. The following sections outline several research directions that build upon this framework and may further expand the capabilities and applications of ELHs.

7.2.1 Multi-Enzyme Functionalized Engineered Living Hydrogels

Expanding biochemical functionality beyond single-enzyme systems represents a natural next step in the development of enzyme-functionalized ELHs. Future studies should investigate the incorporation of multiple enzymes that are independently immobilized within the hydrogel to create localized reaction networks that perform more complex biochemical functions. Coupling sequential and complementary enzymatic pathways would enable localized control over reaction intermediates, introducing additional layers of spatial and kinetic regulation over biochemical processes occurring within the material. For example, combining complementary enzymes such as glucose oxidase (GOx) and lactate oxidase (LOx) with catalase could create a material that disrupts tumor metabolism by depleting glucose and tumor-derived lactate while consuming the harmful hydrogen peroxide produced by both oxidases.^{240, 241} In a bacterial ELH, this system could protect encapsulated bacteria while enabling sustained and localized therapeutic production. Beyond sequential or complementary reactions, multiple enzymes could be incorporated to regulate oxygen, nutrients, metabolites, cellular protection, and therapeutic activity within the

same material. Such systems could ELHs to function in environments where reaction byproducts harm encapsulated cells or where multiple chemical stressors are present.

Rather than functioning solely as protective materials, multi-enzyme ELHs could be further designed to actively regulate the biochemical microenvironment to support and enhance the function of encapsulated microorganisms. Non-ELH studies have shown that urease and carbonic anhydrase together can regulate local pH and bicarbonate availability.²⁴² Translating this system into a bacterial ELH could enable local pH control after gelation, creating a living material that actively responds to acidic conditions while carbonate deposition simultaneously alters hydrogel stiffness rather than relying solely on its initial hydrogel properties. More broadly, these systems could dynamically regulate their internal chemical environment and material properties, enabling ELHs to perform more complex biological functions across biomedical, environmental, and industrial applications.

7.2.2 Advanced Hydrogel Architectures

While this dissertation demonstrated that hydrogel architecture can be used to regulate molecular transport, bacterial confinement, and biomolecule release, the PEG-based systems investigated here represent only a small subset of the hydrogel chemistries available for engineered living materials. Future work should focus on developing expanded hydrogel architectures that can independently control mechanical properties, transport behavior, degradation, and biochemical responsiveness. Such systems would further expand the range of cellular environments that can be created within ELHs while providing additional design flexibility for specific applications.

One promising direction is the development of layered hydrogel that combine multiple polymer chemistries within a single material. For example, integrating PEG-vinyl sulfone and PEG-diacrylate networks could provide a mechanically stable backbone for long-term cell

encapsulation while incorporating a secondary, environmentally responsive network that dynamically modifies transport properties. Covalent crosslinks formed between thiol and vinyl sulfone groups remain stable across a broad range of pH conditions and chemical exposures. In contrast, thiol–acrylate crosslinks are susceptible to multiple degradation pathways, including hydrolysis under acidic, neutral, and basic conditions and degradation in the presence of chemicals such as hydrogen peroxide or specific enzymes. Depending on the polymer chemistry employed, this secondary network could be designed to respond to pH, reactive oxygen species, enzymatic activity, or other biologically relevant stimuli, enabling controlled expansion of the hydrogel while preserving the structural integrity and cellular confinement provided by the primary PEG-vinyl sulfone network.

These hydrogel architectures could also enable multistage therapeutic delivery strategies. Selective degradation of the responsive network could dramatically increase hydrogel mesh size in response to disease-associated stimuli, triggering rapid release of encapsulated therapeutic payloads. Following this initial release, the remaining structural network would retain encapsulated microorganisms or cells that continuously produce and secrete therapeutic biomolecules, effectively transitioning the material from a stimulus-responsive drug delivery platform to a localized living factory. This combination of immediate payload delivery followed by sustained cell-mediated therapeutic production could significantly expand the functionality of ELHs beyond conventional drug delivery systems. More broadly, this approach would extend the design principles established in this dissertation by enabling molecular transport to be dynamically modified without compromising the long-term structural support required for cellular confinement and function.

7.2.3 Expanding Predictive Modeling of Engineered Living Hydrogels

The reaction-diffusion framework developed in this dissertation provides a foundation for predictive modeling. While the current model describes hydrogen peroxide transport coupled with immobilized catalase kinetics, the model can be further developed to incorporate cellular processes that govern the behavior of bacteria within the hydrogel.

For example, incorporating cellular metabolism into the reaction-diffusion model would enable prediction of localized cellular responses that cannot be directly measured experimentally. By coupling experimentally determined nutrient uptake, metabolite production, and microbial growth kinetics with molecular transport, future models could predict spatial variations in cellular activity, viability, and metabolic state throughout the hydrogel. Such models would provide insight into the heterogeneous microenvironments experienced by individual cells while identifying localized transport limitations, nutrient depletion, metabolite accumulation, or other microscale phenomena that are difficult to observe experimentally. This capability would extend the current framework beyond describing only molecular transport and reaction kinetics to predicting how localized chemical environments influence individual cellular behavior.

Future iterations of the model could also incorporate dynamic material properties, including hydrogel swelling, degradation, enzyme deactivation, and changes in transport behavior over time, providing a more comprehensive representation of ELHs under application-relevant conditions. Together, these additions would allow hydrogel geometry, material composition, enzyme loading, and cellular density to be systematically evaluated prior to experimental validation, reducing the need for extensive empirical trial-and-error during material design. By integrating molecular transport, biochemical reactions, cellular metabolism, and evolving material properties within a single design, future models could provide a comprehensive predictive platform

for increasingly complex engineered living hydrogels while reducing reliance on extensive experimental optimization.

7.2.4 Translation to Application-Relevant Systems

The design principles established throughout this dissertation provide a basis for translating ELHs into increasingly complex and application-relevant systems. The regulation of molecular transport, hydrogel architecture, and biochemical functionality, together with quantitative modeling, enables the rational design of living materials tailored to specific biological and engineering challenges. Collectively, these advances provide a versatile framework for ELHs that operate under diverse chemical, biological, and environmental conditions. These design principles are broadly applicable across biomedical, environmental, and industrial technologies. While ELHs have advanced substantially, expanding their use beyond proof-of-concept systems will require addressing limitations that emerge as these materials are scaled, deployed, and operated over extended periods. As hydrogel thickness and cell loading increase, limitations in oxygen and nutrient transport, changes in pH, and waste accumulation can make it more difficult to maintain microbial viability and function over time.^{7, 243} For biomedical applications, more work is needed to keep these materials attached and localized at the intended site while controlling how many bacteria remain in the hydrogel, how microbial products move into the surrounding tissue, and whether released bacteria spread beyond the target area.^{244, 245} Finally, widespread use will require long-term storage methods that preserve cell viability and function without altering hydrogel geometry, transport properties, mechanical integrity, or containment, along with manufacturing methods that produce these materials consistently across batches.^{7, 245}

Chapter 8 - References

- (1) Nguyen, P. Q.; Courchesne, N. M. D.; Duraj-Thatte, A.; Praveschotinunt, P.; Joshi, N. S. Engineered living materials: prospects and challenges for using biological systems to direct the assembly of smart materials. *Advanced Materials* **2018**, *30* (19), 1704847.
- (2) Rodrigo-Navarro, A.; Sankaran, S.; Dalby, M. J.; del Campo, A.; Salmeron-Sanchez, M. Engineered living biomaterials. *Nature Reviews Materials* **2021**, *6* (12), 1175-1190. DOI: 10.1038/s41578-021-00350-8.
- (3) Liu, A. P.; Appel, E. A.; Ashby, P. D.; Baker, B. M.; Franco, E.; Gu, L.; Haynes, K.; Joshi, N. S.; Kloxin, A. M.; Kouwer, P. H. The living interface between synthetic biology and biomaterial design. *Nature materials* **2022**, *21* (4), 390-397.
- (4) Yu, S.; Sun, H.; Li, Y.; Wei, S.; Xu, J.; Liu, J. Hydrogels as promising platforms for engineered living bacteria-mediated therapeutic systems. *Materials today bio* **2022**, *16*, 100435.
- (5) An, B.; Wang, Y.; Huang, Y.; Wang, X.; Liu, Y.; Xun, D.; Church, G. M.; Dai, Z.; Yi, X.; Tang, T.-C. Engineered living materials for sustainability. *Chemical Reviews* **2022**, *123* (5), 2349-2419.
- (6) Liu, X.; Inda, M. E.; Lai, Y.; Lu, T. K.; Zhao, X. Engineered Living Hydrogels. *Adv Mater* **2022**, *34* (26), e2201326. DOI: 10.1002/adma.202201326 From NLM Medline.
- (7) Etter, E. L.; Thormann, S.; Venkatraman, S.; Potluru, S. S.; Nguyen, J. Hydrogel platforms for engineered live biotherapeutics: materials, microbial integration and clinical potential. *RSC Pharmaceutics* **2026**, *3* (2), 305-330, 10.1039/D5PM00304K. DOI: 10.1039/D5PM00304K.
- (8) Tang, T.-C.; Tham, E.; Liu, X.; Yehl, K.; Rovner, A. J.; Yuk, H.; de la Fuente-Nunez, C.; Isaacs, F. J.; Zhao, X.; Lu, T. K. Hydrogel-based biocontainment of bacteria for continuous sensing and computation. *Nature Chemical Biology* **2021**, *17* (6), 724-731. DOI: 10.1038/s41589-021-00779-6.
- (9) Knierim, C.; Enzeroth, M.; Kaiser, P.; Dams, C.; Nette, D.; Seubert, A.; Klingl, A.; Greenblatt, C. L.; Jérôme, V.; Agarwal, S.; Freitag, R.; Greiner, A. Living Composites of Bacteria and Polymers as Biomimetic Films for Metal Sequestration and Bioremediation. *Macromolecular Bioscience* **2015**, *15* (8), 1052-1059. DOI: <https://doi.org/10.1002/mabi.201400538>.
- (10) Knoll, M. T.; Fuderer, E.; Gescher, J. Sprayable biofilm – Agarose hydrogels as 3D matrix for enhanced productivity in bioelectrochemical systems. *Biofilm* **2022**, *4*, 100077. DOI: <https://doi.org/10.1016/j.bioflm.2022.100077>.
- (11) Chen, J. Y.; Xie, P.; Zhang, Z. P. Reduced graphene oxide/polyacrylamide composite hydrogel scaffold as biocompatible anode for microbial fuel cell. *Chemical Engineering Journal* **2019**, *361*, 615-624. DOI: <https://doi.org/10.1016/j.cej.2018.12.116>.
- (12) Zheng, T.; Zhang, M.; Wu, L.; Guo, S.; Liu, X.; Zhao, J.; Xue, W.; Li, J.; Liu, C.; Li, X.; Jiang, Q.; Bao, J.; Zeng, J.; Yu, T.; Xia, C. Upcycling CO₂ into energy-rich long-chain compounds via electrochemical and metabolic engineering. *Nature Catalysis* **2022**, *5* (5), 388-396. DOI: 10.1038/s41929-022-00775-6.
- (13) Wang, X.; Han, F.; Xiao, Z.; Zhou, X.; Liu, X.; Chen, Y.; Li, K.; Li, Y.; Yu, Q.; Zhao, H.; Zhu, M.; Wang, R.; Liu, Z.; Zhong, C. 3-D Printable Living Hydrogels as Portable Bio-energy Devices. *Advanced Materials* **2025**, *37* (18), 2419249. DOI: <https://doi.org/10.1002/adma.202419249>.
- (14) Dey, S.; Sankaran, S. Engineered bacterial therapeutics with material solutions. *Trends in Biotechnology* **2024**, *42* (12), 1663-1676. DOI: 10.1016/j.tibtech.2024.06.011 (accessed 2026/04/13).

- (15) Harimoto, T.; Herrero Quevedo, F.; Zillig, J.; Schreiber, S.; Wu, Y.; Ahn, C. H.; To, T.; Thakur, R.; Tatara, A. M.; Kang, S.; Chen, Z.; Lafuente-Gómez, N.; Hanan, B.; Pauer, A.; Lightbown, S.; Weitz, D. A.; Mooney, D. J. Implantable living materials autonomously deliver therapeutics using contained engineered bacteria. *Science* **2026**, 392 (6799), 729-734. DOI: doi:10.1126/science.aec2071.
- (16) Duraj-Thatte, A. M.; Courchesne, N.-M. D.; Praveschotinunt, P.; Rutledge, J.; Lee, Y.; Karp, J. M.; Joshi, N. S. Genetically Programmable Self-Regenerating Bacterial Hydrogels. *Advanced Materials* **2019**, 31 (40), 1901826. DOI: <https://doi.org/10.1002/adma.201901826>.
- (17) Johnston, T. G.; Yuan, S.-F.; Wagner, J. M.; Yi, X.; Saha, A.; Smith, P.; Nelson, A.; Alper, H. S. Compartmentalized microbes and co-cultures in hydrogels for on-demand bioproduction and preservation. *Nature Communications* **2020**, 11 (1), 563. DOI: 10.1038/s41467-020-14371-4.
- (18) Sugianto, W.; Altin-Yavuzarslan, G.; Tickman, B. I.; Kiattisewee, C.; Yuan, S.-F.; Brooks, S. M.; Wong, J.; Alper, H. S.; Nelson, A.; Carothers, J. M. Gene expression dynamics in input-responsive engineered living materials programmed for bioproduction. *Materials Today Bio* **2023**, 20, 100677. DOI: <https://doi.org/10.1016/j.mtbio.2023.100677>.
- (19) Guo, S.; Dubuc, E.; Rave, Y.; Verhagen, M.; Twisk, S. A. E.; van der Hek, T.; Oerlemans, G. J. M.; van den Oetelaar, M. C. M.; van Hazendonk, L. S.; Bröls, M.; Eijkens, B. V.; Joostens, P. L.; Keij, S. R.; Xing, W.; Nijs, M.; Stalpers, J.; Sharma, M.; Gerth, M.; Boonen, R. J. E. A.; Verduin, K.; Merckx, M.; Voets, I. K.; de Greef, T. F. A. Engineered Living Materials Based on Adhesin-Mediated Trapping of Programmable Cells. *ACS Synthetic Biology* **2020**, 9 (3), 475-485. DOI: 10.1021/acssynbio.9b00404.
- (20) Ben David, N.; Mafi, M.; Nyska, A.; Gross, A.; Greiner, A.; Mizrahi, B. Bacillus subtilis in PVA Microparticles for Treating Open Wounds. *ACS Omega* **2021**, 6 (21), 13647-13653. DOI: 10.1021/acsomega.1c00790.
- (21) Richbourg, N. R.; Wancura, M.; Gilchrist, A. E.; Toubbeh, S.; Harley, B. A. C.; Cosgriff-Hernandez, E.; Peppas, N. A. Precise control of synthetic hydrogel network structure via linear, independent synthesis-swelling relationships. *Science Advances* **2021**, 7 (7), eabe3245. DOI: doi:10.1126/sciadv.abe3245.
- (22) Cruise, G. M.; Scharp, D. S.; Hubbell, J. A. Characterization of permeability and network structure of interfacially photopolymerized poly(ethylene glycol) diacrylate hydrogels. *Biomaterials* **1998**, 19 (14), 1287-1294. DOI: [https://doi.org/10.1016/S0142-9612\(98\)00025-8](https://doi.org/10.1016/S0142-9612(98)00025-8).
- (23) Bryant, S. J.; Bender, R. J.; Durand, K. L.; Anseth, K. S. Encapsulating chondrocytes in degrading PEG hydrogels with high modulus: Engineering gel structural changes to facilitate cartilaginous tissue production. *Biotechnology and Bioengineering* **2004**, 86 (7), 747-755. DOI: <https://doi.org/10.1002/bit.20160>.
- (24) Gomez-Florit, M.; Pardo, A.; Domingues, R. M. A.; Graça, A. L.; Babo, P. S.; Reis, R. L.; Gomes, M. E. Natural-Based Hydrogels for Tissue Engineering Applications. *Molecules* **2020**, 25 (24), 5858.
- (25) Zhao, L.; Zhou, Y.; Zhang, J.; Liang, H.; Chen, X.; Tan, H. Natural Polymer-Based Hydrogels: From Polymer to Biomedical Applications. *Pharmaceutics* **2023**, 15 (10), 2514.
- (26) Peters, J. T.; Wechsler, M. E.; Peppas, N. A. Advanced biomedical hydrogels: molecular architecture and its impact on medical applications. *Regenerative Biomaterials* **2021**, 8 (6). DOI: 10.1093/rb/rbab060 (accessed 7/7/2026).
- (27) Metters, A.; Hubbell, J. Network Formation and Degradation Behavior of Hydrogels Formed by Michael-Type Addition Reactions. *Biomacromolecules* **2005**, 6 (1), 290-301. DOI: 10.1021/bm049607o.

- (28) Lin-Gibson, S.; Jones, R. L.; Washburn, N. R.; Horkay, F. Structure–Property Relationships of Photopolymerizable Poly(ethylene glycol) Dimethacrylate Hydrogels. *Macromolecules* **2005**, *38* (7), 2897-2902. DOI: 10.1021/ma0487002.
- (29) Tibbitt, M. W.; Kloxin, A. M.; Sawicki, L.; Anseth, K. S. Mechanical Properties and Degradation of Chain and Step Polymerized Photodegradable Hydrogels. *Macromolecules* **2013**, *46* (7). DOI: 10.1021/ma302522x.
- (30) Braccini, I.; Pérez, S. Molecular Basis of Ca²⁺-Induced Gelation in Alginates and Pectins: The Egg-Box Model Revisited. *Biomacromolecules* **2001**, *2* (4), 1089-1096. DOI: 10.1021/bm010008g.
- (31) Normand, V.; Muller, S.; Ravey, J.-C.; Parker, A. Gelation Kinetics of Gelatin: A Master Curve and Network Modeling. *Macromolecules* **2000**, *33* (3), 1063-1071. DOI: 10.1021/ma9909455.
- (32) Sæther, H. V.; Holme, H. K.; Maurstad, G.; Smidsrød, O.; Stokke, B. T. Polyelectrolyte complex formation using alginate and chitosan. *Carbohydrate Polymers* **2008**, *74* (4), 813-821. DOI: <https://doi.org/10.1016/j.carbpol.2008.04.048>.
- (33) Sawhney, A. S.; Pathak, C. P.; Hubbell, J. A. Bioerodible hydrogels based on photopolymerized poly(ethylene glycol)-co-poly(α-hydroxy acid) diacrylate macromers. *Macromolecules* **1993**, *26* (4), 581-587. DOI: 10.1021/ma00056a005.
- (34) Anseth, K. S.; Kline, L. M.; Walker, T. A.; Anderson, K. J.; Bowman, C. N. Reaction Kinetics and Volume Relaxation during Polymerizations of Multiethylene Glycol Dimethacrylates. *Macromolecules* **1995**, *28* (7), 2491-2499. DOI: 10.1021/ma00111a050.
- (35) Elisseeff, J.; Anseth, K.; Sims, D.; McIntosh, W.; Randolph, M.; Langer, R. Transdermal photopolymerization for minimally invasive implantation. *Proceedings of the National Academy of Sciences* **1999**, *96* (6), 3104-3107. DOI: doi:10.1073/pnas.96.6.3104.
- (36) Burdick, J. A.; Anseth, K. S. Photoencapsulation of osteoblasts in injectable RGD-modified PEG hydrogels for bone tissue engineering. *Biomaterials* **2002**, *23* (22), 4315-4323. DOI: [https://doi.org/10.1016/S0142-9612\(02\)00176-X](https://doi.org/10.1016/S0142-9612(02)00176-X).
- (37) O'Brien, A. K.; Bowman, C. N. Impact of Oxygen on Photopolymerization Kinetics and Polymer Structure. *Macromolecules* **2006**, *39* (7), 2501-2506. DOI: 10.1021/ma051863l.
- (38) Mironi-Harpaz, I.; Wang, D. Y.; Venkatraman, S.; Seliktar, D. Photopolymerization of cell-encapsulating hydrogels: Crosslinking efficiency versus cytotoxicity. *Acta Biomaterialia* **2012**, *8* (5), 1838-1848. DOI: <https://doi.org/10.1016/j.actbio.2011.12.034>.
- (39) Hahn, M. S.; Taite, L. J.; Moon, J. J.; Rowland, M. C.; Ruffino, K. A.; West, J. L. Photolithographic patterning of polyethylene glycol hydrogels. *Biomaterials* **2006**, *27* (12), 2519-2524. DOI: <https://doi.org/10.1016/j.biomaterials.2005.11.045>.
- (40) Sakai, T.; Matsunaga, T.; Yamamoto, Y.; Ito, C.; Yoshida, R.; Suzuki, S.; Sasaki, N.; Shibayama, M.; Chung, U.-i. Design and fabrication of a high-strength hydrogel with ideally homogeneous network structure from tetrahedron-like macromonomers. *Macromolecules* **2008**, *41* (14), 5379-5384.
- (41) Lutolf, M. P.; Hubbell, J. A. Synthesis and Physicochemical Characterization of End-Linked Poly(ethylene glycol)-co-peptide Hydrogels Formed by Michael-Type Addition. *Biomacromolecules* **2003**, *4* (3), 713-722. DOI: 10.1021/bm025744e.
- (42) Zhong, M.; Wang, R.; Kawamoto, K.; Olsen, B. D.; Johnson, J. A. Quantifying the impact of molecular defects on polymer network elasticity. *Science* **2016**, *353* (6305), 1264-1268. DOI: doi:10.1126/science.aag0184.

- (43) Kharkar, P. M.; Rehmann, M. S.; Skeens, K. M.; Maverakis, E.; Kloxin, A. M. Thiol–ene Click Hydrogels for Therapeutic Delivery. *ACS Biomaterials Science & Engineering* **2016**, 2 (2), 165-179. DOI: 10.1021/acsbiomaterials.5b00420.
- (44) Hiemstra, C.; van der Aa, L. J.; Zhong, Z.; Dijkstra, P. J.; Feijen, J. Rapidly in Situ-Forming Degradable Hydrogels from Dextran Thiols through Michael Addition. *Biomacromolecules* **2007**, 8 (5), 1548-1556. DOI: 10.1021/bm061191m.
- (45) Darling, N. J.; Hung, Y.-S.; Sharma, S.; Segura, T. Controlling the kinetics of thiol-maleimide Michael-type addition gelation kinetics for the generation of homogenous poly(ethylene glycol) hydrogels. *Biomaterials* **2016**, 101, 199-206. DOI: <https://doi.org/10.1016/j.biomaterials.2016.05.053>.
- (46) Chatani, S.; Nair, D. P.; Bowman, C. N. Relative reactivity and selectivity of vinyl sulfones and acrylates towards the thiol–Michael addition reaction and polymerization. *Polymer Chemistry* **2013**, 4 (4), 1048-1055. DOI: 10.1039/c2py20826a (accessed 7/13/2026).
- (47) Gennari, A.; Wedgwood, J.; Lallana, E.; Francini, N.; Tirelli, N. Thiol-based michael-type addition. A systematic evaluation of its controlling factors. *Tetrahedron* **2020**, 76 (47), 131637. DOI: <https://doi.org/10.1016/j.tet.2020.131637>.
- (48) Zustiak, S. P.; Leach, J. B. Hydrolytically degradable poly(ethylene glycol) hydrogel scaffolds with tunable degradation and mechanical properties. *Biomacromolecules* **2010**, 11 (5), 1348-1357. DOI: 10.1021/bm100137q From NLM.
- (49) Ehrbar, M.; Rizzi, S. C.; Schoenmakers, R. G.; San Miguel, B.; Hubbell, J. A.; Weber, F. E.; Lutolf, M. P. Biomolecular Hydrogels Formed and Degraded via Site-Specific Enzymatic Reactions. *Biomacromolecules* **2007**, 8 (10), 3000-3007. DOI: 10.1021/bm070228f.
- (50) Lin, C.-C.; Anseth, K. S. Controlling Affinity Binding with Peptide-Functionalized Poly(ethylene glycol) Hydrogels. *Advanced Functional Materials* **2009**, 19 (14), 2325-2331. DOI: <https://doi.org/10.1002/adfm.200900107>.
- (51) Bhusari, S.; Sankaran, S.; del Campo, A. Regulating Bacterial Behavior within Hydrogels of Tunable Viscoelasticity. *Advanced Science* **2022**, 9 (17), 2106026. DOI: <https://doi.org/10.1002/advs.202106026>.
- (52) Bush, B. G.; Shapiro, J. M.; DelRio, F. W.; Cook, R. F.; Oyen, M. L. Mechanical measurements of heterogeneity and length scale effects in PEG-based hydrogels. *Soft Matter* **2015**, 11 (36), 7191-7200. DOI: 10.1039/c5sm01210d (accessed 7/13/2026).
- (53) Axpe, E.; Chan, D.; Offeddu, G. S.; Chang, Y.; Merida, D.; Hernandez, H. L.; Appel, E. A. A Multiscale Model for Solute Diffusion in Hydrogels. *Macromolecules* **2019**, 52 (18), 6889-6897. DOI: 10.1021/acs.macromol.9b00753.
- (54) Richbourg, N. R.; Peppas, N. A. Solute diffusion and partitioning in multi-arm poly(ethylene glycol) hydrogels. *Journal of Materials Chemistry B* **2023**, 11 (2), 377-388. DOI: 10.1039/d2tb02004a (accessed 7/13/2026).
- (55) Majer, G.; Southan, A. Adenosine triphosphate diffusion through poly(ethylene glycol) diacrylate hydrogels can be tuned by cross-link density as measured by PFG-NMR. *The Journal of Chemical Physics* **2017**, 146 (22). DOI: 10.1063/1.4984979 (accessed 7/13/2026).
- (56) Zustiak, S. P.; Boukari, H.; Leach, J. B. Solute diffusion and interactions in cross-linked poly(ethylene glycol) hydrogels studied by Fluorescence Correlation Spectroscopy. *Soft Matter* **2010**, 6 (15), 3609-3618, 10.1039/C0SM00111B. DOI: 10.1039/C0SM00111B.
- (57) Lu, S.; Anseth, K. S. Release Behavior of High Molecular Weight Solutes from Poly(ethylene glycol)-Based Degradable Networks. *Macromolecules* **2000**, 33 (7), 2509-2515. DOI: 10.1021/ma9915024.

- (58) Rehmann, M. S.; Skeens, K. M.; Kharkar, P. M.; Ford, E. M.; Maverakis, E.; Lee, K. H.; Kloxin, A. M. Tuning and Predicting Mesh Size and Protein Release from Step Growth Hydrogels. *Biomacromolecules* **2017**, *18* (10), 3131-3142. DOI: 10.1021/acs.biomac.7b00781.
- (59) Kalairaj, M. S.; George, I.; George, S. M.; Farfán, S. a. E.; Lee, Y. J.; Rivera-Tarazona, L. K.; Wang, S.; Abdelrahman, M. K.; Tasmim, S.; Dana, A.; Zimmern, P. E.; Subashchandrabose, S.; Ware, T. H. Controlled Release of Microorganisms from Engineered Living Materials. *ACS Applied Materials & Interfaces* **2025**, *17* (28), 40326-40339. DOI: 10.1021/acsami.5c11155 (accessed 7/25/2026).
- (60) Zhu, J. Bioactive modification of poly(ethylene glycol) hydrogels for tissue engineering. *Biomaterials* **2010**, *31* (17), 4639-4656. DOI: <https://doi.org/10.1016/j.biomaterials.2010.02.044>.
- (61) Barrett-Catton, E.; Ross, M. L.; Asuri, P. Multifunctional Hydrogel Nanocomposites for Biomedical Applications. *Polymers* **2021**, *13* (6), 856.
- (62) Fullenkamp, D. E.; Rivera, J. G.; Gong, Y.-k.; Lau, K. H. A.; He, L.; Varshney, R.; Messersmith, P. B. Mussel-inspired silver-releasing antibacterial hydrogels. *Biomaterials* **2012**, *33* (15), 3783-3791. DOI: <https://doi.org/10.1016/j.biomaterials.2012.02.027>.
- (63) Zhou, J.; Yang, X.; Liu, W.; Wang, C.; Shen, Y.; Zhang, F.; Zhu, H.; Sun, H.; Chen, J.; Lam, J.; Mikos, A. G.; Wang, C. Injectable OPF/graphene oxide hydrogels provide mechanical support and enhance cell electrical signaling after implantation into myocardial infarct. *Theranostics* **2018**, *8* (12), 3317-3330, Research Paper. DOI: 10.7150/thno.25504.
- (64) Steinbeck, L.; Braunmiller, D. L.; Wolff, H. J. M.; Huettche, V.; Wang, J.; Wessling, M.; Crassous, J. J.; Linkhorst, J. Magnetically Actuable Complex-Shaped Microgels for Spatio-Temporal Flow Control. *Advanced Materials Technologies* **2023**, *8* (14), 2300044. DOI: <https://doi.org/10.1002/admt.202300044>.
- (65) Andreopoulos, F. M.; Roberts, M. J.; Bentley, M. D.; Harris, J. M.; Beckman, E. J.; Russell, A. J. Photoimmobilization of organophosphorus hydrolase within a PEG-based hydrogel. *Biotechnology and Bioengineering* **1999**, *65* (5), 579-588. DOI: [https://doi.org/10.1002/\(SICI\)1097-0290\(19991205\)65:5<579::AID-BIT11>3.0.CO;2-Y](https://doi.org/10.1002/(SICI)1097-0290(19991205)65:5<579::AID-BIT11>3.0.CO;2-Y).
- (66) Marco-Dufort, B.; Janczy, J. R.; Hu, T.; Lütolf, M.; Gatti, F.; Wolf, M.; Woods, A.; Tetter, S.; Sridhar, B. V.; Tibbitt, M. W. Thermal stabilization of diverse biologics using reversible hydrogels. *Science Advances* **2022**, *8* (31), eabo0502. DOI: doi:10.1126/sciadv.abo0502.
- (67) Mancini, R. J.; Paluck, S. J.; Bat, E.; Maynard, H. D. Encapsulated Hydrogels by E-beam Lithography and Their Use in Enzyme Cascade Reactions. *Langmuir* **2016**, *32* (16), 4043-4051. DOI: 10.1021/acs.langmuir.6b00560.
- (68) Tomatsu, I.; Peng, K.; Kros, A. Photoresponsive hydrogels for biomedical applications. *Advanced Drug Delivery Reviews* **2011**, *63* (14), 1257-1266. DOI: <https://doi.org/10.1016/j.addr.2011.06.009>.
- (69) Qureshi, D.; Nayak, S. K.; Maji, S.; Anis, A.; Kim, D.; Pal, K. Environment sensitive hydrogels for drug delivery applications. *European Polymer Journal* **2019**, *120*, 109220. DOI: <https://doi.org/10.1016/j.eurpolymj.2019.109220>.
- (70) Aimetti, A. A.; Machen, A. J.; Anseth, K. S. Poly(ethylene glycol) hydrogels formed by thiol-ene photopolymerization for enzyme-responsive protein delivery. *Biomaterials* **2009**, *30* (30), 6048-6054. DOI: <https://doi.org/10.1016/j.biomaterials.2009.07.043>.
- (71) Kloxin, A. M.; Kasko, A. M.; Salinas, C. N.; Anseth, K. S. Photodegradable Hydrogels for Dynamic Tuning of Physical and Chemical Properties. *Science* **2009**, *324* (5923), 59-63. DOI: doi:10.1126/science.1169494.

- (72) DeForest, C. A.; Anseth, K. S. Cytocompatible click-based hydrogels with dynamically tunable properties through orthogonal photoconjugation and photocleavage reactions. *Nature Chemistry* **2011**, *3* (12), 925-931. DOI: 10.1038/nchem.1174.
- (73) Griffin, D. R.; Kasko, A. M. Photosensitive Delivery of Model Therapeutics from Hydrogels. *ACS Macro Letters* **2012**, *1* (11), 1330-1334. DOI: 10.1021/mz300366s.
- (74) Azagarsamy, M. A.; Anseth, K. S. Wavelength-Controlled Photocleavage for the Orthogonal and Sequential Release of Multiple Proteins. *Angewandte Chemie International Edition* **2013**, *52* (51), 13803-13807. DOI: <https://doi.org/10.1002/anie.201308174>.
- (75) Ilg, P. Stimuli-responsive hydrogels cross-linked by magnetic nanoparticles. *Soft Matter* **2013**, *9* (13), 3465-3468. DOI: 10.1039/c3sm27809c (accessed 7/14/2026).
- (76) Xue, B.; Qin, M.; Wang, T.; Wu, J.; Luo, D.; Jiang, Q.; Li, Y.; Cao, Y.; Wang, W. Electrically Controllable Actuators Based on Supramolecular Peptide Hydrogels. *Advanced Functional Materials* **2016**, *26* (48), 9053-9062. DOI: <https://doi.org/10.1002/adfm.201603947>.
- (77) Le, L.; Altin-Yavuzarslan, G.; Klatt, E. C.; Sadaba, N.; Berg, A. C.; Karanjia, A. V.; Brooks, S. M.; Zalatan, J. G.; Pozzo, L. D.; Carothers, J. M.; Alper, H. S.; Nelson, A. Bioproduction, bioprotection, and biocontainment in multi-kingdom microbial systems with 3D spatial control. *Biofabrication* **2026**, *18* (2), 025028. DOI: 10.1088/1758-5090/ae55cd.
- (78) Bao, Z.; Yang, S.; Hu, D.; Liu, J.; Jiang, B.; Sui, X.; Qi, Q.; Li, L.; Zhao, G. Programmable Semi-Interpenetrating Living Materials With Robust Stability for Versatile Bioremediation and Biotherapeutics. *Advanced Science* **2026**, *13* (29), e24320. DOI: <https://doi.org/10.1002/advs.202524320>.
- (79) Tadimarri, V. S.; Tyagi, T. A.; Duong, C. N.; Rasheed, S.; Müller, R.; Sankaran, S. Adaptations of Gram-Negative and Gram-Positive Probiotic Bacteria in Engineered Living Materials. *ACS Biomaterials Science & Engineering* **2025**, *11* (6), 3773-3784. DOI: 10.1021/acsbmaterials.5c00325.
- (80) Ueberham, M.; Danneberg, C.; Wagner, L.-M.; Pompe, T. Coupling pH-Responsive Dyes to Agarose Hydrogels for Monitoring Metabolic States of Encapsulated Phototrophic Microbial Consortia. *ACS Applied Bio Materials* **2025**, *8* (12), 10855-10865. DOI: 10.1021/acsbam.5c01556.
- (81) Fattahi, N.; Reed, J.; Heronemus, E.; Fernando, P.; Hansen, R.; Parameswaran, P. Polyethylene glycol hydrogel coatings for protection of electroactive bacteria against chemical shocks. *Bioelectrochemistry* **2024**, *156*, 108595. DOI: <https://doi.org/10.1016/j.bioelechem.2023.108595>.
- (82) Reed, J. A.; Retterer, S. T.; Hansen, R. R. Photodegradable Hydrogel Matrices for Spatiotemporal Control of Bacteria Transport and Delivery. *ACS Applied Materials & Interfaces* **2025**, *17* (37), 51919-51930. DOI: 10.1021/acsbam.5c14670.
- (83) Gutierrez, M. M.; Reed, J. A.; McElroy, R. A.; Hansen, R. R. Bacteria encapsulation into polyethylene glycol hydrogels using Michael-type addition reactions. *MRS Advances* **2024**, *9* (19), 1520-1526. DOI: 10.1557/s43580-024-00940-y.
- (84) Reed, J. A.; Gutierrez, M. M.; Dougherty, D. A.; Zaman, A. B.; Hansen, R. R. PEG-Based Living Hydrogels Engineered for Tunable Production of Bioactive Lipopeptides. *ACS Applied Polymer Materials* **2026**. DOI: 10.1021/acsbam.6c00761.
- (85) Semmling, B.; Nagel, S.; Sternberg, K.; Weitschies, W.; Seidlitz, A. Impact of different tissue-simulating hydrogel compartments on in vitro release and distribution from drug-eluting stents. *European Journal of Pharmaceutics and Biopharmaceutics* **2014**, *87* (3), 570-578. DOI: <https://doi.org/10.1016/j.ejpb.2014.04.010>.

- (86) Pimenta, A. F. R.; Ascenso, J.; Fernandes, J. C. S.; Colaço, R.; Serro, A. P.; Saramago, B. Controlled drug release from hydrogels for contact lenses: Drug partitioning and diffusion. *International Journal of Pharmaceutics* **2016**, *515* (1), 467-475. DOI: <https://doi.org/10.1016/j.ijpharm.2016.10.047>.
- (87) Liu, D. E.; Kotsmar, C.; Nguyen, F.; Sells, T.; Taylor, N. O.; Prausnitz, J. M.; Radke, C. J. Macromolecule Sorption and Diffusion in HEMA/MAA Hydrogels. *Industrial & Engineering Chemistry Research* **2013**, *52* (50), 18109-18120. DOI: 10.1021/ie402148u.
- (88) Amsden, B. Solute Diffusion within Hydrogels. Mechanisms and Models. *Macromolecules* **1998**, *31* (23), 8382-8395. DOI: 10.1021/ma980765f.
- (89) Richbourg, N. R.; Ravikumar, A.; Peppas, N. A. Solute Transport Dependence on 3D Geometry of Hydrogel Networks. *Macromolecular Chemistry and Physics* **2021**, *222* (16), 2100138. DOI: <https://doi.org/10.1002/macp.202100138>.
- (90) Kotsmar, C.; Sells, T.; Taylor, N.; Liu, D. E.; Prausnitz, J. M.; Radke, C. J. Aqueous Solute Partitioning and Mesh Size in HEMA/MAA Hydrogels. *Macromolecules* **2012**, *45* (22), 9177-9187. DOI: 10.1021/ma3018487.
- (91) Van Hove, A. H.; G. Beltejar, M.-J.; Benoit, D. S. W. Development and in vitro assessment of enzymatically-responsive poly(ethylene glycol) hydrogels for the delivery of therapeutic peptides. *Biomaterials* **2014**, *35* (36), 9719-9730. DOI: <https://doi.org/10.1016/j.biomaterials.2014.08.019>.
- (92) Huynh, C. T.; Nguyen, M. K.; Tonga, G. Y.; Longé, L.; Rotello, V. M.; Alsberg, E. Photocleavable Hydrogels for Light-Triggered siRNA Release. *Advanced Healthcare Materials* **2016**, *5* (3), 305-310. DOI: <https://doi.org/10.1002/adhm.201500778>.
- (93) Siepmann, J.; Siepmann, F. Modeling of diffusion controlled drug delivery. *J Control Release* **2012**, *161* (2), 351-362. DOI: 10.1016/j.jconrel.2011.10.006 From NLM.
- (94) Wenger, L.; Hubbuch, J. Investigation of Lysozyme Diffusion in Agarose Hydrogels Employing a Microfluidics-Based UV Imaging Approach. *Frontiers in Bioengineering and Biotechnology* **2022**, *Volume 10 - 2022*, Original Research. DOI: 10.3389/fbioe.2022.849271.
- (95) Hettiaratchi, M. H.; Schudel, A.; Rouse, T.; García, A. J.; Thomas, S. N.; Guldberg, R. E.; McDevitt, T. C. A rapid method for determining protein diffusion through hydrogels for regenerative medicine applications. *APL Bioengineering* **2018**, *2* (2). DOI: 10.1063/1.4999925 (accessed 7/24/2026).
- (96) Kamholz, A. E.; Schilling, E. A.; Yager, P. Optical Measurement of Transverse Molecular Diffusion in a Microchannel. *Biophysical Journal* **2001**, *80* (4), 1967-1972. DOI: 10.1016/S0006-3495(01)76166-8 (accessed 2026/07/23).
- (97) Canal, T.; Peppas, N. A. Correlation between mesh size and equilibrium degree of swelling of polymeric networks. *J Biomed Mater Res* **1989**, *23* (10), 1183-1193. DOI: 10.1002/jbm.820231007 From NLM.
- (98) Piciooreanu, C.; Loosdrecht, M. C. M. v.; Heijnen, J. J. Modelling the effect of oxygen concentration on nitrite accumulation in a biofilm airlift suspension reactor. *Water Science and Technology* **1997**, *36* (1), 147-156. ProQuest One Academic.
- (99) Charbonneau, M. R.; Isabella, V. M.; Li, N.; Kurtz, C. B. Developing a new class of engineered live bacterial therapeutics to treat human diseases. *Nat Commun* **2020**, *11* (1), 1738. DOI: 10.1038/s41467-020-15508-1 From NLM Medline.
- (100) Jin, K.; Huang, Y.; Che, H.; Wu, Y. Engineered Bacteria for Disease Diagnosis and Treatment Using Synthetic Biology. *Microb Biotechnol* **2025**, *18* (1), e70080. DOI: 10.1111/1751-7915.70080 From NLM Medline.

- (101) Xu, Q.; Ali, S.; Afzal, M.; Nizami, A. S.; Han, S.; Dar, M. A.; Zhu, D. Advancements in bacterial chemotaxis: Utilizing the navigational intelligence of bacteria and its practical applications. *Sci Total Environ* **2024**, *931*, 172967. DOI: 10.1016/j.scitotenv.2024.172967 From NLM Medline.
- (102) Li, Z.; Wang, Y.; Liu, J.; Rawding, P.; Bu, J.; Hong, S.; Hu, Q. Chemically and Biologically Engineered Bacteria-Based Delivery Systems for Emerging Diagnosis and Advanced Therapy. *Adv Mater* **2021**, *33* (38), e2102580. DOI: 10.1002/adma.202102580 From NLM Medline.
- (103) Jiang, Z.; Li, M.; McClements, D. J.; Liu, X.; Liu, F. Recent advances in the design and fabrication of probiotic delivery systems to target intestinal inflammation. *Food Hydrocolloids* **2022**, *125*. DOI: 10.1016/j.foodhyd.2021.107438.
- (104) Andrade, F.; Roca-Melendres, M. M.; Durán-Lara, E. F.; Rafael, D.; Schwartz Jr, S. Stimuli-responsive hydrogels for cancer treatment: The role of pH, light, ionic strength and magnetic field. *Cancers* **2021**, *13* (5), 1164.
- (105) Ruskowitz, E. R.; DeForest, C. A. Photoresponsive biomaterials for targeted drug delivery and 4D cell culture. *Nature Reviews Materials* **2018**, *3* (2). DOI: 10.1038/natrevmats.2017.87.
- (106) Kloxin, A. M. K., A.M.; Salinas, C.N.; Anseth, K.S. Photodegradable Hydrogels for Dynamic Tuning of Physical and Chemical Properties. *Science* **2009**, *324* (59-63). DOI: 10.1126/science.1169494.
- (107) Pidhatika, B.; Rodenstein, M.; Chen, Y.; Rakhmatullina, E.; Mühlebach, A.; Acikgöz, C.; Textor, M.; Konradi, R. Comparative stability studies of poly(2-methyl-2-oxazoline) and poly(ethylene glycol) brush coatings. *Biointerphases* **2012**, *7* (1-4), 1. DOI: 10.1007/s13758-011-0001-y From NLM.
- (108) Kolewe, K. W.; Zhu, J.; Mako, N. R.; Nonnenmann, S. S.; Schiffman, J. D. Bacterial Adhesion Is Affected by the Thickness and Stiffness of Poly(ethylene glycol) Hydrogels. *ACS Appl Mater Interfaces* **2018**, *10* (3), 2275-2281. DOI: 10.1021/acsami.7b12145.
- (109) Lee, S.; Tong, X.; Yang, F. Effects of the poly (ethylene glycol) hydrogel crosslinking mechanism on protein release. *Biomaterials science* **2016**, *4* (3), 405-411.
- (110) Zhou, K.; Feng, M.; Mao, H.; Gu, Z. Photoclick polysaccharide-based bioinks with an extended biofabrication window for 3D embedded bioprinting. *Biomaterials Science* **2022**, *10* (16), 4479-4491.
- (111) Nair, D. P.; Podgórski, M.; Chatani, S.; Gong, T.; Xi, W.; Fenoli, C. R.; Bowman, C. N. The Thiol-Michael Addition Click Reaction: A Powerful and Widely Used Tool in Materials Chemistry. *Chemistry of Materials* **2013**, *26* (1), 724-744. DOI: 10.1021/cm402180t.
- (112) Hoyle, C. E.; Bowman, C. N. Thiol-ene click chemistry. *Angewandte Chemie International Edition* **2010**, *49* (9), 1540-1573.
- (113) Matsunaga, T.; Sakai, T.; Akagi, Y.; Chung, U.-i.; Shibayama, M. Structure characterization of tetra-PEG gel by small-angle neutron scattering. *Macromolecules* **2009**, *42* (4), 1344-1351.
- (114) Gutierrez, M. M.; Reed, J. A.; McElroy, R. A.; Hansen, R. R. Bacteria encapsulation into polyethylene glycol hydrogels using Michael-type addition reactions. *MRS Advances* **2024**, 1-7.
- (115) Matsumoto, S.; Yamaguchi, S.; Ueno, S.; Komatsu, H.; Ikeda, M.; Ishizuka, K.; Iko, Y.; Tabata, K. V.; Aoki, H.; Ito, S.; Noji, H.; Hamachi, I. Photo gel-sol/sol-gel transition and its patterning of a supramolecular hydrogel as stimuli-responsive biomaterials. *Chemistry* **2008**, *14* (13), 3977-3986. DOI: 10.1002/chem.200701904 From NLM Medline.
- (116) van der Vlies, A. J.; Barua, N.; Nieves-Otero, P. A.; Platt, T. G.; Hansen, R. R. On Demand Release and Retrieval of Bacteria from Microwell Arrays Using Photodegradable Hydrogel Membranes. *Acs Appl Bio Mater* **2019**, *2* (1), 266-276. DOI: 10.1021/acsabm.8b00592.

- (117) Fattahi, N.; Nieves-Otero, P. A.; Masigol, M.; Van Der Vlies, A. J.; Jensen, R. S.; Hansen, R. R.; Platt, T. G. Photodegradable hydrogels for rapid screening, isolation, and genetic characterization of bacteria with rare phenotypes. *Biomacromolecules* **2020**, *21* (8), 3140-3151.
- (118) Masigol, M.; Radaha, E. L.; Kannan, A. D.; Salberg, A. G.; Fattahi, N.; Parameswaran, P.; Hansen, R. R. Polymer Surface Dissection for Correlated Microscopic and Compositional Analysis of Bacterial Aggregates during Membrane Biofouling. *ACS Applied Bio Materials* **2022**, *5* (1), 134-145. DOI: 10.1021/acsabm.1c00971.
- (119) Barua, N.; Herken, A. M.; Stern, K. R.; Reese, S.; Powers, R. L.; Morrell-Falvey, J. L.; Platt, T. G.; Hansen, R. R. Simultaneous Discovery of Positive and Negative Interactions Among Rhizosphere Bacteria Using Microwell Recovery Arrays. *Front Microbiol* **2021**, *11*, 601788. DOI: 10.3389/fmicb.2020.601788.
- (120) Barua, N.; Herken, A. M.; Melendez-Velador, N.; Platt, T. G.; Hansen, R. R. Photo-addressable microwell devices for rapid functional screening and isolation of pathogen inhibitors from bacterial strain libraries. *Biomicrofluidics* **2024**, *18* (1). DOI: 10.1063/5.0188270 (accessed 10/20/2025).
- (121) Barua, N.; Clouse, K. M.; Ruiz Diaz, D. A.; Wagner, M. R.; Platt, T. G.; Hansen, R. R. Screening the maize rhizobiome for consortia that improve *Azospirillum brasilense* root colonization and plant growth outcomes. *Frontiers in Sustainable Food Systems* **2023**, *7*. DOI: 10.3389/fsufs.2023.1106528.
- (122) Yan, B.; Boyer, J. C.; Habault, D.; Branda, N. R.; Zhao, Y. Near infrared light triggered release of biomacromolecules from hydrogels loaded with upconversion nanoparticles. *J Am Chem Soc* **2012**, *134* (40), 16558-16561. DOI: 10.1021/ja308876j.
- (123) Zhang, X.; Al-Dossary, A.; Hussain, M.; Setlow, P.; Li, J. Applications of *Bacillus subtilis* Spores in Biotechnology and Advanced Materials. *Appl Environ Microbiol* **2020**, *86* (17). DOI: 10.1128/AEM.01096-20 From NLM Medline.
- (124) Kaspar, F.; Neubauer, P.; Gimpel, M. Bioactive Secondary Metabolites from *Bacillus subtilis*: A Comprehensive Review. *J Nat Prod* **2019**, *82* (7), 2038-2053. DOI: 10.1021/acs.jnatprod.9b00110 From NLM.
- (125) Liu, A. P.; Appel, E. A.; Ashby, P. D.; Baker, B. M.; Franco, E.; Gu, L.; Haynes, K.; Joshi, N. S.; Kloxin, A. M.; Kouwer, P. H. J.; Mittal, J.; Morsut, L.; Noireaux, V.; Parekh, S.; Schulman, R.; Tang, S. K. Y.; Valentine, M. T.; Vega, S. L.; Weber, W.; Stephanopoulos, N.; Chaudhuri, O. The living interface between synthetic biology and biomaterial design. *Nat Mater* **2022**, *21* (4), 390-397. DOI: 10.1038/s41563-022-01231-3 From NLM Medline.
- (126) Yang, W. J.; Pranantyo, D.; Neoh, K. G.; Kang, E. T.; Teo, S. L.; Rittschof, D. Layer-by-layer click deposition of functional polymer coatings for combating marine biofouling. *Biomacromolecules* **2012**, *13* (9), 2769-2780. DOI: 10.1021/bm300757e.
- (127) Magin, C. M.; Finlay, J. A.; Clay, G.; Callow, M. E.; Callow, J. A.; Brennan, A. B. Antifouling performance of cross-linked hydrogels: refinement of an attachment model. *Biomacromolecules* **2011**, *12* (4), 915-922. DOI: 10.1021/bm101229v.
- (128) Gutierrez, M. M.; Cameron-Harp, M. V.; Chakraborty, P. P.; Stallbaumer-Cyr, E. M.; Morrow, J. A.; Hansen, R. R.; Derby, M. M. Investigating a microbial approach to water conservation: Effects of *Bacillus subtilis* and Surfactin on evaporation dynamics in loam and sandy loam soils. *Frontiers in Sustainable Food Systems* **2022**, *6*. DOI: 10.3389/fsufs.2022.959591.
- (129) Salek, M. M.; Carrara, F.; Zhou, J.; Stocker, R.; Jimenez-Martinez, J. Multiscale porosity microfluidics to study bacterial transport in heterogeneous chemical landscapes. *Advanced Science* **2024**, *11* (20), 2310121.

- (130) Smercina, D.; Zambare, N.; Hofmockel, K.; Sadler, N.; Bredeweg, E. L.; Nicora, C.; Markillie, L. M.; Aufrecht, J. Synthetic Soil Aggregates: Bioprinted Habitats for High-Throughput Microbial Metaphenomics. *Microorganisms* **2022**, *10* (5), 944.
- (131) Ma, L.; Shi, Y.; Siemianowski, O.; Yuan, B.; Egner, T. K.; Mirnezami, S. V.; Lind, K. R.; Ganapathysubramanian, B.; Venditti, V.; Cademartiri, L. Hydrogel-based transparent soils for root phenotyping in vivo. *Proceedings of the National Academy of Sciences* **2019**, *116* (22), 11063-11068.
- (132) Fattahi, N.; Barua, N.; van der Vlies, A. J.; Hansen, R. R. Photodegradable Hydrogel Interfaces for Bacteria Screening, Selection, and Isolation. *JoVE* **2021**, *177*, e63048. DOI: 10.3791/63048.
- (133) Kim, S.; Masum, F.; Kim, J.-K.; Chung, H. J.; Jeon, J. S. On-chip phenotypic investigation of combinatory antibiotic effects by generating orthogonal concentration gradients. *Lab on a Chip* **2019**, *19* (6), 959-973.
- (134) Meyvis, T.; De Smedt, S.; Demeester, J.; Hennink, W. Influence of the degradation mechanism of hydrogels on their elastic and swelling properties during degradation. *Macromolecules* **2000**, *33* (13), 4717-4725.
- (135) Johnston, I. D.; McCluskey, D. K.; Tan, C. K.; Tracey, M. C. Mechanical characterization of bulk Sylgard 184 for microfluidics and microengineering. *Journal of Micromechanics and Microengineering* **2014**, *24* (3), 035017.
- (136) Siepmann, J.; Siepmann, F. Modeling of diffusion controlled drug delivery. *Journal of Controlled Release* **2012**, *161* (2), 351-362. DOI: <https://doi.org/10.1016/j.jconrel.2011.10.006>.
- (137) Chien, T.; Harimoto, T.; Kepecs, B.; Gray, K.; Coker, C.; Hou, N.; Pu, K.; Azad, T.; Nolasco, A.; Pavlicova, M.; Danino, T. Enhancing the tropism of bacteria via genetically programmed biosensors. *Nat Biomed Eng* **2022**, *6* (1), 94-104. DOI: 10.1038/s41551-021-00772-3 From NLM.
- (138) Prindle, A.; Samayoa, P.; Razinkov, I.; Danino, T.; Tsimring, L. S.; Hasty, J. A sensing array of radically coupled genetic ‘biopixels’. *Nature* **2012**, *481* (7379), 39-44. DOI: 10.1038/nature10722.
- (139) Forbes, N. S. Engineering the perfect (bacterial) cancer therapy. *Nature Reviews Cancer* **2010**, *10* (11), 785-794. DOI: 10.1038/nrc2934.
- (140) Pliszczyk, D.; Bourgeois, S.; Bordes, C.; Valour, J.-P.; Mazoyer, M.-A.; Orecchioni, A.; Nakache, E.; Lantéri, P. Improvement of an encapsulation process for the preparation of pro-and prebiotics-loaded bioadhesive microparticles by using experimental design. *European journal of pharmaceutical sciences* **2011**, *44* (1-2), 83-92.
- (141) Zustiak, S. P.; Leach, J. B. Hydrolytically Degradable Poly(Ethylene Glycol) Hydrogel Scaffolds with Tunable Degradation and Mechanical Properties. *Biomacromolecules* **2010**, *11* (5), 1348-1357. DOI: 10.1021/bm100137q.
- (142) Rijns, L.; Rutten, M. G.; Vreken, A. F.; Aldana, A. A.; Baker, M. B.; Dankers, P. Y. Mimicking the extracellular world: From natural to fully synthetic matrices utilizing supramolecular biomaterials. *Nanoscale* **2024**, *16* (35), 16290-16312.
- (143) Pidhatika, B.; Rodenstein, M.; Chen, Y.; Rakhmatullina, E.; Mühlebach, A.; Acikgöz, C.; Textor, M.; Konradi, R. Comparative stability studies of poly (2-methyl-2-oxazoline) and poly (ethylene glycol) brush coatings. *Biointerphases* **2012**, *7* (1), 1.
- (144) Ekblad, T.; Bergström, G.; Ederth, T.; Conlan, S. L.; Mutton, R.; Clare, A. S.; Wang, S.; Liu, Y.; Zhao, Q.; D’Souza, F.; Donnelly, G. T.; Willemsen, P. R.; Pettitt, M. E.; Callow, M. E.; Callow, J. A.; Liedberg, B. Poly(ethylene glycol)-Containing Hydrogel Surfaces for Antifouling

Applications in Marine and Freshwater Environments. *Biomacromolecules* **2008**, 9 (10), 2775-2783. DOI: 10.1021/bm800547m.

(145) Hern, D. L.; Hubbell, J. A. Incorporation of adhesion peptides into nonadhesive hydrogels useful for tissue resurfacing. *Journal of Biomedical Materials Research* **1998**, 39 (2), 266-276. DOI: [https://doi.org/10.1002/\(SICI\)1097-4636\(199802\)39:2<266::AID-JBM14>3.0.CO;2-B](https://doi.org/10.1002/(SICI)1097-4636(199802)39:2<266::AID-JBM14>3.0.CO;2-B).

(146) Cui, J.; Liu, L.; Chen, B.; Hu, J.; Song, M.; Dai, H.; Wang, X.; Geng, H. A comprehensive review on the inherent and enhanced antifouling mechanisms of hydrogels and their applications. *International Journal of Biological Macromolecules* **2024**, 265, 130994. DOI: <https://doi.org/10.1016/j.ijbiomac.2024.130994>.

(147) Lin, C.-C.; Anseth, K. S. PEG Hydrogels for the Controlled Release of Biomolecules in Regenerative Medicine. *Pharmaceutical Research* **2009**, 26 (3), 631-643. DOI: 10.1007/s11095-008-9801-2.

(148) Andrade, F.; Roca-Melendres, M. M.; Durán-Lara, E. F.; Rafael, D.; Schwartz, S. Stimuli-Responsive Hydrogels for Cancer Treatment: The Role of pH, Light, Ionic Strength and Magnetic Field. *Cancers* **2021**, 13 (5), 1164.

(149) Liao, H.; Munoz-Pinto, D.; Qu, X.; Hou, Y.; Grunlan, M. A.; Hahn, M. S. Influence of hydrogel mechanical properties and mesh size on vocal fold fibroblast extracellular matrix production and phenotype. *Acta Biomaterialia* **2008**, 4 (5), 1161-1171. DOI: <https://doi.org/10.1016/j.actbio.2008.04.013>.

(150) Correa, S.; Grosskopf, A. K.; Lopez Hernandez, H.; Chan, D.; Yu, A. C.; Stapleton, L. M.; Appel, E. A. Translational Applications of Hydrogels. *Chemical Reviews* **2021**, 121 (18), 11385-11457. DOI: 10.1021/acs.chemrev.0c01177.

(151) Leconte, A.; Tournant, L.; Muchembled, J.; Paucellier, J.; Héquet, A.; Deracinois, B.; Deweer, C.; Krier, F.; Deleu, M.; Oste, S.; Jacques, P.; Coutte, F. Assessment of Lipopeptide Mixtures Produced by *Bacillus subtilis* as Biocontrol Products against Apple Scab (*Venturia inaequalis*). *Microorganisms* **2022**, 10 (9), 1810.

(152) Caulier, S.; Nannan, C.; Gillis, A.; Licciardi, F.; Bragard, C.; Mahillon, J. Overview of the Antimicrobial Compounds Produced by Members of the *Bacillus subtilis* Group. *Frontiers in Microbiology* **2019**, Volume 10 - 2019, Review. DOI: 10.3389/fmicb.2019.00302.

(153) Hsieh, F.-C.; Li, M.-C.; Lin, T.-C.; Kao, S.-S. Rapid Detection and Characterization of Surfactin-Producing *Bacillus subtilis* and Closely Related Species Based on PCR. *Current Microbiology* **2004**, 49 (3), 186-191. DOI: 10.1007/s00284-004-4314-7.

(154) Savitskaya, I. S.; Shokatayeva, D. H.; Kistaubayeva, A. S.; Ignatova, L. V.; Digel, I. E. Antimicrobial and wound healing properties of a bacterial cellulose based material containing *B. subtilis* cells. *Heliyon* **2019**, 5 (10), e02592. DOI: <https://doi.org/10.1016/j.heliyon.2019.e02592>.

(155) Cao, X.-h.; Wang, A.-h.; Wang, C.-l.; Mao, D.-z.; Lu, M.-f.; Cui, Y.-q.; Jiao, R.-z. Surfactin induces apoptosis in human breast cancer MCF-7 cells through a ROS/JNK-mediated mitochondrial/caspase pathway. *Chemico-Biological Interactions* **2010**, 183 (3), 357-362. DOI: <https://doi.org/10.1016/j.cbi.2009.11.027>.

(156) Fattahi, N.; Barua, N.; van der Vlies, A. J.; Hansen, R. R. Photodegradable hydrogel interfaces for bacteria screening, selection, and isolation. *Journal of Visualized Experiments* **2021**, (177).

(157) Barua, N.; Clouse, K. M.; Ruiz Diaz, D. A.; Wagner, M. R.; Platt, T. G.; Hansen, R. R. Screening the maize rhizobiome for consortia that improve *Azospirillum brasilense* root

colonization and plant growth outcomes. *Frontiers in Sustainable Food Systems* **2023**, Volume 7 - 2023, Original Research. DOI: 10.3389/fsufs.2023.1106528.

(158) Morton, E. R.; Fuqua, C. Laboratory Maintenance of *Agrobacterium*. *Current Protocols in Microbiology* **2012**, 24 (1), 3D.1.1-3D.1.6. DOI: <https://doi.org/10.1002/9780471729259.mc03d01s24>.

(159) Leighton, T.; Doi, R. H. The stability of messenger ribonucleic acid during sporulation in *Bacillus subtilis*. *Journal of Biological Chemistry* **1971**, 246 (10), 3189-3195.

(160) Miller, J. H. Experiments in molecular genetics. (*No Title*) **1972**.

(161) Han, C.; Zhang, X.; Pang, G.; Zhang, Y.; Pan, H.; Li, L.; Cui, M.; Liu, B.; Kang, R.; Xue, X.; Sun, T.; Liu, J.; Chang, J.; Zhao, P.; Wang, H. Hydrogel microcapsules containing engineered bacteria for sustained production and release of protein drugs. *Biomaterials* **2022**, 287, 121619. DOI: <https://doi.org/10.1016/j.biomaterials.2022.121619>.

(162) Tao, S.; Zhang, S.; Wei, K.; Maniura-Weber, K.; Li, Z.; Ren, Q. An Injectable Living Hydrogel with Embedded Probiotics as a Novel Strategy for Combating Multifaceted Pathogen Wound Infections. *Advanced Healthcare Materials* **2024**, 13 (27), 2400921. DOI: <https://doi.org/10.1002/adhm.202400921>.

(163) Ming, Z.; Han, L.; Bao, M.; Zhu, H.; Qiang, S.; Xue, S.; Liu, W. Living Bacterial Hydrogels for Accelerated Infected Wound Healing. *Advanced Science* **2021**, 8 (24), 2102545. DOI: <https://doi.org/10.1002/advs.202102545>.

(164) Buchanan, R. L.; Cygnarowicz, M. L. A mathematical approach toward defining and calculating the duration of the lag phase. *Food Microbiology* **1990**, 7 (3), 237-240.

(165) Sankaran, S.; del Campo, A. Optoregulated Protein Release from an Engineered Living Material. *Advanced Biosystems* **2019**, 3 (2), 1800312. DOI: <https://doi.org/10.1002/adbi.201800312>.

(166) Yang, H.; Yu, H.; Shen, Z. A novel high-throughput and quantitative method based on visible color shifts for screening *Bacillus subtilis* THY-15 for surfactin production. *Journal of Industrial Microbiology and Biotechnology* **2015**, 42 (8), 1139-1147. DOI: 10.1007/s10295-015-1635-4 (accessed 5/23/2025).

(167) Thermo Fisher, S. *LIVE/DEAD™ BacLight™ Bacterial Viability Kits*; Thermo Fisher Scientific, 2004. <https://assets.thermofisher.com/TFS-Assets/LSG/manuals/mp07007.pdf>.

(168) Soyuer, K.; Bilen Ozyurek, S. An eco-friendly approach to biosurfactant production using low-cost wastes. *Journal of Dispersion Science and Technology* **2024**, 45 (8), 1599-1612. DOI: 10.1080/01932691.2023.2225581.

(169) Russell, A.; Loosemore, M. Effect of phenol on *Bacillus subtilis* spores at elevated temperatures. *Applied Microbiology* **1964**, 12 (5), 403-406.

(170) Laflamme, C.; Lavigne, S.; Ho, J.; Duchaine, C. Assessment of bacterial endospore viability with fluorescent dyes. *Journal of Applied Microbiology* **2004**, 96 (4), 684-692.

(171) Kroger, S. M.; Hill, L.; Jain, E.; Stock, A.; Bracher, P. J.; He, F.; Zustiak, S. P. Design of Hydrolytically Degradable Polyethylene Glycol Crosslinkers for Facile Control of Hydrogel Degradation. *Macromolecular Bioscience* **2020**, 20 (10), 2000085. DOI: <https://doi.org/10.1002/mabi.202000085>.

(172) Nardecchia, I.; Lechelon, M.; Gori, M.; Donato, I.; Preto, J.; Floriani, E.; Jaeger, S.; Mailfert, S.; Marguet, D.; Ferrier, P.; Pettini, M. Detection of long-range electrostatic interactions between charged molecules by means of fluorescence correlation spectroscopy. *Physical Review E* **2017**, 96 (2), 022403. DOI: 10.1103/PhysRevE.96.022403.

- (173) Scheidweiler, D.; Bordoloi, A. D.; Jiao, W.; Sentchilo, V.; Bollani, M.; Chhun, A.; Engel, P.; de Anna, P. Spatial structure, chemotaxis and quorum sensing shape bacterial biomass accumulation in complex porous media. *Nature Communications* **2024**, *15* (1), 191. DOI: 10.1038/s41467-023-44267-y.
- (174) Boedicker, J. Q.; Vincent, M. E.; Ismagilov, R. F. Microfluidic Confinement of Single Cells of Bacteria in Small Volumes Initiates High-Density Behavior of Quorum Sensing and Growth and Reveals Its Variability. *Angewandte Chemie International Edition* **2009**, *48* (32), 5908-5911. DOI: <https://doi.org/10.1002/anie.200901550>.
- (175) Li, P.; Dou, X.; Feng, C.; Müller, M.; Chang, M. W.; Frettlöh, M.; Schönherr, H. Isolated Reporter Bacteria in Supramolecular Hydrogel Microwell Arrays. *Langmuir* **2017**, *33* (31), 7799-7809. DOI: 10.1021/acs.langmuir.7b00749.
- (176) Gao, M.; Zheng, H.; Ren, Y.; Lou, R.; Wu, F.; Yu, W.; Liu, X.; Ma, X. A crucial role for spatial distribution in bacterial quorum sensing. *Scientific Reports* **2016**, *6* (1), 34695. DOI: 10.1038/srep34695.
- (177) Rahman, F. B.; Sarkar, B.; Moni, R.; Rahman, M. S. Molecular genetics of surfactin and its effects on different sub-populations of *Bacillus subtilis*. *Biotechnology Reports* **2021**, *32*, e00686. DOI: <https://doi.org/10.1016/j.btre.2021.e00686>.
- (178) Kinsinger, R. F.; Shirk, M. C.; Fall, R. Rapid Surface Motility in *Bacillus subtilis* Is Dependent on Extracellular Surfactin and Potassium Ion. *Journal of Bacteriology* **2003**, *185* (18), 5627-5631. DOI: doi:10.1128/jb.185.18.5627-5631.2003.
- (179) Meena, K. R.; Sharma, A.; Kanwar, S. S. Antitumoral and Antimicrobial Activity of Surfactin Extracted from *Bacillus subtilis* KLP2015. *International Journal of Peptide Research and Therapeutics* **2020**, *26* (1), 423-433. DOI: 10.1007/s10989-019-09848-w.
- (180) Alborno, R. V.; Oyarzún, D.; Burgess, K. Optimisation of surfactin yield in *Bacillus* using data-efficient active learning and high-throughput mass spectrometry. *Computational and Structural Biotechnology Journal* **2024**, *23*, 1226-1233. DOI: 10.1016/j.csbj.2024.02.012 (accessed 2025/10/29).
- (181) Rivera-Tarazona, L. K.; Kalairaj, M. S.; Corazao, T.; Javed, M.; Zimmermann, P. E.; Subashchandrabose, S.; Ware, T. H. Controlling shape morphing and cell release in engineered living materials. *Biomaterials advances* **2022**, *143*, 213182.
- (182) Hansen, R. H.; Timm, A. C.; Timm, C. M.; Bible, A. N.; Morrell-Falvey, J. L.; Pelletier, D. A.; Simpson, M. L.; Doktycz, M. J.; Retterer, S. T. Stochastic assembly of bacteria in microwell arrays reveals the importance of confinement in community development. *PLoS One* **2016**, *11* (5), e0155080.
- (183) González, L. M.; Mukhitov, N.; Voigt, C. A. Resilient living materials built by printing bacterial spores. *Nature Chemical Biology* **2020**, *16* (2), 126-133. DOI: 10.1038/s41589-019-0412-5.
- (184) Pinto, C. A.; Veiga, V.; Ganjeh, A. M.; Tavares, J.; Matos, G.; Lima, V.; Saraiva, J. A. *Bacillus subtilis* spores' inactivation kinetics by hyperbaric storage at room temperature and dependence of pH and nutrient availability. *International Journal of Food Microbiology* **2025**, *441*, 111322. DOI: <https://doi.org/10.1016/j.ijfoodmicro.2025.111322>.
- (185) Cano, R. J.; Borucki, M. K. Revival and identification of bacterial spores in 25- to 40-million-year-old Dominican amber. *Science* **1995**, *268* (5213), 1060-1064. DOI: 10.1126/science.7538699 From NLM.

- (186) Hu, F.; Liu, Y.; Li, S. Rational strain improvement for surfactin production: enhancing the yield and generating novel structures. *Microb Cell Fact* **2019**, *18* (1), 42. DOI: 10.1186/s12934-019-1089-x From NLM.
- (187) An, B.; Wang, Y.; Huang, Y.; Wang, X.; Liu, Y.; Xun, D.; Church, G. M.; Dai, Z.; Yi, X.; Tang, T.-C.; Zhong, C. Engineered Living Materials For Sustainability. *Chemical Reviews* **2023**, *123* (5), 2349-2419. DOI: 10.1021/acs.chemrev.2c00512.
- (188) Rivera-Tarazona, L. K.; Campbell, Z. T.; Ware, T. H. Stimuli-responsive engineered living materials. *Soft Matter* **2021**, *17* (4), 785-809.
- (189) Gutenberger, G. M.; Holgate, O. M.; Arnold, W. A.; Guest, J. S.; Novak, P. J. Polyethylene glycol as a robust, biocompatible encapsulant for two-stage treatment of food and beverage wastewater. *Environmental Science: Water Research & Technology* **2024**, *10* (2), 467-479.
- (190) Charbonneau, M. R.; Isabella, V. M.; Li, N.; Kurtz, C. B. Developing a new class of engineered live bacterial therapeutics to treat human diseases. *Nature Communications* **2020**, *11* (1), 1738. DOI: 10.1038/s41467-020-15508-1.
- (191) Zhou, S.; Gravekamp, C.; Bermudes, D.; Liu, K. Tumour-targeting bacteria engineered to fight cancer. *Nature Reviews Cancer* **2018**, *18* (12), 727-743. DOI: 10.1038/s41568-018-0070-z.
- (192) Etter, E. L.; Thormann, S.; Venkatraman, S.; Potluru, S. S.; Nguyen, J. Hydrogel platforms for engineered live biotherapeutics: materials, microbial integration and clinical potential. *RSC pharmaceuticals* **2026**.
- (193) Luchan, J.; Choi, C.; Carrier, R. L. Reactive oxygen species limit intestinal mucosa-bacteria homeostasis in vitro. *Scientific Reports* **2021**, *11* (1), 23727. DOI: 10.1038/s41598-021-02080-x.
- (194) Halliwell, B.; Clement, M. V.; Long, L. H. Hydrogen peroxide in the human body. *FEBS Letters* **2000**, *486* (1), 10-13. DOI: [https://doi.org/10.1016/S0014-5793\(00\)02197-9](https://doi.org/10.1016/S0014-5793(00)02197-9).
- (195) Yao, Y.; Zhang, H.; Wang, Z.; Ding, J.; Wang, S.; Huang, B.; Ke, S.; Gao, C. Reactive oxygen species (ROS)-responsive biomaterials mediate tissue microenvironments and tissue regeneration. *Journal of Materials Chemistry B* **2019**, *7* (33), 5019-5037. DOI: 10.1039/c9tb00847k (accessed 7/29/2026).
- (196) Liu, X.; Huang, D.; Zhu, C.; Zhu, F.; Zhu, X.; Zhou, D. Production of reactive oxygen species during redox manipulation and its potential impacts on activated sludge wastewater treatment processes. *Environmental science & technology* **2024**, *58* (52), 23042-23052.
- (197) Mosquera-Romero, S.; PrévotEAU, A.; Vanwonterghem, I.; Arends, J. B.; Dominguez, L.; Rousseau, D. P.; Rabaey, K. Hydrogen peroxide in bioelectrochemical systems negatively affects microbial current generation. *Journal of Applied Electrochemistry* **2021**, *51* (10), 1463-1478.
- (198) Wang, Z.; Li, D.; Shi, Y.; Sun, Y.; Okeke, S. I.; Yang, L.; Zhang, W.; Zhang, Z.; Shi, Y.; Xiao, L. Recent Implementations of Hydrogel-Based Microbial Electrochemical Technologies (METs) in Sensing Applications. *Sensors* **2023**, *23* (2), 641.
- (199) Hahn, M. M.; González, J. F.; Gunn, J. S. Salmonella Biofilms Tolerate Hydrogen Peroxide by a Combination of Extracellular Polymeric Substance Barrier Function and Catalase Enzymes. *Frontiers in Cellular and Infection Microbiology* **2021**, *Volume 11 - 2021*, Original Research. DOI: 10.3389/fcimb.2021.683081.
- (200) Kolewe, K. W.; Zhu, J.; Mako, N. R.; Nonnenmann, S. S.; Schiffman, J. D. Bacterial Adhesion Is Affected by the Thickness and Stiffness of Poly(ethylene glycol) Hydrogels. *ACS Applied Materials & Interfaces* **2018**, *10* (3), 2275-2281. DOI: 10.1021/acsami.7b12145.
- (201) Dowds, B. C. A. The oxidative stress response in *Bacillus subtilis*. *FEMS Microbiology Letters* **1994**, *124* (3), 255-263. DOI: 10.1111/j.1574-6968.1994.tb07294.x (accessed 4/11/2026).

- (202) Wang, X.; Wang, Q. Enzyme-Laden Bioactive Hydrogel for Biocatalytic Monitoring and Regulation. *Accounts of Chemical Research* **2021**, *54* (5), 1274-1287. DOI: 10.1021/acs.accounts.0c00832.
- (203) Shannon, M. R.; Zhou, B.; Perriman, A. W. Leveraging the Power of Enzymes in Engineered Dead and Living Materials. *Advanced Functional Materials* **2024**, *34* (44), 2404522. DOI: <https://doi.org/10.1002/adfm.202404522>.
- (204) Jeevarathinam, A. S.; Guo, F.; Williams, T.; Smolen, J. A.; Hyde, J. A.; McShane, M. J.; de Figueiredo, P.; Alge, D. L. Enzyme functionalized microgels enable precise regulation of dissolved oxygen and anaerobe culture. *Materials Today Bio* **2021**, *9*, 100092. DOI: <https://doi.org/10.1016/j.mtbio.2020.100092>.
- (205) Meyer, J.; Meyer, L.-E.; Kara, S. Enzyme immobilization in hydrogels: A perfect liaison for efficient and sustainable biocatalysis. *Engineering in Life Sciences* **2022**, *22* (3-4), 165-177. DOI: <https://doi.org/10.1002/elsc.202100087>.
- (206) Mohsin, M. Z.; Omer, R.; Huang, J.; Mohsin, A.; Guo, M.; Qian, J.; Zhuang, Y. Advances in engineered *Bacillus subtilis* biofilms and spores, and their applications in bioremediation, biocatalysis, and biomaterials. *Synthetic and Systems Biotechnology* **2021**, *6* (3), 180-191. DOI: <https://doi.org/10.1016/j.synbio.2021.07.002>.
- (207) Kaspar, F.; Neubauer, P.; Gimpel, M. Bioactive Secondary Metabolites from *Bacillus subtilis*: A Comprehensive Review. *Journal of Natural Products* **2019**, *82* (7), 2038-2053. DOI: 10.1021/acs.jnatprod.9b00110.
- (208) Paez, J. I.; Farrukh, A.; Valbuena-Mendoza, R.; Włodarczyk-Biegun, M. K.; Del Campo, A. Thiol-methylsulfone-based hydrogels for 3D cell encapsulation. *ACS applied materials & interfaces* **2020**, *12* (7), 8062-8072.
- (209) Jansen, L. E.; Negrón-Piñero, L. J.; Galarza, S.; Peyton, S. R. Control of thiol-maleimide reaction kinetics in PEG hydrogel networks. *Acta Biomaterialia* **2018**, *70*, 120-128. DOI: <https://doi.org/10.1016/j.actbio.2018.01.043>.
- (210) Engasser, J.-M.; Horvath, C. Diffusion and Kinetics with Immobilized Enzymes¹ The authors' research and the preparation of this chapter were supported by Grants No. GM 20993 and CA 17245 from the National Institutes of Health, U.S. Public Health Service. In *Applied Biochemistry and Bioengineering*, Wingard, L. B., Katchalski-Katzir, E., Goldstein, L. Eds.; Vol. 1; Elsevier, 1976; pp 127-220.
- (211) Tse, P. H. S.; Leypoldt, J. K.; Gough, D. A. Determination of the intrinsic kinetic constants of immobilized glucose oxidase and catalase. *Biotechnology and Bioengineering* **1987**, *29* (6), 696-704. DOI: <https://doi.org/10.1002/bit.260290606>.
- (212) Thermo Fisher, S. Amine-Reactive Probe Labeling Protocol. (accessed 2026/07/03).
- (213) Kirkpatrick, B. E.; Rubio, M. T.; Yendamuri, T.; Elmer, N. V.; Benoit, D. S. W.; Guymon, C. A.; Anseth, K. S.; Hebner, T. S. Structure–reactivity based control of radical-mediated degradation in thiol–Michael hydrogels. *Journal of Materials Chemistry B* **2025**, *13* (33), 10177-10186. DOI: 10.1039/d5tb01237f (accessed 7/3/2026).
- (214) Nair, D. P.; Podgórski, M.; Chatani, S.; Gong, T.; Xi, W.; Fenoli, C. R.; Bowman, C. N. The thiol-Michael addition click reaction: a powerful and widely used tool in materials chemistry. *Chemistry of Materials* **2014**, *26* (1), 724-744.
- (215) Nam, C.; Zimudzi, T. J.; Geise, G. M.; Hickner, M. A. Increased Hydrogel Swelling Induced by Absorption of Small Molecules. *ACS Applied Materials & Interfaces* **2016**, *8* (22), 14263-14270. DOI: 10.1021/acsami.6b02069.

- (216) Kitano, H.; Ichikawa, K.; Ide, M.; Fukuda, M.; Mizuno, W. Fourier Transform Infrared Study on the State of Water Sorbed to Poly(ethylene glycol) Films. *Langmuir* **2001**, *17* (6), 1889-1895. DOI: 10.1021/la0008986.
- (217) Ide, M.; Yoshikawa, D.; Maeda, Y.; Kitano, H. State of Water inside and at the Surface of Poly(ethylene glycol) Films Examined by FT-IR. *Langmuir* **1999**, *15* (4), 926-929. DOI: 10.1021/la980239h.
- (218) Yan, B.; Zhang, Y.; Wei, C.; Xu, Y. Facile synthesis of ROS-responsive biodegradable main chain poly(carbonate-thioether) copolymers. *Polymer Chemistry* **2018**, *9* (7), 904-911. DOI: 10.1039/c7py01908d (accessed 7/3/2026).
- (219) Jeanmaire, D.; Laliturai, J.; Almalik, A.; Carampin, P.; d'Arcy, R.; Lallana, E.; Evans, R.; Winpenny, R. E.; Tirelli, N. Chemical specificity in REDOX-responsive materials: the diverse effects of different Reactive Oxygen Species (ROS) on polysulfide nanoparticles. *Polymer Chemistry* **2014**, *5* (4), 1393-1404.
- (220) Napoli, A.; Valentini, M.; Tirelli, N.; Müller, M.; Hubbell, J. A. Oxidation-responsive polymeric vesicles. *Nature Materials* **2004**, *3* (3), 183-189. DOI: 10.1038/nmat1081.
- (221) Tan, J.; Li, C.; De Bruycker, K.; Zhang, G.; Gu, J.; Zhang, Q. Recyclable cross-linked hydroxythioether particles with tunable structures via robust and efficient thiol-epoxy dispersion polymerizations. *RSC advances* **2017**, *7* (82), 51763-51772.
- (222) Sarapas, J. M.; Tew, G. N. Poly (ether-thioethers) by thiol-ene click and their oxidized analogues as lithium polymer electrolytes. *Macromolecules* **2016**, *49* (4), 1154-1162.
- (223) Touchet, T.; Briggs, S.; Graul, L.; Maitland, D. J. Development and Characterization of Oxidatively Responsive Thiol-Ene Networks for Bone Graft Applications. *ACS Applied Bio Materials* **2022**, *5* (6), 2633-2642. DOI: 10.1021/acsabm.2c00103.
- (224) Smith, M. A.; Kalana, U. I.; Sterbinsky, G. E.; Wu, T.; Kiesewetter, M. K.; Hayes, D. Determining sulfur speciation in oxidatively crosslinked degradable polymers using sulfur K-edge X-ray absorption spectroscopy. *Journal of Polymer Science* **2024**, *62* (18), 4315-4325.
- (225) Tjell, A. Ø.; Almdal, K. Diffusion rate of hydrogen peroxide through water-swelled polyurethane membranes. *Sensing and Bio-Sensing Research* **2018**, *21*, 35-39. DOI: <https://doi.org/10.1016/j.sbsr.2018.10.001>.
- (226) Offeddu, G. S.; Axpe, E.; Harley, B. A. C.; Oyen, M. L. Relationship between permeability and diffusivity in polyethylene glycol hydrogels. *AIP Advances* **2018**, *8* (10). DOI: 10.1063/1.5036999 (accessed 5/11/2026).
- (227) Vats, K.; Marsh, G.; Harding, K.; Zampetakis, I.; Waugh, R. E.; Benoit, D. S. W. Nanoscale physicochemical properties of chain- and step-growth polymerized PEG hydrogels affect cell-material interactions. *Journal of Biomedical Materials Research Part A* **2017**, *105* (4), 1112-1122. DOI: <https://doi.org/10.1002/jbm.a.36007>.
- (228) Jürgen-Lohmann, D. L.; Legge, R. L. Immobilization of bovine catalase in sol-gels. *Enzyme and Microbial Technology* **2006**, *39* (4), 626-633. DOI: <https://doi.org/10.1016/j.enzmictec.2005.11.015>.
- (229) Hamilton, B. K.; Gardner, C. R.; Colton, C. K. Effect of diffusional limitations on lineweaver-burk plots for immobilized enzymes. *AIChE Journal* **1974**, *20* (3), 503-510. DOI: <https://doi.org/10.1002/aic.690200310>.
- (230) Abdel-Mageed, H. M.; Abd El Aziz, A. E.; Abdel Raouf, B. M.; Mohamed, S. A.; Nada, D. Antioxidant-biocompatible and stable catalase-based gelatin-alginate hydrogel scaffold with thermal wound healing capability: immobilization and delivery approach. *3 Biotech* **2022**, *12* (3), 73. DOI: 10.1007/s13205-022-03131-4.

- (231) Arica, M. Y.; Öktem, H. A.; Öktem, Z.; Tuncel, S. A. Immobilization of catalase in poly(isopropylacrylamide-co-hydroxyethylmethacrylate) thermally reversible hydrogels. *Polymer International* **1999**, *48* (9), 879-884. DOI: [https://doi.org/10.1002/\(SICI\)1097-0126\(199909\)48:9<879::AID-PI237>3.0.CO;2-N](https://doi.org/10.1002/(SICI)1097-0126(199909)48:9<879::AID-PI237>3.0.CO;2-N).
- (232) Wu, Y.-S.; Ngai, S.-C.; Goh, B.-H.; Chan, K.-G.; Lee, L.-H.; Chuah, L.-H. Anticancer Activities of Surfactin and Potential Application of Nanotechnology Assisted Surfactin Delivery. *Frontiers in Pharmacology* **2017**, *Volume 8 - 2017*, Review. DOI: 10.3389/fphar.2017.00761.
- (233) Pham, T. T.; Tran, P. L.; Phung, C. D.; Nguyen, H. T.; Nguyen, C. H.; Yong, C. S.; Kim, J. O.; Yook, S.; Jeong, J.-H. Surface-Triggered In Situ Gelation for Tunable Conformal Hydrogel Coating of Therapeutic Cells and Biomedical Devices. *Advanced Functional Materials* **2021**, *31* (21), 2010169. DOI: <https://doi.org/10.1002/adfm.202010169>.
- (234) Araújo-Gomes, N.; Zoetebier-Liszka, B.; van Loo, B.; Becker, M.; Nijhuis, S.; Smink, A. M.; de Haan, B. J.; de Vos, P.; Karperien, M.; Leijten, J. Microfluidic Generation of Thin-Shelled Polyethylene Glycol-Tyramine Microgels for Non-Invasive Delivery of Immunoprotected β -Cells. *Advanced Healthcare Materials* **2024**, *13* (25), 2301552. DOI: <https://doi.org/10.1002/adhm.202301552>.
- (235) Richbourg, N. R.; Peppas, N. A. High-Throughput FRAP Analysis of Solute Diffusion in Hydrogels. *Macromolecules* **2021**, *54* (22), 10477-10486. DOI: 10.1021/acs.macromol.1c01752 (accessed 7/29/2026).
- (236) Bonvillain, R. W.; Painter, R. G.; Ledet, E. M.; Wang, G. Comparisons of resistance of CF and Non-CF pathogens to Hydrogen Peroxide and Hypochlorous Acid Oxidants In Vitro. *BMC Microbiology* **2011**, *11* (1), 112. DOI: 10.1186/1471-2180-11-112.
- (237) Botin, T.; Ramirez-Chamorro, L.; Vidic, J.; Langella, P.; Martin-Verstraete, I.; Chatel, J.-M.; Auger, S. The Tolerance of Gut Commensal *Faecalibacterium* to Oxidative Stress Is Strain Dependent and Relies on Detoxifying Enzymes. *Applied and Environmental Microbiology* **2023**, *89* (7), e00606-00623. DOI: doi:10.1128/aem.00606-23.
- (238) Alcorn, T. M.; Zheng, H. Y.; Gunther, M. R.; Hassett, D. J.; Cohen, M. S. Variation in hydrogen peroxide sensitivity between different strains of *Neisseria gonorrhoeae* is dependent on factors in addition to catalase activity. *Infection and Immunity* **1994**, *62* (5), 2138-2140. DOI: doi:10.1128/iai.62.5.2138-2140.1994.
- (239) Aoyagi, R.; Sato, R.; Terada, A.; Tokuyama, H. Novel composite gel beads for the immobilization of ammonia-oxidizing bacteria: Fabrication, characterization, and biokinetic analysis. *Chemical Engineering Journal* **2018**, *342*, 260-265. DOI: <https://doi.org/10.1016/j.cej.2018.02.087>.
- (240) Xiao, Y.-P.; Chen, P.-H.; Lei, S.; Bai, F.; Fu, L.-H.; Lin, J.; Huang, P. Biocatalytic Depletion of Tumorigenic Energy Sources Driven by Cascade Reactions for Efficient Antitumor Therapy. *Angewandte Chemie International Edition* **2022**, *61* (42), e202204584. DOI: <https://doi.org/10.1002/anie.202204584>.
- (241) Choi, H.; Yeo, M.; Kang, Y.; Kim, H. J.; Park, S. G.; Jang, E.; Park, S. H.; Kim, E.; Kang, S. Lactate oxidase/catalase-displaying nanoparticles efficiently consume lactate in the tumor microenvironment to effectively suppress tumor growth. *Journal of Nanobiotechnology* **2023**, *21* (1), 5. DOI: 10.1186/s12951-022-01762-6.
- (242) Dhami, N. K.; Reddy, M. S.; Mukherjee, A. Synergistic Role of Bacterial Urease and Carbonic Anhydrase in Carbonate Mineralization. *Applied Biochemistry and Biotechnology* **2014**, *172* (5), 2552-2561. DOI: 10.1007/s12010-013-0694-0.

- (243) Mao, S.; Bai, B.; Ye, X.; Lin, Y. Living Hydrogels: Harnessing Microorganism–Material Synergy for Next-Generation Therapeutics. *Advanced Science* **2026**, *13* (25), e21766. DOI: <https://doi.org/10.1002/advs.202521766>.
- (244) Harimoto, T.; Jung, W.-H.; Mooney, D. J.; Harimoto, T.; Jung, W.-H.; Mooney, D. J. Delivering living medicines with biomaterials. *Nature reviews*. **2025**, *10* (3), 191-210. DOI: 10.1038/s41578-024-00766-y.
- (245) Desai, K.; Mekontso, J. I.; Deshpande, K.; Trujillo, S. Preclinical Assessment of Living Therapeutic Materials: State-of-Art and Challenges. *ACS Biomaterials Science & Engineering* **2025**, *11* (5), 2584-2600. DOI: 10.1021/acsbiomaterials.5c00247 (accessed 7/30/2026).
- (246) Thermo Fisher, S. Peptones, Supplements and Feeds: Technical Reference Guide. 1 ed.; Thermo Fisher Scientific: Waltham, MA, USA, 2019; p 61.
- (247) Cavallo, A.; Madaghiele, M.; Masullo, U.; Lionetto, M. G.; Sannino, A. Photo-crosslinked poly (ethylene glycol) diacrylate (PEGDA) hydrogels from low molecular weight prepolymer: swelling and permeation studies. *Journal of Applied Polymer Science* **2017**, *134* (2).
- (248) Wilkins, D. K.; Grimshaw, S. B.; Receveur, V.; Dobson, C. M.; Jones, J. A.; Smith, L. J. Hydrodynamic Radii of Native and Denatured Proteins Measured by Pulse Field Gradient NMR Techniques. *Biochemistry* **1999**, *38* (50), 16424-16431. DOI: 10.1021/bi991765q.
- (249) Fattahi, N.; Reed, J.; Heronemus, E.; Fernando, P.; Hansen, R.; Parameswaran, P. Polyethylene glycol hydrogel coatings for protection of electroactive bacteria against chemical shocks. *Bioelectrochemistry* **2024**, *156*. DOI: 10.1016/j.bioelechem.2023.108595.
- (250) Cruise, G. M.; Scharp, D. S.; Hubbell, J. A. Characterization of permeability and network structure of interfacially photopolymerized poly(ethylene glycol) diacrylate hydrogels. *Biomaterials*. **1998**, *19* (14), 1287-1294. DOI: 10.1016/S0142-9612(98)00025-8.

Appendix A - List of Abbreviations

Abbreviation	Name
ACA-PEG-SCM	Acrylamide-poly(ethylene glycol)-succinimidyl carboxymethyl ester
AF488	Alexa Fluor 488
AF633	Alexa Fluor 633
ATCC	American Type Culture Collection
ATGN	Ammonium, tryptone, glucose, and nutrient phosphate medium
<i>B. subtilis</i>	<i>Bacillus subtilis</i>
BLC	Bovine liver catalase
BTB	Bromothymol blue
CFCF	Cell-free culture fluid
CNMS	Center for Nanophase Materials Sciences
CPC	Cetylpyridinium chloride
DI	Deionized
DMSO	Dimethyl sulfoxide
<i>E. coli</i>	<i>Escherichia coli</i>
ELH	Engineered living hydrogel
ELM	Engineered living material
EPS	Extracellular polymeric substances
FITC	Fluorescein isothiocyanate
FTIR	Fourier-transform infrared spectroscopy
GFP	Green fluorescent protein
GRAS	Generally recognized as safe
H ₂ O ₂	Hydrogen peroxide
MEC	Microbial electrolysis cell
MFC	Microbial fuel cell
MIC	Minimum inhibitory concentration
MW	Molecular weight
MxC	Microbial electrochemical cell
NHS	N-hydroxysuccinimide
NMR	Nuclear magnetic resonance
OD ₆₀₀	Optical density at 600 nm
<i>o</i> -NB	<i>o</i> -nitrobenzyl
ORNL	Oak Ridge National Laboratory
PAA	Poly(acrylic acid)
PBS	Phosphate-buffered saline
PDMS	Polydimethylsiloxane
<i>pd</i> -PEG	Photodegradable poly(ethylene glycol)
PEG	Poly(ethylene glycol)
PEGDA	Poly(ethylene glycol) diacrylate
PEG- <i>o</i> -NB-DA	Poly(ethylene glycol) <i>o</i> -nitrobenzyl diacrylate
PEGTT	Poly(ethylene glycol) tetrathiol
PEGVS	Poly(ethylene glycol) vinyl sulfone
PFOS	Trichloro(1H,1H,2H,2H,-perfluorooctyl)silane
PVA	Poly(vinyl alcohol)

RIE	Reactive ion etching
ROI	Region of interest
ROS	Reactive oxygen species
RP-HPLC	Reverse-phase high-performance liquid chromatography
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SG	Schaeffer's glucose medium
SMART	Stimulus-responsive hydrogels
TLFM	Time-lapse fluorescence microscopy
TRITC	Tetramethylrhodamine isothiocyanate
TSB	Tryptic soy broth
UV	Ultraviolet
UV-Vis	Ultraviolet-visible spectroscopy

Appendix B - Supporting Information for Chapter 4

B.1 Estimated tryptic soy broth (TSB) diffusion coefficient method

TSB is a complex mixture of casein/soy peptones, sodium chloride, and natural sugars, with the main carbon and nitrogen sources required for growth coming from glucose and tryptone (digest of casein), respectively.²⁴⁶ The diffusion coefficient of glucose has previously been measured through similar PEG hydrogels ($1.29 \times 10^{-6} \text{ cm}^2/\text{s}$).²⁴⁷ Difco tryptone is a digest of casein protein and is the primary nitrogen source in TSB; its peptide molecular weight breakdown is given on page 61 of the Thermo-Fisher technical guide to peptones, supplements, and feeds.²⁴⁶

To estimate the diffusion coefficient (D_0) for each peptide molecular weight range in water, the Stokes-Einstein equation was used:⁵³

$$D_o = \frac{k_b T}{6\pi\eta R_h} \quad (1)$$

where k_b is the Boltzmann constant, T is the absolute temperature, and η is the dynamic viscosity of the solvent (water), and R_h is the average hydrodynamic radius for each peptide molecular weight range. Equation 5 in Wilkins et al.²⁴⁸ was used to calculate R_h for each unfolded/denatured peptide for each molecular weight range shown as:

$$R_h = (2.21 \pm 1.07)N^{0.57 \pm 0.02} \quad (2)$$

Where N is the number of residues in the polypeptide chain. While this equation is in terms of amino acid residues instead of molecular weight, the average weight of an amino acid (110 Da) is used to estimate the number of residues. The middle value of each peptide range (125 Da for <250 Da and 1,250 for 500-2,000 Da, etc.) was used for each size. The weighted average diffusion coefficient ($\overline{D}_0$) was calculated with Equation 3:

$$\overline{D}_0 = \sum \left(\frac{M_i}{100} * D_i \right) \quad (3)$$

where M_i is the mass fraction for each size range. With this, $\overline{D_0}$ was calculated to be $5.09 \times 10^{-6} \text{ cm}^2/\text{s}$. Assuming an average reduction in diffusivity of 88% through the hydrogel, which has been measured with other small molecules through these hydrogels,²⁴⁹ the effective diffusion of Difco Tryptone (D_{eff}) through the hydrogel is estimated to be $6.11 \times 10^{-7} \text{ cm}^2/\text{s}$. With these diffusion coefficients, a Python script was created to model passive diffusive transport of glucose and tryptone components through the microfluidic device channel. The model uses Fick's second law:

$$\frac{\partial c_a}{\partial t} = D_{\text{eff}} \left(\frac{\partial^2 c_a}{\partial x^2} \right) \quad (4)$$

and assumes constant concentrations of each component at the source well ($x=0 \text{ } \mu\text{m}$) and sink well ($x=3000 \text{ } \mu\text{m}$) as boundary conditions. The resulting concentration profiles across the channel over 48 hrs are provided for Alexa 594 dye (Fig. B.1 below) which matches experimental data (Fig. 4.2 in main manuscript), for tryptone (Fig. B.2 below), and finally for glucose (Fig. B.3 below). The model shows that 24 hrs was sufficient time for glucose and tryptone to form a linear, steady-state gradient across the hydrogel in the actual chemotaxis experiments.

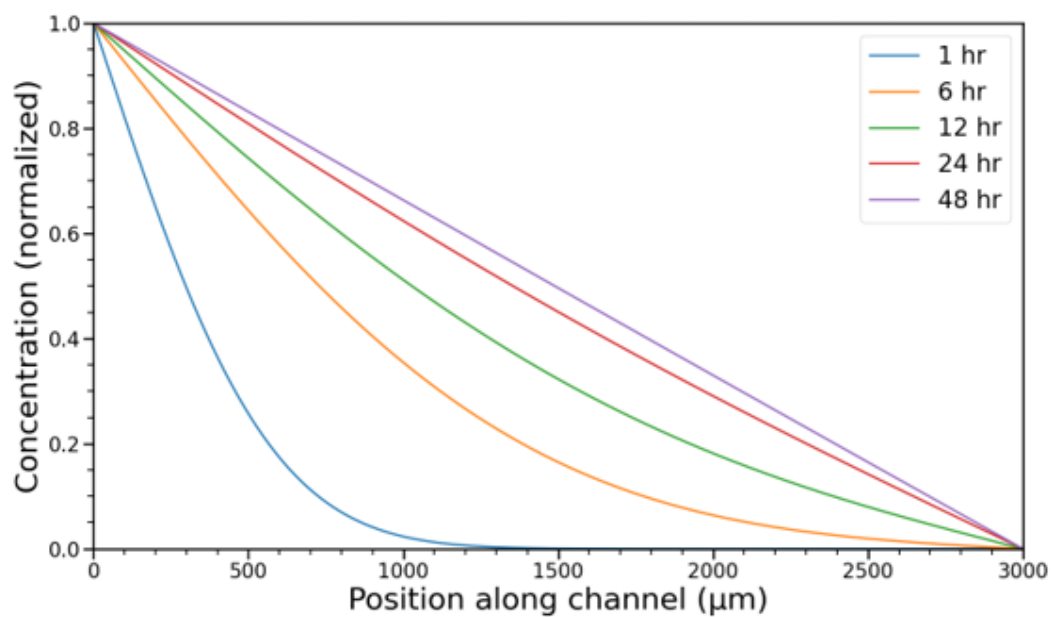


Figure B.1. Simulated concentration profile of Alexa Fluor 594 dye through microfluidic channel according to Fick's Second Law.

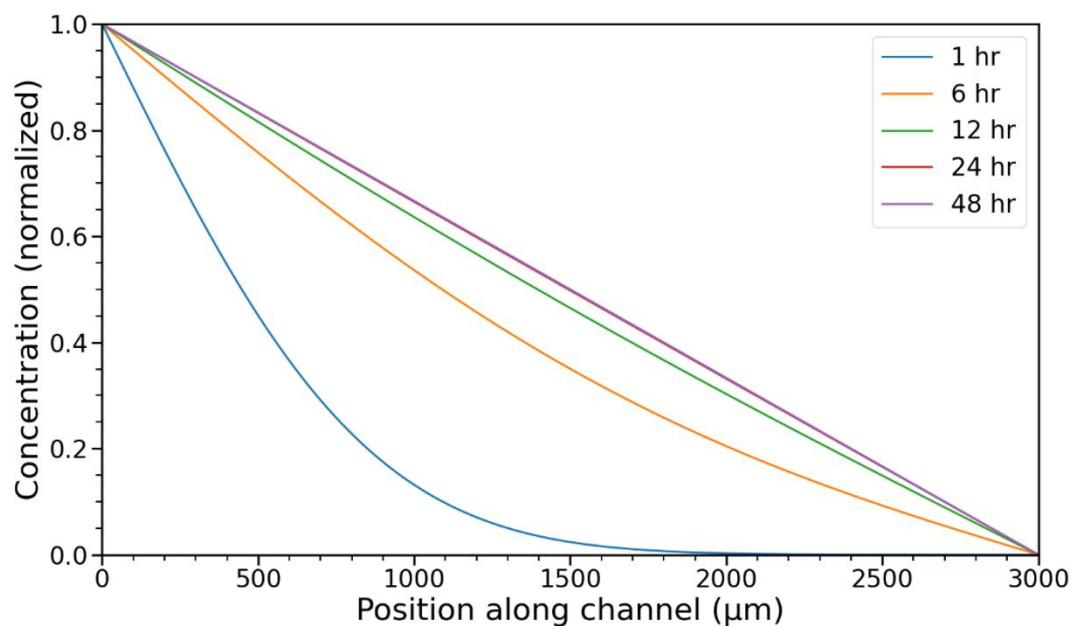


Figure B.2. Simulated concentration profile of difco tryptone through microfluidic channel according to Fick's Second Law.

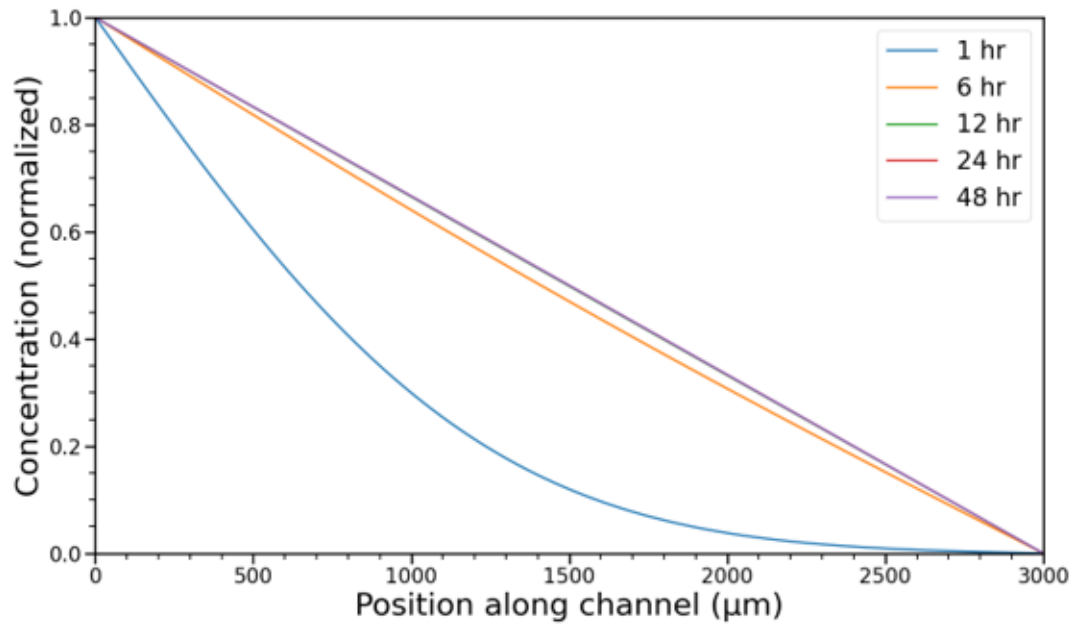


Figure B.3. Simulated concentration profile of glucose through microfluidic channel according to Fick's Second Law.

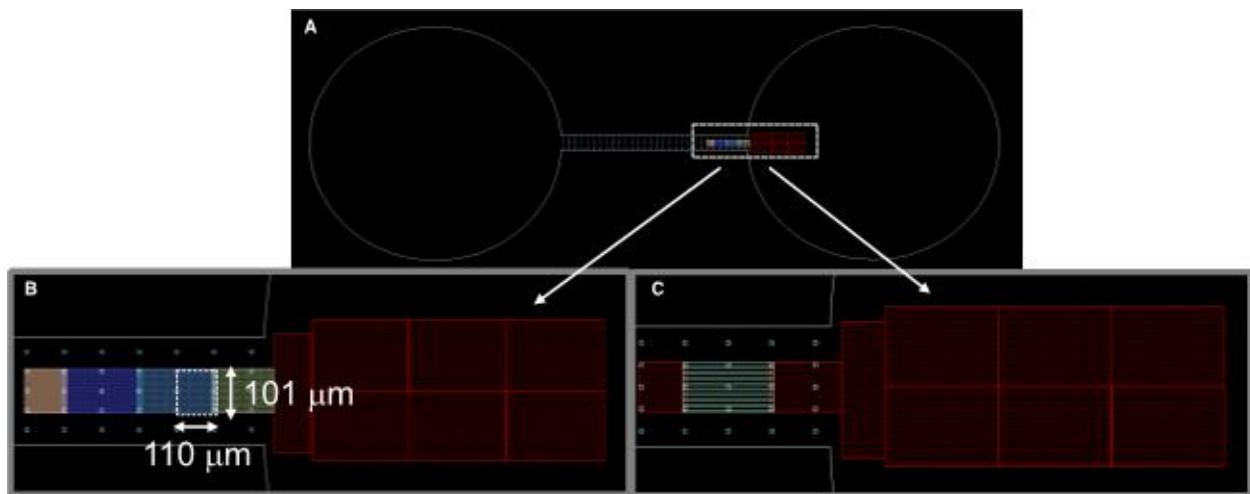


Figure B.4. (A) CAD design of entire microfluidic device in layout editor. Boxes show areas exposed with 365 nm ultraviolet light. Zoomed in view of the region of interest for the (B) degradation ladder experiments and (C) selective delivery experiment.

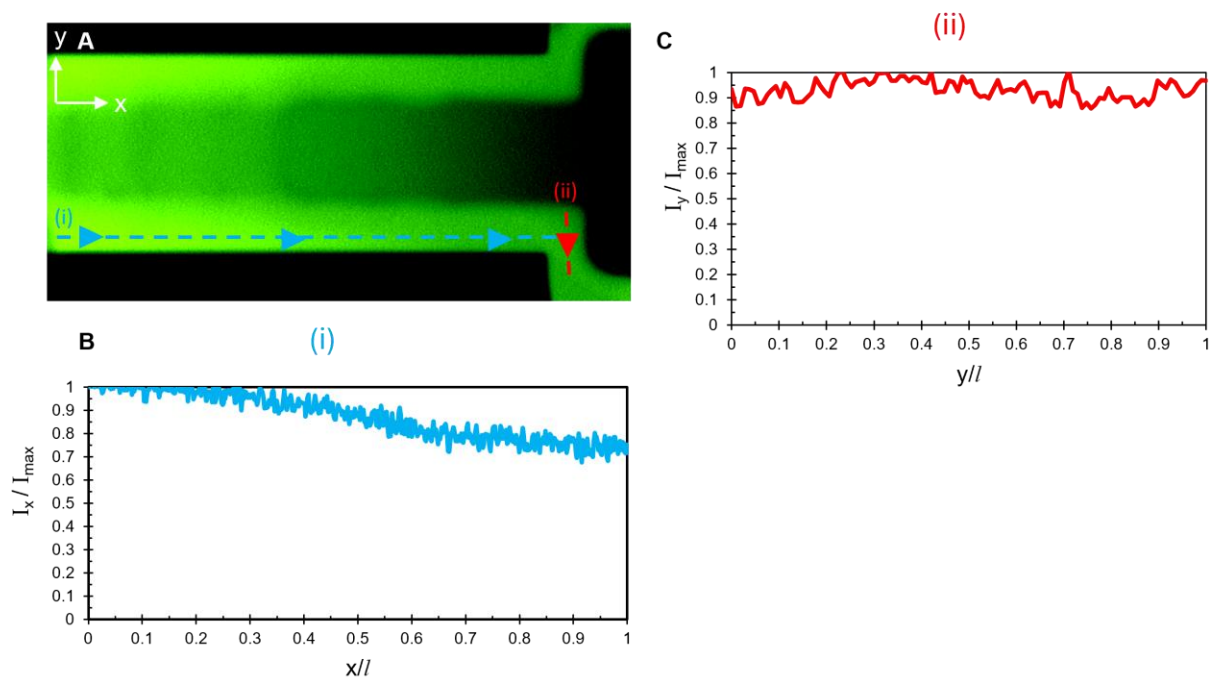


Figure B.5. (A) Image of non-degraded hydrogel regions after staining with Fluorescein-5-Maleimide. Normalized intensity profile in the (B) x-direction and (C) y-direction.

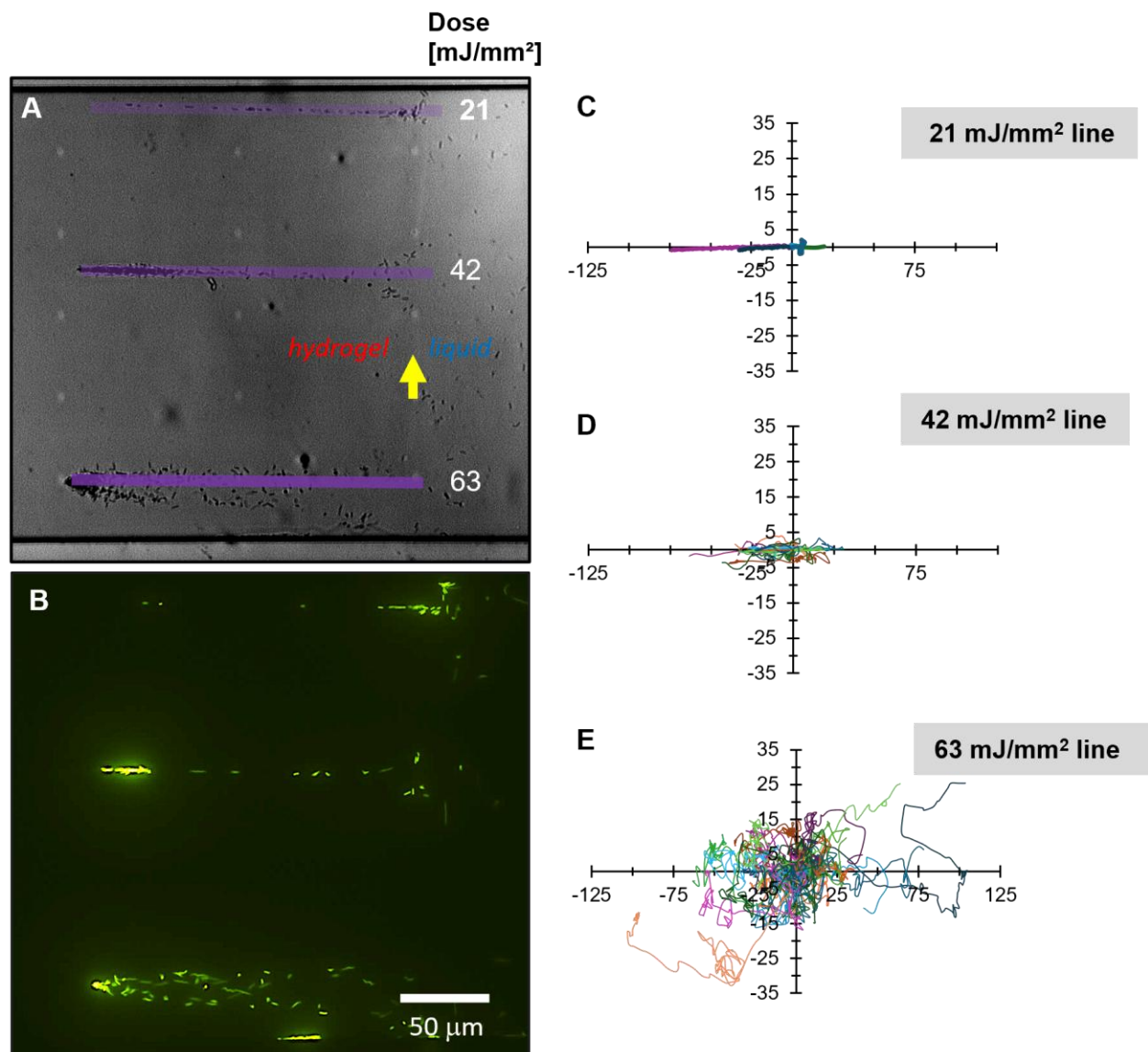


Figure B.6. Image of (A) degradation map and (B) fluorescent image showing bacteria protrude into partially degraded regions. Individual cell trajectories and rose plot for (C) 21 mJ/mm^2 line, (D) 42 mJ/mm^2 line, and (E) 63 mJ/mm^2 line.

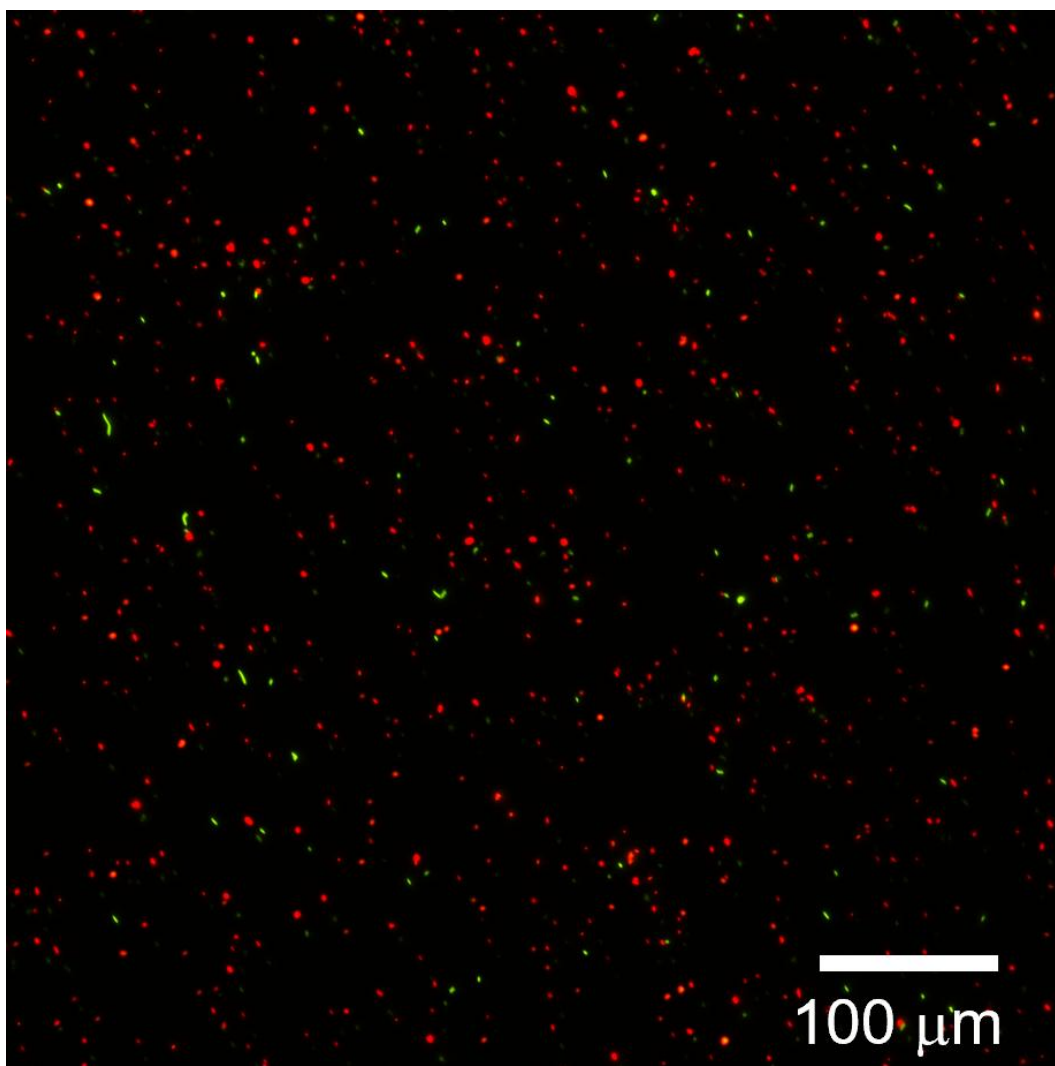


Figure B.7. Red-green fluorescent image of *B. subtilis*-GFP cells (green) mixed with 1 mm diameter polystyrene beads (red) at a ~10:1 bead:cell ratio. This solution was added to the sink well.

Appendix C - Supporting Information for Chapter 5

C.1 Average mesh size calculation using equilibrium swelling theory⁹⁷:

C1.1 Mass swelling ratio (Q_m):

$$Q_m = \frac{M_s}{M_d} \quad (1)$$

Where:

M_d = mass of dry hydrogel

M_s = mass of swollen hydrogel

C1.2 Volumetric swelling ratio (Q_v):

$$Q_v = 1 + \frac{\rho_p}{\rho_s} * (Q_m - 1) \quad (2)$$

Where:

ρ_p = polymer density (1.125 g / cm³)

ρ_s = solvent density (water, 1.000 g / cm³)

C1.3 Polymer volume fraction (v_2):

$$v_2 = Q_v^{-1} \quad (3)$$

C1.4 Molecular weight between crosslinks (M_c):

$$\frac{1}{M_c} = \frac{2}{M_n} - \frac{\bar{v}}{V_1} * \frac{\ln(1-v_2) + v_2 + \chi v_2^2}{v_2^{1/3} - \frac{v_2}{2}} \quad (4)$$

Where:

M_n = average molecular weight of the un-crosslinked gel (molecular weight of 2-arm PEGDA plus 2 * (1/4) of the 4-arm PEGSH molecular weight). For example, for PEGDA_{2k} – PEGSH_{5k} the M_n would be 2,000 + 2 * (1,250) = 4,500

$\bar{v}$ = specific volume of the polymer ($\frac{\rho_s}{\rho_p}$, 0.8889)

V_1 = molar volume of the solvent (water, 18 cm³ / mol)

χ = polymer-solvent interaction parameter (0.426 for PEG-water)^{141, 250}

C1.5 Number of bonds in the crosslink (n):

$$n = 2 * \frac{M_c}{M_r} (5)$$

Where M_r = molecular weight of the repeat unit (44 g/mol for PEG)

C1.6 Root-mean-square end-to-end distance of the unperturbed polymer chain $(r_o^{1/2})^2$:

$$(r_o^{1/2})^2 = l \sqrt{C_n n} (6)$$

Where:

l is the average bond length (0.146 nm)¹⁴¹

C_n is the Flory characteristic ratio of the polymer (typically 4.0 for PEG)⁵⁸

C1.7 Mesh size (ξ) calculation:

$$\xi \text{ (nm)} = v_2^{-1/3} * (r_o^2)^{1/2} (7)$$

C2 Hydrogel length calculation for diffusion coefficient measurement:

C2.1 Estimation of initial gel mass

Hydrogel precursor densities were estimated from the weighted density contributions from each of precursor stock solutions. Although hydrogels were prepared from a nominal 200 μ L precursor volume, formulation-specific densities were used to convert measured gel masses into volumes during equilibrium swelling calculations.

PEGDA and PEGSH stock solutions were prepared in water. Stock solution mass concentrations were estimated from the molecular weights given from the manufacturer specific to the polymer lot and the corresponding molar stock concentrations. For example:

$$49 \text{ mM } 10\text{kDa PEGDA} * 11,000 \frac{\text{g}}{\text{mol}} = 0.539 \text{ g/mL}$$

$$20 \text{ mM } 10\text{kDa PEGSH} * 10,897 \frac{\text{g}}{\text{mol}} = 0.2179 \text{ g/mL}$$

$$\rho_{gel} = \sum \frac{\rho_i * V_i}{V_{gel}} \quad (8)$$

Where:

ρ_{gel} = specific density of the gel formulation

ρ_i = density of the precursor

V_i = volume of precursor added

V_{gel} = volume of the gel

Initial gel mass ($m_{gel,i}$) can then be calculated by:

$$m_{gel,i} = \rho_{gel} * V_{gel} \quad (9)$$

C2.2 Estimation of the swollen hydrogel volume

Equilibrium swollen masses were used from the average mesh size determination to estimate swollen hydrogel volumes and subsequent hydrogel thickness used for diffusion coefficient estimation.

$$Q_m = \frac{m_{gel,f}}{m_{gel,i}} \quad (10)$$

The swollen volume of the hydrogel was then calculated using:

$$V_{gel,f} = Q_v * V_{initial} \quad (11)$$

Where Q_v is calculated using equation 4.

C2.3 Hydrogel length estimation

The internal cross-sectional area of the cylindrical glass vials used to estimate the swollen hydrogel length. To calculate the glass jar inner diameter, 1 mL of tryptic soy broth (TSB) was added to an empty vial and the liquid height was measured from the internal vial bottom to the bottom of the liquid meniscus. The internal vial radius was calculated to be 0.4955 cm, corresponding to an internal diameter of 0.991 cm. The internal glass jar cross-sectional area ($A_{c,jar}$) was then calculated to be 0.771 cm². The length of swollen hydrogel ($L_{gel,f}$) can then be calculated by:

$$L_{gel,f} = \frac{V_{gel,f}}{A_{c,jar}} (12)$$

Table C.1. Estimated hydrogel densities, initial masses, and swollen thicknesses

Hydrogel formulation	Estimated formulation density (ρ_{gel}, g/mL)	Estimated initial hydrogel mass ($m_{gel,i}$, mg)	Estimated swollen thickness ($L_{gel,f}$, cm)
2kDA/5kSH (~9.5 nm mesh)	1.006	201.2	0.520
10kDA/10kSH (~15 nm mesh)	1.020	204.0	0.889
10kDA/20kSH (~19 nm mesh)	1.026	205.2	1.259

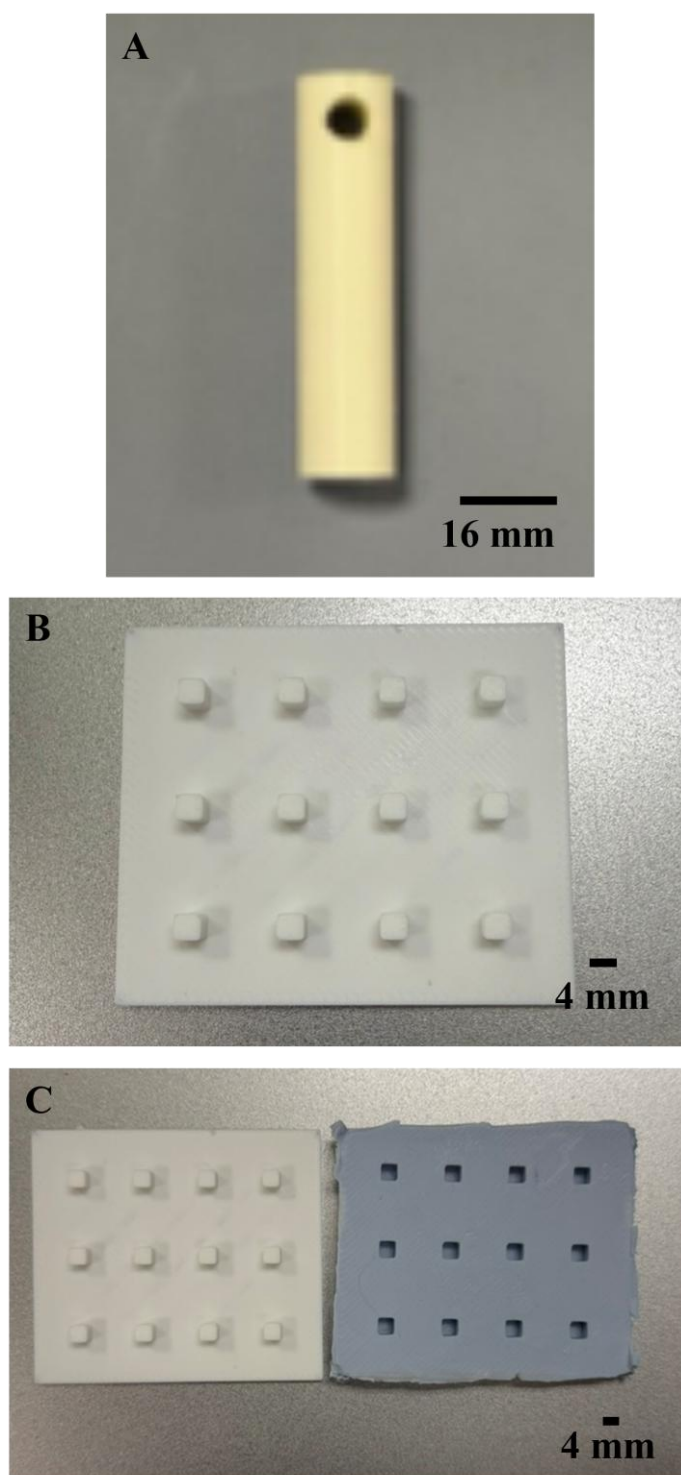


Figure C.1. (A) Image of 3D-printed attachments to measure Young's modulus and (B and C) the plate/mold to create 4 mm hydrogel cubes.

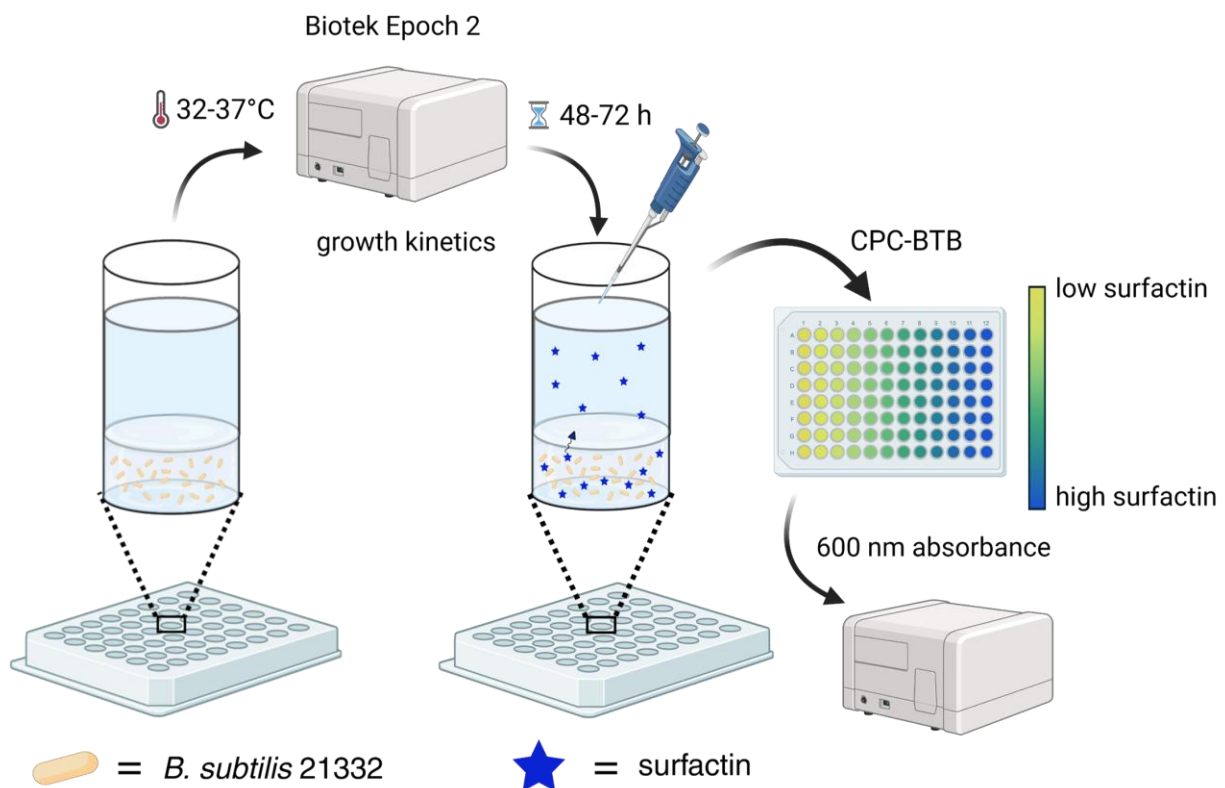


Figure C.2. Experimental schematic of supernatant taken from above the hydrogel in 48-well plates to measure surfactin production from *B. subtilis* encapsulated in PEG hydrogels. Figure was completed using Biorender software.

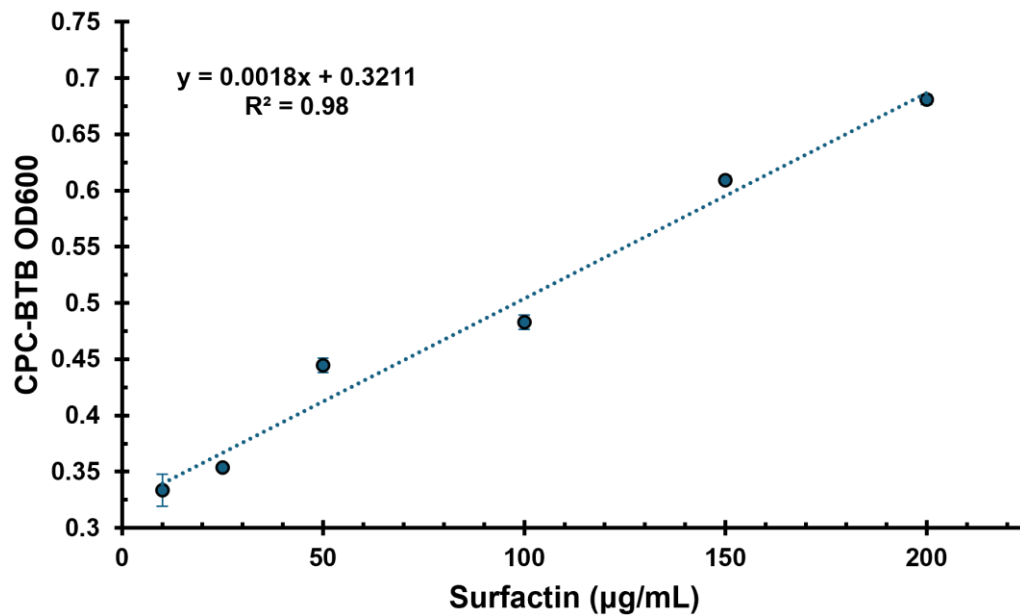


Figure C.3. Standard calibration curve relating absolute surfactin concentration to absorbance at 600 nm using purified surfactin dissolved in M9 minimal media.

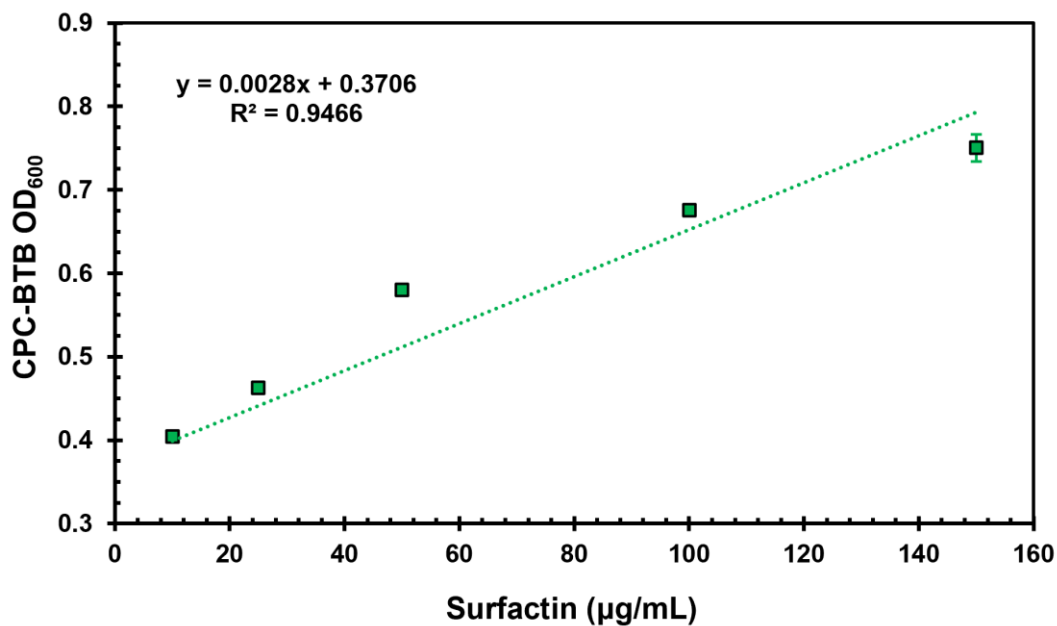


Figure C.4. Standard calibration curve relating absolute surfactin concentration to absorbance at 600 nm using purified surfactin dissolved in tryptic soy broth (TSB) media.

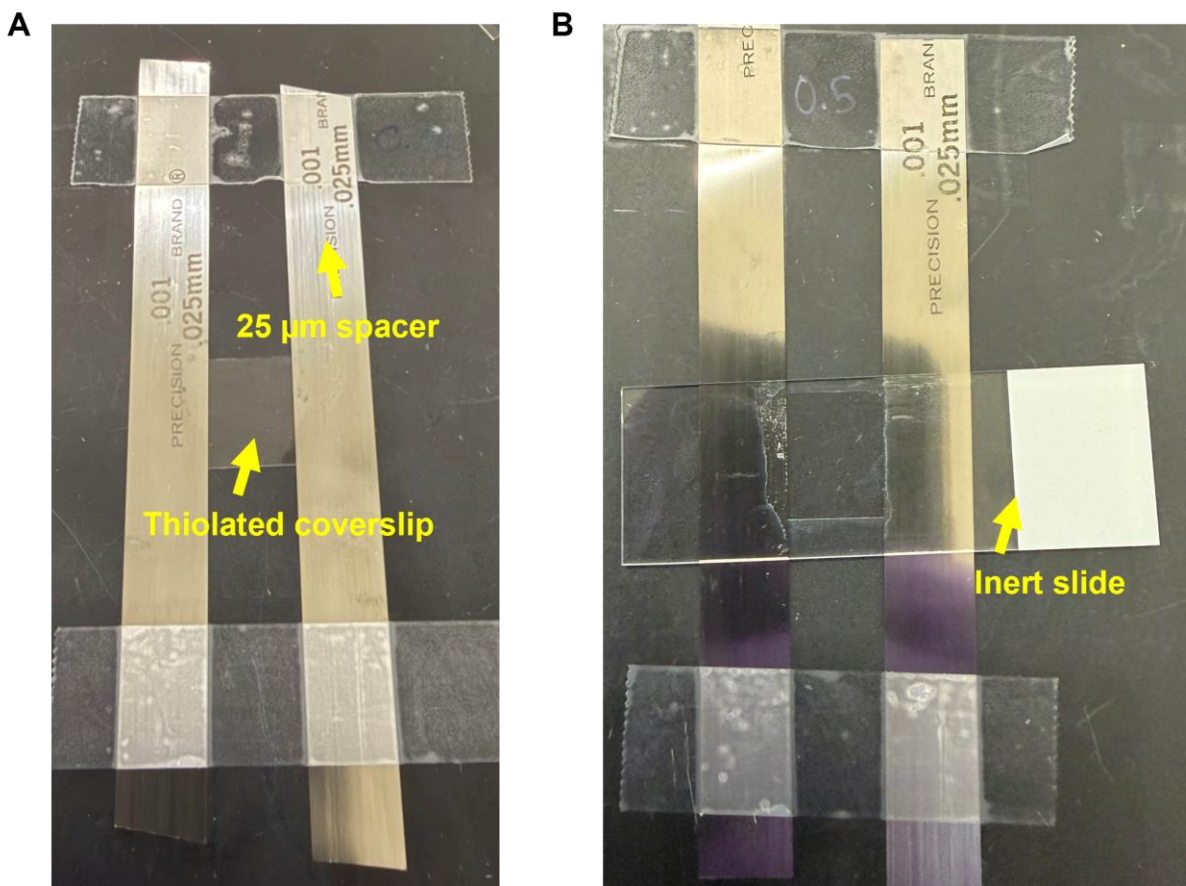


Figure C.5. (A) Thiolated coverslip taped under 25 μm spacers to control hydrogel thickness. (B) hydrophobic/inert glass slide with hydrogel pressed onto the spacers.

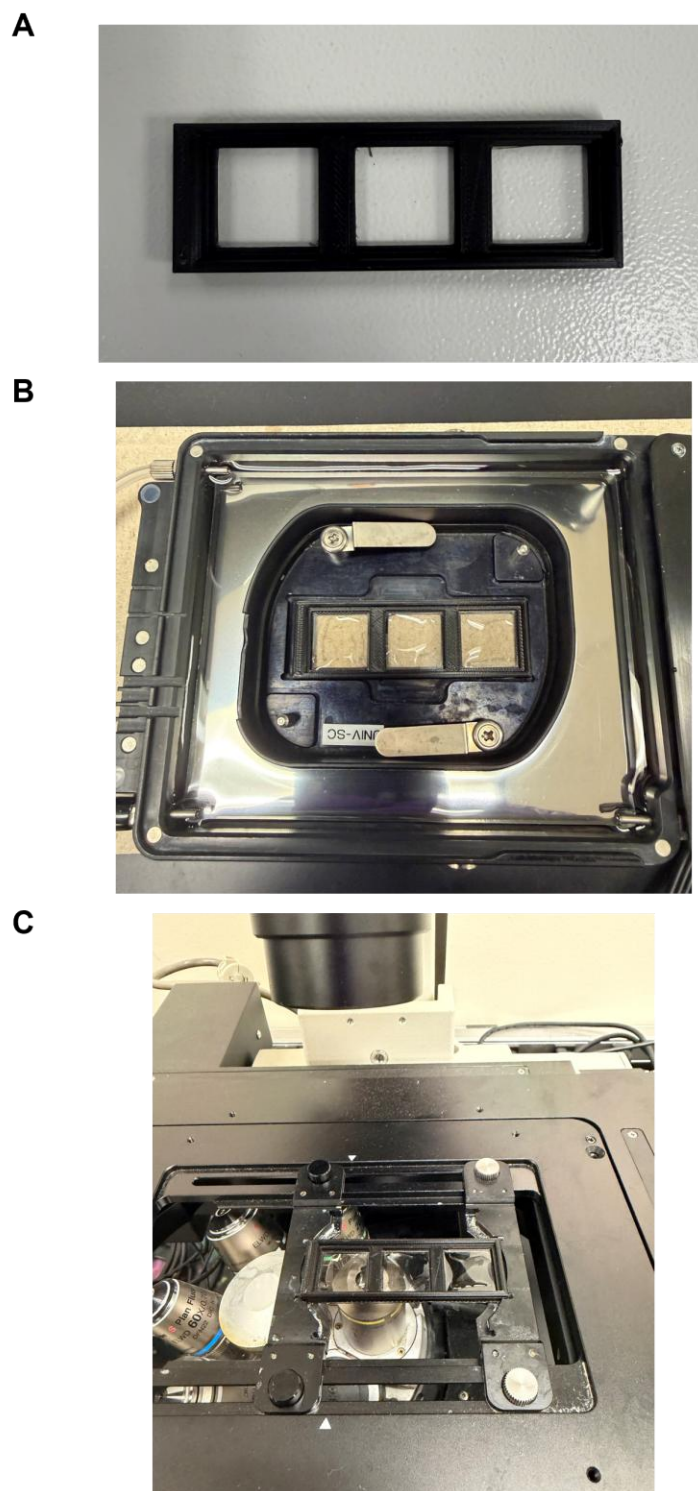


Figure C.6. (A) 3D-printed coverslip holder to culture hydrogels on a coverslip. Each holder has room for three coverslips. (B) Coverslip holder inside the live cell incubation chamber. (C) View of imaging setup. The coverslip holder fits in the microscope stage allowing imaging without disturbing the hydrogels.

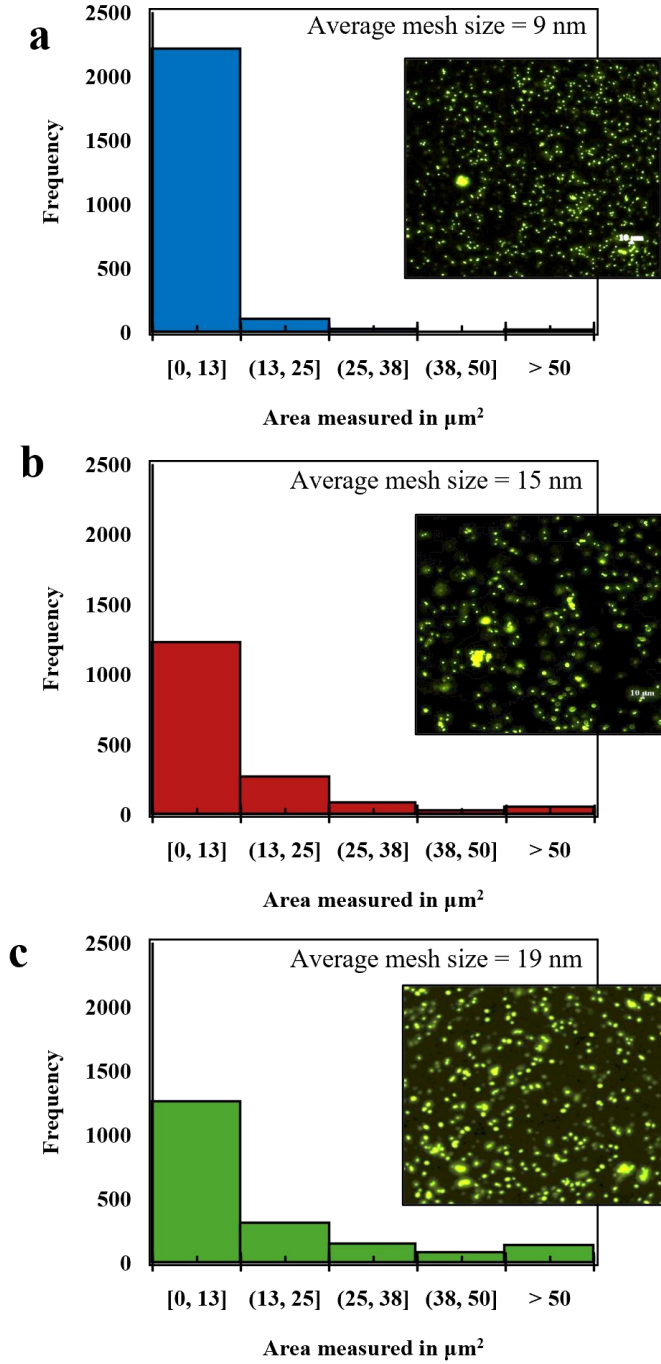


Figure C.7. Effect of hydrogel mesh size on encapsulated *B. subtilis*-GFP from (a) PEGDA_{2k}–PEGTT_{5k}, (b) PEGDA_{10k}–PEGTT_{10k}, and (c) PEGDA_{10k}–PEGTT_{20k} hydrogels as depicted by the histogram (left) and corresponding fluorescence microscopy (right). Data is based on (n=3) independent hydrogels for each mesh size.

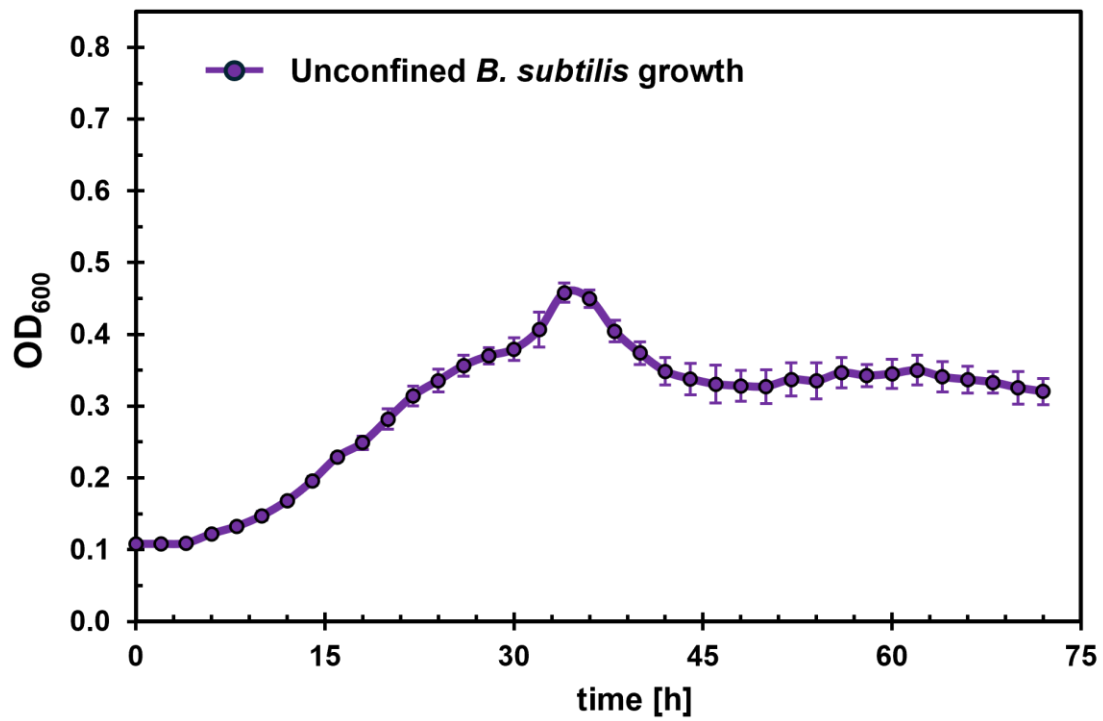


Figure C.8. Growth kinetics of unconfined *B. subtilis* in M9 minimal media culture under identical culture conditions as in confined hydrogels.

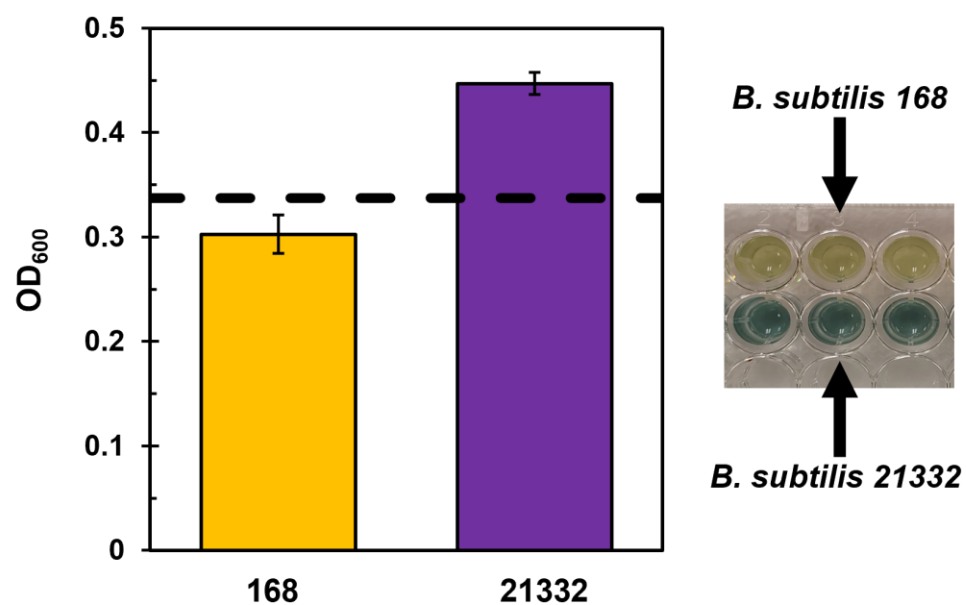


Figure C.9. Specificity test of the CPC-BTB colorimetric assay comparing cell-free culture fluid of *B. subtilis* strain 168 (non-surfactin producing), *B. subtilis* strain 21332 (surfactin producing), and background (black dashed line)

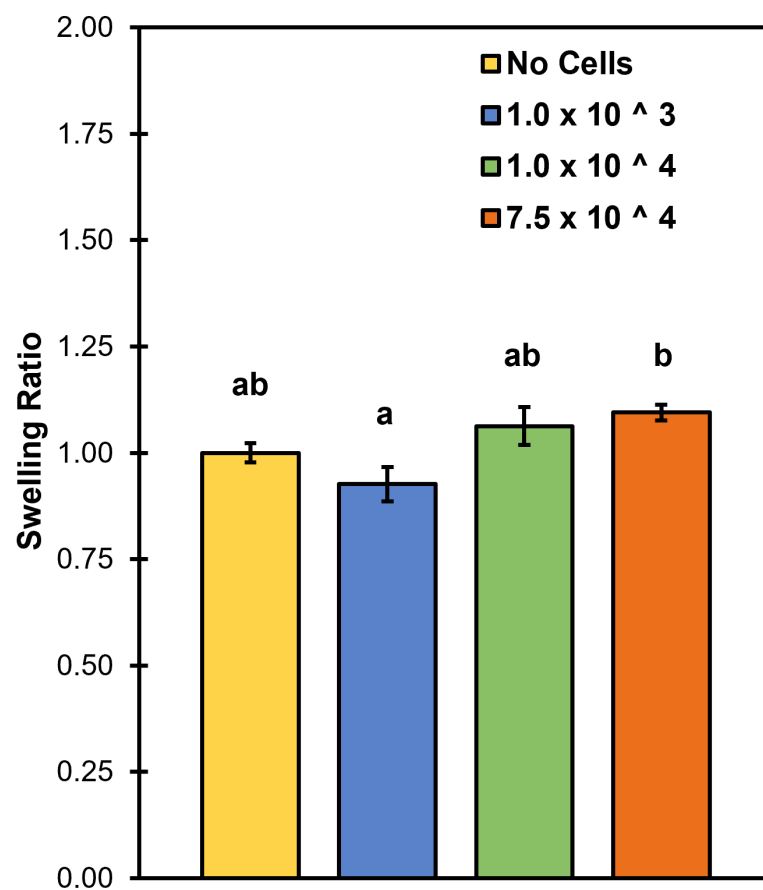


Figure C.10. Equilibrium swelling ratios of H9.5 hydrogels with low, medium, and high initial *B. subtilis* cell densities. Error bars indicate standard deviation (n=3) and differing letters indicate significance ($p < 0.05$) by Tukey's test.

Appendix D - Supporting Information for Chapter 6

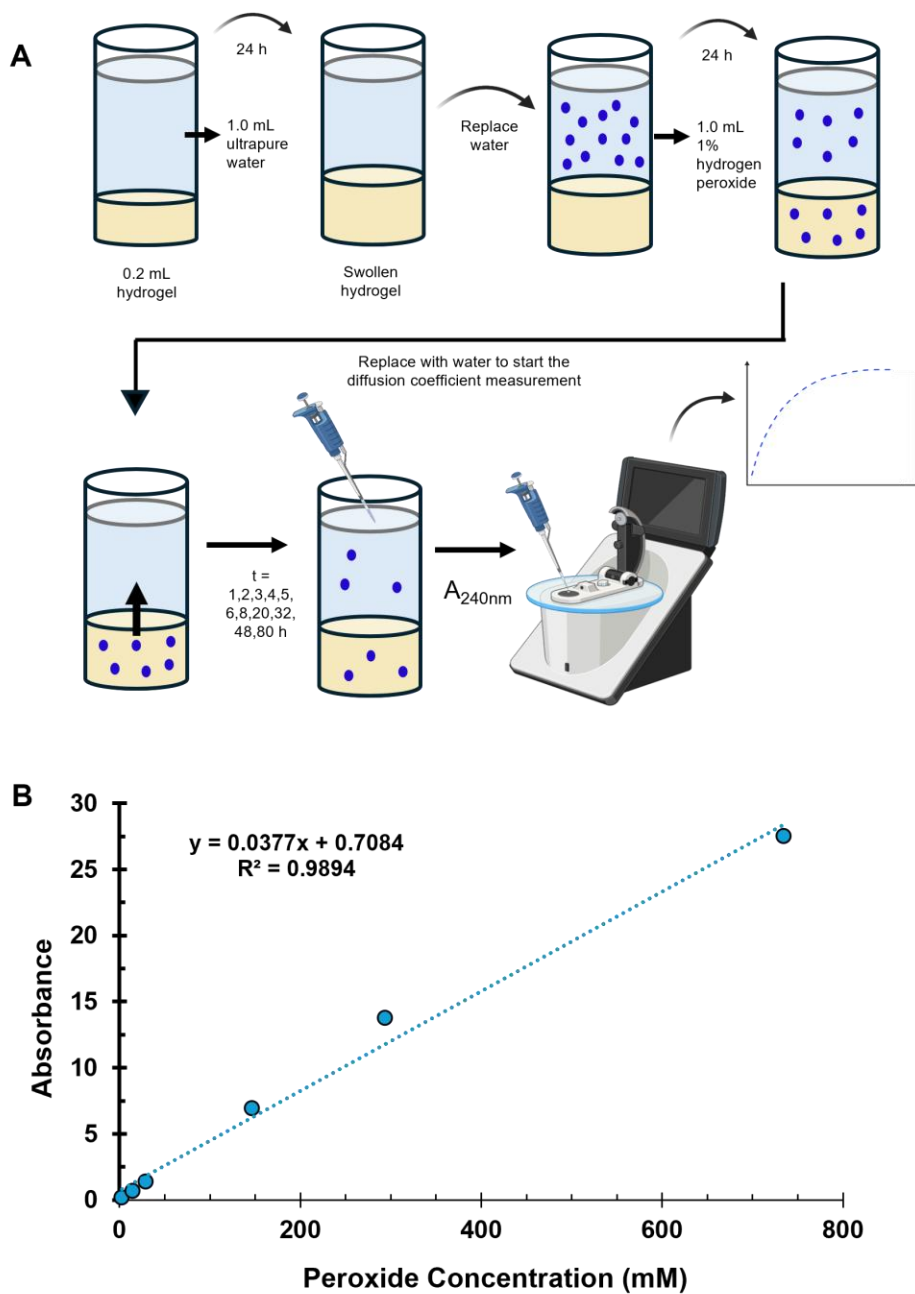


Figure D.1. (A) Experimental schematic of H₂O₂ effective diffusion coefficient. H₂O₂ concentration from release was quantified using UV-Vis absorbance at 240 nm. (B) Calibration curve used to convert absorbance measured by UV-Vis to concentration using Beer's law

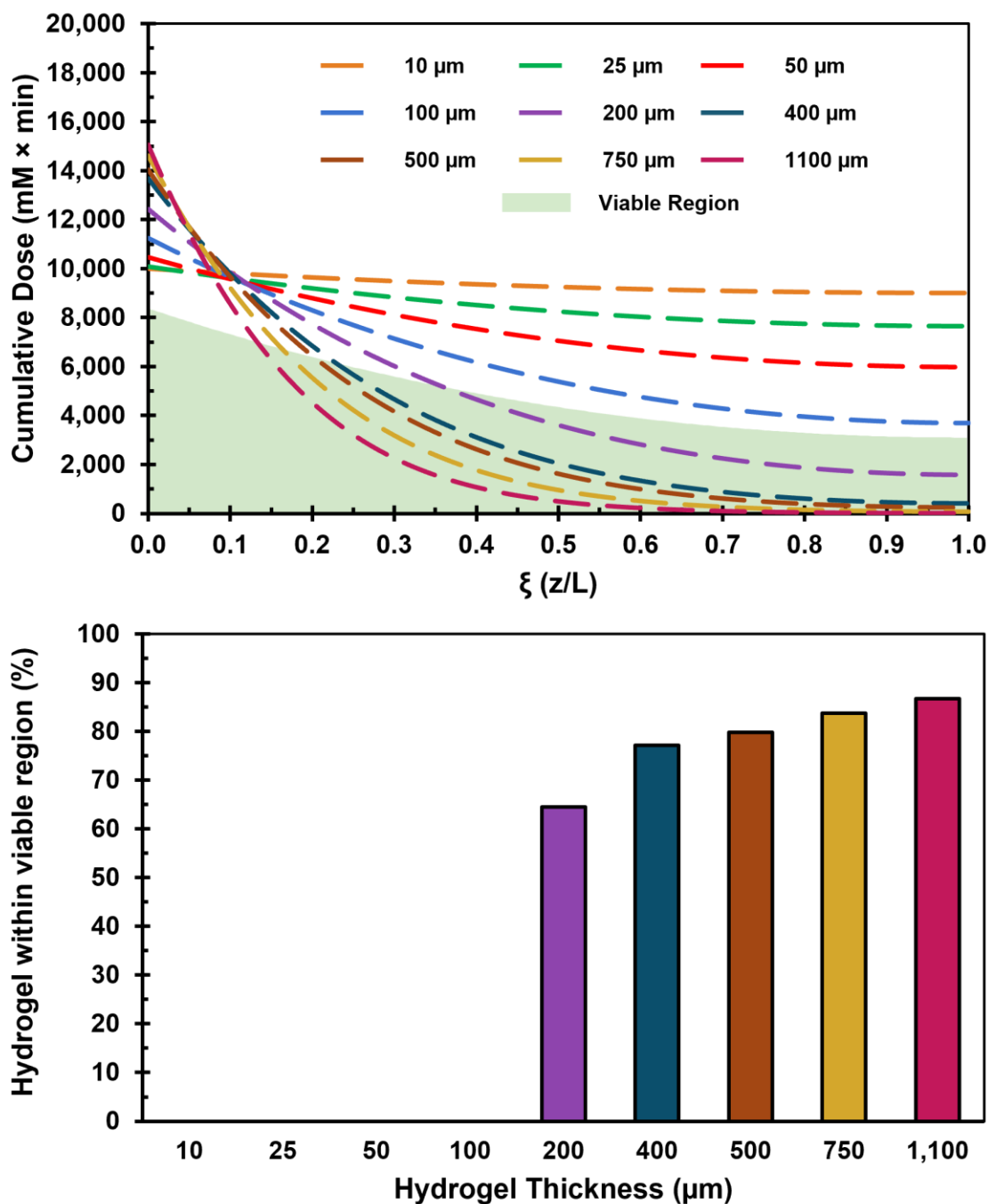


Figure D.2. (Top) H₂O₂ exposure profiles across normalized hydrogel depth as a function of hydrogel thickness. (Bottom) Percentage of the hydrogel with cumulative H₂O₂ exposure below the empirically defined exposure threshold, where bacterial recovery was previously observed.

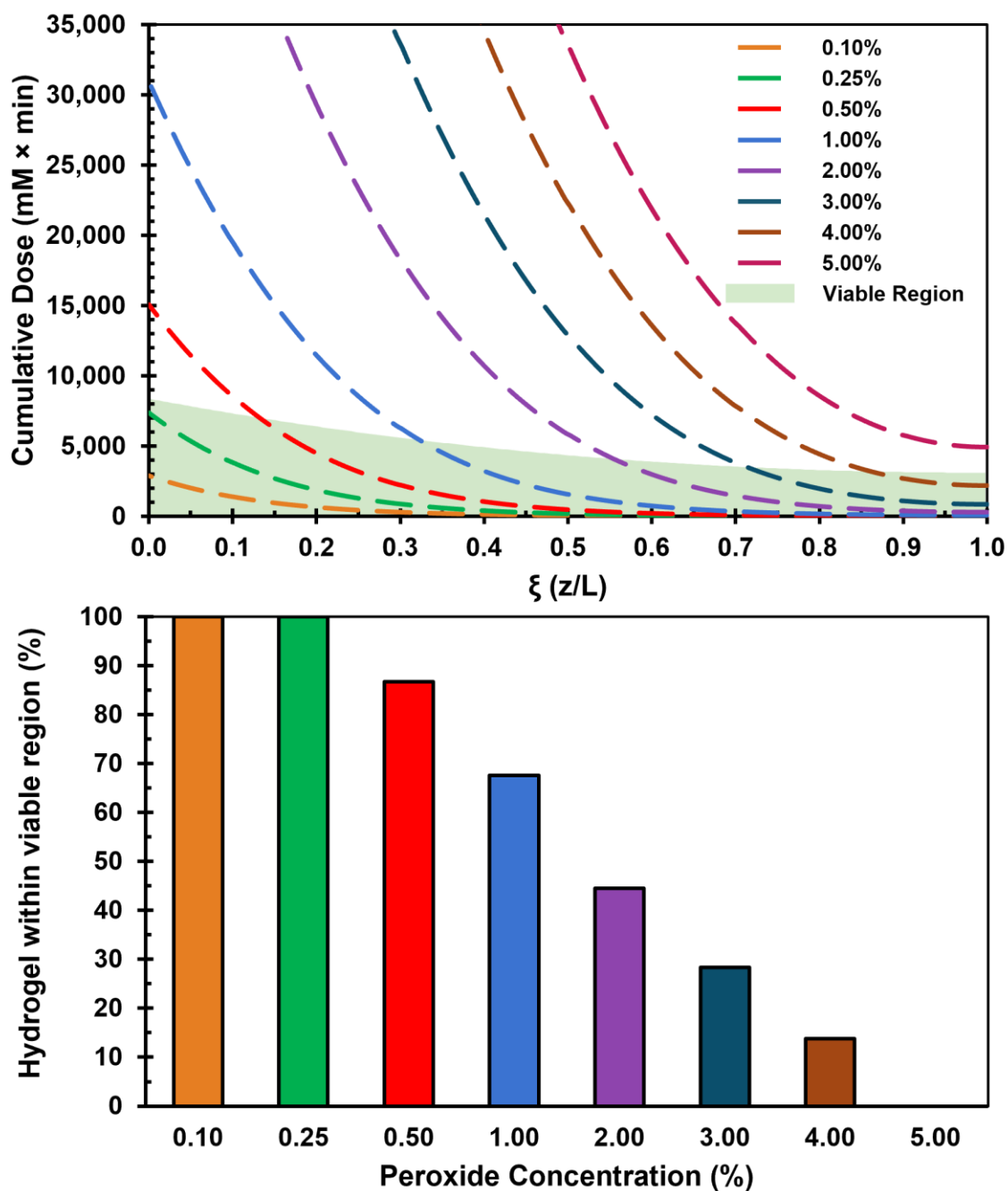


Figure D.3. Top) H₂O₂ exposure profiles across normalized hydrogel depth as a function of external peroxide concentration. (Bottom) Percentage of the hydrogel with cumulative H₂O₂ exposure below the empirically defined exposure threshold, where bacterial recovery was previously observed.