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3D Printing Enzyme Mediated Interpenetrating-Network Biohybrid Materials with Shape Changing Properties
3D Printing Enzyme Mediated Interpenetrating-Network Biohybrid Materials with Shape Changing Properties

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Abstract

In this thesis, a novel shape-changing interpenetrating network-producing 3D bioink is described that is the combination of a calcium-crosslinked alginate network and an N,N'-methylenebisacrylamide-crosslinked poly(N-isopropylacrylamide) (PNIPAm) network. The printing protocol incorporates a bienzymatic initiation system, utilizing horseradish peroxidase and glucose oxidase, and is entirely performed at room temperature under aerobic conditions. The 3D structures produced using the ink retain the contractile thermosensitive properties of PNIPAm single networks, and certain printability metric performances of the ink are comparable to state-of-the-art commercial formulations. Also investigated and discussed are the ink’s print dimension limitations, property tunability, internal structure, and mechanical properties. Furthermore, the ink is inoculated with *E. coli* cells to produce 3D printable biohybrid materials and two functional prototypes are explored. The first is a bioremediation device capable of hydrolysing organophosphates via genetically engineered, phosphotriesterase-producing cells. The second prototype explored is an engineered living material, where the native redox activity of unmodified *E. coli* cells is integrated into the ink’s curing system. Living cell-initiation has previously not been combined with 3D bioprinting. As a further iteration, this second prototype material is repeated with modified *E. coli* cells, transformed to overexpress the artificial oxidoreductase, C45. Here, C45 is demonstrated to be capable of catalysing the formation of a polymeric material, which is the first example of a synthetic enzyme being able to do so.
Acknowledgements

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I would finally like to thank my family. Mum, Dad, Lucy, Grandma, and Grandpa, who have all always been there for me, and trusted me to know what I’m doing. When really, I often don’t.
Author’s Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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# Table of Contents

**Chapter 1: Introduction**

1. **Biohybrid materials**
   1.1 Biohybrid functional materials/bioreactors
   1.2 Biohybrid materials in human cell culture applications
   1.3 Biohybrid materials in soft robotics
   1.4 Engineered Living Materials

2. **Radical polymerisations**
   2.1 Enzyme-mediated polymerisations
      2.1.1 Horseradish peroxidase
      2.1.2 Glucose oxidase

3. **Hydrogels**
   3.1 Alginate
   3.2 Poly(N-isopropylacrylamide) (PNIPAm)

4. **Bioprinting and bioinks**
   4.1 Inkjet bioprinting
   4.2 Laser-assisted bioprinting
   4.3 Stereolithography bioprinting
   4.4 Extrusion bioprinting
   4.5 Strategies for hydrogel bioink reinforcement

5. **Material properties of representative hydrogels**

6. **Thesis aims and objectives, structure, and major outcomes**

7. **References**

**Chapter 2: Methods and Materials**

2.1 General interpenetrating network bioink preparation
Chapter 1: Introduction

2.1.1 Dual Asymmetric Centrifuge (DAC) ................................................................. 52

2.2 3D printing ........................................................................................................... 52

2.2.1 G-code ............................................................................................................. 54

2.3 Printability assays ............................................................................................... 55

2.3.1 Printing uniformity assays .............................................................................. 56

2.3.2 Filament collapse assay ................................................................................... 58

2.4 Contractile thermosensitivity assays .................................................................. 59

2.5 Ink characterisation techniques .......................................................................... 60

2.5.1 Rheology .......................................................................................................... 60

2.5.2 Cryo scanning electron microscopy ................................................................. 61

2.5.3 Compression testing ........................................................................................ 63

2.6 Phosphotriesterase plasmid construction, transformation, and expression ........ 65

2.6.1 Phosphotriesterase plasmid construction ........................................................ 66

2.6.2 PTE plasmid transformation into competent cells ............................................ 66

2.6.3 Lysogeny broth (LB) agar plate preparation ..................................................... 66

2.6.4 Bioprinted organophosphate-degrading bacterial microreactor assay ............... 66

2.6.5 Confocal microscopy of E. coli-laden bioink ..................................................... 69

2.7 Casting E. coli-mediated single networks ............................................................ 70

2.8 C45 plasmid construction, transformation, and expression ............................... 71

2.8.1 C45 plasmid construction ................................................................................ 72

2.8.2 C45 expression, whole cell aliquotation, and purification ............................... 72

2.8.2.1 UV/vis characterisation of C45 .................................................................. 74

2.8.3 Casting isolated-C45 initiated single networks ............................................... 74

2.8.4 3D printing IPN ink with a C45-based initiation system ................................... 75

2.8.5 Casting C45-E. coli mediated single networks ............................................... 75

2.9 Statistical significance analysis .......................................................................... 75

2.10 References .......................................................................................................... 76
Chapter 3: Preparation and Characterisation of a 3D-Printable Shape-Changing Enzyme-Mediated Interpenetrating Network

3.1 Introduction .................................................................................................................. 79

3.2 Results and discussion................................................................................................. 80

3.2.1 The IPN ink 3D printing protocol .............................................................................. 80

3.2.1.1 The effect of the wash crosslinking step on shape fidelity .................................... 84

3.2.1.2 Dimension limitations of the IPN ink wash crosslinking protocol ......................... 87

3.2.1.3 Printing hollow IPN constructs .............................................................................. 92

3.2.2 IPN pre-gel rheology ............................................................................................... 95

3.2.3 Printability assays ...................................................................................................... 98

3.2.3.1 Printing uniformity assay ..................................................................................... 98

3.2.3.2 Filament collapse test .......................................................................................... 100

3.2.4 IPN thermosensitive contractile properties ................................................................ 101

3.2.4.1 TS repeatability assay .......................................................................................... 101

3.2.4.2 Alginate concentration’s effect on TS .................................................................. 103

3.2.4.3 IPN Print thermosensitivity kinetics assay .......................................................... 104

3.2.5 Cryo-scanning electron microscopy of IPN prints .................................................... 105

3.2.6 Compression testing of the IPN gel .......................................................................... 109

3.3 Conclusions and further work ..................................................................................... 114

3.4 Acknowledgement of collaboration ............................................................................. 116

3.5 References .................................................................................................................... 117

Chapter 4: Printing the Interpenetrating Network Ink with Living Cells

4.1 Introduction .................................................................................................................... 124

4.2 Results and discussion................................................................................................. 127

4.2.1 Printing an organophosphate-degrading biohybrid material via an E. coli laden IPN ink ........................................................................................................... 127

4.2.1.1 E. coli laden IPN print integrity ............................................................................. 129

4.2.1.2 E. coli laden IPN print thermosensitivity ............................................................... 130
Chapter 1: Introduction

4.2.1.3 Paraoxon assay of bioprinted constructs ................................................................. 131
4.2.2 Utilizing E. coli as a source of redox potential ............................................................. 134
4.2.2.1 Utilizing C45-expressing E. coli as a source of redox potential .............................. 137

4.3 Conclusions and further work .......................................................................................... 141

4.4 Acknowledgement of collaboration ................................................................................. 143

4.5 References ....................................................................................................................... 144

Chapter 5: Concluding Remarks and Summary ..................................................................... 150

Chapter 6: Appendix A .......................................................................................................... 151

Chapter 7: Appendix B .......................................................................................................... 157
### Chapter 1: Introduction

#### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcAc</td>
<td>Acetylacetone</td>
</tr>
<tr>
<td>BM</td>
<td>Biohybrid material</td>
</tr>
<tr>
<td>CTS</td>
<td>Contractile thermosensitivity</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELM</td>
<td>Engineered living material</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GOx</td>
<td>Glucose oxidase</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IPN</td>
<td>Interpenetrating network</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>LCST</td>
<td>Lower critical solution temperature</td>
</tr>
<tr>
<td>MBA</td>
<td>N, N’-Methylenebisacrylamide</td>
</tr>
<tr>
<td>Mq</td>
<td>Deionised water</td>
</tr>
<tr>
<td>OP</td>
<td>Organophosphate</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PNIPAm</td>
<td>Poly(N-isopropylacrylamide)</td>
</tr>
<tr>
<td>PTE</td>
<td>Phosphotriesterase</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>TS</td>
<td>Thermosensitivity</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UV-vis</td>
<td>Ultraviolet-visible</td>
</tr>
<tr>
<td>XG</td>
<td>Xanthan gum</td>
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Chapter 1: Introduction

1.1 Biohybrid materials

Biohybrid materials are composites that contain both biological and non-biological components. They aim to combine the versatile autonomous and adaptive behaviors of organic systems with the tractability of synthetic materials, such as polymers/gels/ceramics/metals etc., to produce bespoke constructs with novel mechanical and physico-chemical behaviours. The biological component of a biohybrid material could be isolated biomolecules (e.g., DNA or proteins), assemblies of biomolecules, bioconjugates, whole living cells, entire tissues, or a combination of any of these. These biological components could contribute to the construction, maintenance, or repair of the main material matrix, or they could reinforce, or add auxiliary properties to the material. They may even direct the material to grow or react in a certain way as a response to an environmental stimulus. The biological component may even be removed or killed at a certain point to leave behind a uniquely templated material free from continued maintenance or biohazards.

1.1.1 Biohybrid functional materials/bioreactors

One of the most common classes of biohybrid materials involves the immobilization of a functional organic component (usually whole cells, or enzymes) within an inorganic matrix to create a biohybrid functional material. If this function is to facilitate a chemical reaction, then the material can be even more specifically identified as a bioreactor. Some biohybrid functional materials simply utilize unmodified biological components for the purpose of encapsulating or concentrating their endogenous behaviors. For example, recent work has been published on bioprocess intensification constructs made from 3D bioprinting yeast within a poly(ethylene glycol) diacrylate-based (PEGDA) bioink (Figure 1-1A). The printed lattice bioscaffolds exhibited greater ethanol production in the presence of glucose than analogous bulk constructs due to the improved mass transport through the porous structures. Authors in other papers have used unmodified Bacillus subtilis spores for their hygroscopic actuation
Chapter 1: Introduction

properties. These spores are dormant cells with a protein shell that is capable of reversibly swelling depending on the environmental relative humidity, changing their diameter to as much as 12%. By laminating these spores onto one side of a flexible synthetic substrate it’s possible to use humidity to induce differential strain and drive curvature. It is even possible to pattern the spores onto the substrate using a lithography-based patterning technique. Chen et al. demonstrated that by combining many of these devices together their combined actuation can be used as a hydration-driven engine to drive miniature vehicles (Figure 1-1B).

There is also research in biohybrid functional materials that utilize genetically modified organisms. This is due to the additional versatility of these systems, and the potential of using recombinant DNA technology to overexpress selected enzymes. Synthetic gene networks, enabled by synthetic biology, have been designed that are capable of sensing, responding, computing, and recording. For example, Liu et al. published research fabricating flexible hydrogel-elastomer wearables that hosted programmed cells (Figure 1-1C). Also, Chen et al. used a cell-free system that utilised a synthetic gene network and cell free extracts embedded into a paper substrate for the development of portable, low-cost, rapid virus sensors.
Chapter 1: Introduction

Figure 1-1: Biohybrid functional material examples. A, Bioprinted yeast cells demonstrating ethanol production in the presence of glucose. Printed lattice bioreactors demonstrated increased activity vs. bulk structures due to the improved mass transport. B, Spore-actuated lamella structures. By depositing a dense suspension of spores onto one side of an elastomer substrate humidity can be used to drive curvature. By combining many of these structures, authors have fabricated devices with humidity-driven rotation. C, Wearable biohybrid material encapsulating living genetically modified E. coli cells. Cells are capable of fluorescing in response to sensing signalling molecules. Figure adapted from Elsevier, PNAS, and ACS.3,4,6,10

Beyond casting and 3D printing, biohybrid materials have also been fabricated using electrospinning to produce polymer-microbe fibers. Liu et al. demonstrated the encapsulation of three different types of bio-technology relevant genera (Psuedomonas, Zymomonas and Escheria) that all remained viable for over 1 week within the fibers.12 This represents a platform for the electrospinning of novel bio-hybrid
material or thin-film catalysts. Other examples displaying the range of biohybrid materials include the incorporation of fungi into polymeric materials to create antibiotic-releasing,\cite{13} and self-cleaning surfaces,\cite{14} bricks with bacteria that can glow in the dark, adsorb pollution, and change colour when wet,\cite{15} and a conductive composite film made from the fermentation of beer yeast in the presence of carbon nanotubes.\cite{16}

1.1.2 Biohybrid materials in human cell culture applications

Naturally, biohybrid materials also play key roles in biomaterials, tissue engineering, artificial organ, and regenerative medicine research.\cite{17,18} Living bone itself is a composite of inorganic minerals and collagen proteins.\cite{19} Systems for the 3D culture of human cells in synthetic materials typically involve scaffolds, 3D porous solid biomaterials, that attempt to recapitulate the properties of native tissue and/or stimulate the regeneration of the original tissue.\cite{20} Here, scaffolds must be: (i) biocompatible, (ii) promote cell-scaffold interactions, (iii) perfusable to gases, nutrients, and cell regulatory factors, and (iv), optionally, biodegradable as to be substituted by tissue regeneration. Many are modified with various biochemical enhancements to improve cell-adhesion or to increase the adsorption of biochemcials that promote stem cell differentiation etc.\cite{21,22} Hydrogels are a common scaffold material target as they recapitulate many of the important properties of natural tissues, can be biodegradable, and are tractable to chemical augmentation.\cite{23,24} However, many other types of synthetic scaffold have been explored, examples include poly(lactic acid) grafts made using high pressure carbon dioxide mixing and electrospun polycaprolactone fibres.\cite{25,26}

Organ-on-a-chip (OOAC) devices are also an important branch of biohybrid materials, and they are listed by the world economic forum as a “Top Ten Emerging Technology”.\cite{27} OOACs are biomimetic systems that attempt to recapitulate the environments of a physiological organ in order to study the physiology and pathology of tissues in vitro, including how they might respond to a particular drug or environmental stress.\cite{28,29} There is even research into their potential utility in the temporary support of damaged organs.\cite{30} They are typically microfluidic PDMS devices which have internal connected chambers incubating living human cells.\cite{31} The microfluidic system must supplement the cells with
nutrients, remove waste, and sometimes induce organ polarity.\textsuperscript{32,33} The OOAC model must also recapitulate any physiological concentration gradients and dynamic mechanical stresses to correctly differentiate and maintain appropriate tissues.\textsuperscript{34–36} Recently, OOAC fabrication has incorporated bioprinting technology to enable faster, simpler and more flexible manufacturing.\textsuperscript{37}

1.1.3 Biohybrid materials in soft robotics

Another theme within biohybrid materials is the use of living cell actuation for biohybrid soft robotics.\textsuperscript{38} Traditional robotic components such as metal skeletons and printed circuit boards are very rigid, inelastic and completely antithetical to natural biological tissues. By combining biological actuators, such as contractile cells, with soft synthetic materials, it’s possible to build actuatatable constructs with soft morphologies.\textsuperscript{39} This allows construct designs to be much more biomimetic, which has several advantages. Materials that are highly compliant can mitigate unpredictability and can translate simple types of actuations into complex motion. They are also more compatible with soft biological tissues.\textsuperscript{40} Cardiomyocyte cells are a common biological actuator. For example, Tanaka \textit{et al.} incorporated a cardiomyocyte sheet onto a hollow elastomer sphere to create a sub-centimetre heart-like pump, and Parker \textit{et al.} used anisotropic cardiomyocyte configurations patterned onto an elastomeric substrate to give it jellyfish-like stroke locomotion.\textsuperscript{41,42} The other major biological actuators used in soft robotics are skeletal muscles, parallel bundles of muscle fibers, and motile microorganisms.\textsuperscript{43–45} However, enduring challenges for living-cell actuated robotics remain, such as extended cell viability and poor performance in extreme conditions. Also, the lack of multi-scale 3D fabrication techniques required to pattern or induce vascularization in larger scale constructs, or produce hierarchical interfacial structures between biological tissues and inorganic materials that avoid stress loci.\textsuperscript{46}

1.1.4 Engineered Living Materials

Engineered living materials (ELMs) are an emerging research area within the field of biohybrid materials. For a biomaterial to qualify as an ELM, its biological component must be whole living cells and the cells must build or assemble at least part of the material itself, or modulate the material properties in some manner.\textsuperscript{1} A natural example of an ELM material would be coral, which is essentially
Chapter 1: Introduction

a calcium carbonate structure assembled by marine organisms from ions in their environment. The organisms do not create the ions themselves, but rather produce an enzyme, carbonic anhydrase, which catalyses the precipitation of calcium carbonate from exogenous Ca$^{2+}$ and CO$_3^{2-}$ ions. An understated literature example of an ELM is the recently developed mycelium materials made from culturing fungus on a medium of agricultural waste. The mycelium grows around the substrate particles, binding them together and the material’s properties (for example, stiffness) can be tuned via the mixture of fungal species used as well as the substrate composition. The material can be cast in moulds and has potential as an environmentally friendly, biodegradable packaging alternative. There is also the potential for biotemplating inorganic nanostructures, such as gold nanowires or platinum nanoparticles, into the mycelium to expand the properties of the material to include conductivity and catalysis etc.

The ultimate aim of ELMs is to create entirely bespoke constructs that are self-assembled, maintained, and augmented by genetically engineered organisms. Programmed devices with control systems, embedded intelligence, and extracellular matrix-cell relationships mimetic of living biology but with novel functionality. Eventually they will produce materials, designed and patterned across multiple length scales, that encapsulate entire communities of completely synthetic protocells with their own designer genomes and proteomes. However, as ELMs remain a nascent research area, most of the contemporary literature is focused on the use of recombinant bacteria to build synthetic bacterial biofilms, i.e., using genetically engineered bacteria to express a fusion variant of one of its native major extracellular matrix (ECM) proteins. Specific ECM protein targets are chosen for their tolerance for chemical augmentation, and the advantage of the approach is that it hijacks the native ECM assembly machinery to self-assemble novel biofilms that now display an engineered functionality. Some examples of this strategy will completely delete the gene coding for an ECM protein and then transform the bacteria with an inducible plasmid containing the engineered fusion variant. This allows ECM generation, and its new functionality, to be dormant until the introduction of a small chemical inducer. Huang et al. in a paper published in Nature Chemical Biology demonstrated this process using *Bacillus subtilis* transformed with an IPTG-inducible plasmid encoding for a TasA amyloid protein fusion variant. Engineered biofilm functionality included triggerable fluoresce and bioremediation of
organophosphates via a 2-step degradation cascade performed by co-cultured strains. The authors realized a large library of TasA fusion variant biofilms, and it was noted that different fusion variants would also result in different biofilm architectures. Duraj-Thatte et al. in 2019 detailed transforming bacteria to express ECM protein fusion variants with mucin adhesive enzymes to generate a self-renewing hydrogel that was able to persist in a murine gastrointestinal tract despite mucin turnover. By using different mucin adhesive enzymes they were also able to tailor the ELMs location and longevity within the gut. This has the potential to be a novel wound healing material for an area of the body extremely poor at retaining foreign materials.

1.2 Radical polymerisations

Polymers are long chain molecules synthesized via the polymerisation reaction of small molecules called monomers. Radical polymerisations are a type of polymerisation reaction where the polymer is formed by a propagating radical species; a molecule with an unpaired valence electron that tends to be highly chemically reactive. Due to this nature of polymer growth, where unsaturated monomers join onto growing polymer active sites one at a time, radical polymerisations are classed as chain-growth polymerisation (Figure 1-2). This is in contrast to step growth polymerisations where multi-functional monomers combine to form dimers, then trimers, then oligomers, and eventually polymers via polyaddition or polycondensation reactions.
Chapter 1: Introduction

Chain growth polymerisation

Step growth polymerisation

Figure 1-2: A representation of chain and step growth polymerisation mechanisms. In chain growth polymerisation polymers grow via the addition of unsaturated monomers onto the ends of growing polymer chains one at a time. In step growth polymerisations multifunctional polymers combine to form dimers, then trimers, then oligomers, which eventually link up to become long polymer chains.

The typical steps in a chain growth polymerisation are initiation, propagation, and termination. These are all illustrated in a representative styrene radical polymerisation scheme in Figure 1-3. Initiation refers to the initial generation of a chain carrier molecule, typically a highly reactive intermediary such as a radical or an ion. This chain carrier then undergoes propagation reactions where monomers are added one at a time onto the growing polymer chain which becomes one unit longer, and generates a new active centre, with each step. In termination, two radicals mutually react to become an inactive polymer. However, during chain growth polymerisation, chain transfer reactions can also occur whereby the active centre of a growing polymer chain is transferred to another molecule. This other molecule can be a monomer, another polymer, or a solvent molecule. Chain transfer reactions reduce the average molecular weight of the final polymer product and therefore are usually undesirable, but they can be induced deliberately via the addition of a chain transfer agent (a molecule with at least one weak chemical bond which can therefore facilitate the reaction). In the absence of any chain transfer or termination reactions, a chain growth polymerisation is termed as a living polymerisation. Here, polymerisation occurs until all the monomer is consumed and can be reactivated upon the addition of new monomer. This affords an extremely high amount of control and allows the synthesis of
Chapter 1: Introduction

complicated, pre-determined, well-defined polymer architectures such as block and graft copolymers, comb networks, and functional colloid composites.\(^{63}\)

\[
\begin{align*}
\text{Initiation:} & \quad \begin{array}{c}
\text{Styrene} \\
+ \quad \text{Initiator} \quad \text{R}^* \\
\rightarrow \quad \text{Styrene Radical}\end{array} \\
\text{Propagation:} & \quad \begin{array}{c}
\text{Styrene Radical} \\
+ \quad \text{Styrene} \quad \rightarrow \quad \text{Styrene Dimer} \\
\text{Styrene Dimer} \\
+ \quad \text{Styrene} \quad \rightarrow \quad \text{Styrene Trimer}\end{array} \\
\text{Termination:} & \quad \begin{array}{c}
\text{Styrene Dimer} \\
+ \quad \text{Styrene} \quad \rightarrow \quad \text{Styrene Tetramer}\end{array}
\end{align*}
\]

Figure 1-3: Radical polymerisation scheme for the synthesis of polystyrene from styrene monomers. Displayed are the initiation, propagation, and termination steps typical in a chain growth polymerisation. The red arrows in the first propagation step highlight the reaction mechanism for radical propagation.

There are many types of radical initiation and initiators, and it is worth noting that not all monomers can be initiated with every type of initiator. A common type of radical initiating molecule is one that will undergo homolytic cleavage of one of its bonds under thermal decomposition or photolysis (photoinitiators). Thermally decomposing initiators are most often organic peroxides or azo compounds. Photoinitiators are most often metal iodides, metal alkyls, or also azo compounds.\(^{64}\) Redox reactions can also be used to generate radicals. For example, using a disulphide or persulfate in combination with a metal reducing agent.\(^{65,66}\) It’s also possible to use ionizing radiation (\(\alpha-, \beta-, \gamma-,\) or \(x-\))
rays) to eject an electron from an initiating species to produce a radical. Other methodologies for radical
generation include electrochemical electrolysis, sonication, and plasma ionisation.\(^{67}\)

An important caveat with radical polymerisations is their susceptibility to oxygen inhibition. Initiator
and growing polymer radicals are known to react with molecular oxygen to form peroxy radicals.\(^{68}\) As
these peroxy radicals are relatively stable, they do not readily reinitiate polymerisation. Therefore,
oxygen essentially quenches radicals and by extension radical polymerisations.

### 1.2.1 Enzyme-mediated polymerisations

Enzymes are proteins (which are a large class of biological polymers comprising of long chains of
amino acids that fold into energetically favourable, precise, dynamic 3D structures) that catalyse
biochemical reactions. Almost all metabolic processes depend on enzymes, and their ability to operate
efficiently under physiological conditions is essential for life to sustain itself.\(^{69}\) In enzyme-mediated
polymerisations (EMPs), enzymes are taken outside of their native biological context and are utilized
in *in vitro* polymer synthesis. As EMPs have remarkable efficiencies under mild conditions, they have
attracted a lot of interest due to their environmental friendliness and potential for *in situ* polymerisations
of biomedical devices.\(^{70}\) Most EMPs are chain-growth free-radical initiated and operate *via* the enzyme,
 generally an oxidase or peroxidase, concurrently reducing a substrate whilst oxidizing a monomer or
radical mediator (Figure 1-4).\(^{71}\) Horseradish peroxidase (HRP) is by far the most utilized enzyme in
EMP research, but glucose oxidase (GOx), and laccase are also very commonly employed.\(^{72}\)
Chapter 1: Introduction

Initiation: \[ 
H_2O_2 + 2 \text{R} \xrightarrow{\text{HRP}} 2\text{H}_2\text{O} + 2 \text{R}^* \]

\[ \text{R} = \text{AcAc} \]

\[ \text{R}^* + \text{M} \xrightarrow{} \text{R} + \text{M}^* \]

Propagation: \[ M_n^* + \text{M} \xrightarrow{} M_{n+1}^* \]

Termination: \[ M_n^* + M_m^* \xrightarrow{} M_{n+m}^* \]

Oxygen inhibition: \[ M_n^* + \text{O}_2 \xrightarrow{} M_n\text{OO}^* \]

Figure 1-4: Reaction scheme for a representative HRP-based EMP displaying the initiation, propagation, termination, and oxygen inhibition steps for a chain-growth polymerisation. Only the first initiation step involves the enzyme. \( R = \) radical mediator (acetylacetone [AcAc]), \( M = \) monomer, \( M_n \) or \( M_m \) = polymer with \( n \) or \( m \) units respectively, \( O = \) oxygen.

Due to oxygen inhibition, EMPs can experience a lengthy initial induction period (e.g., up to an hour), where no polymerisation occurs.\(^\text{32}\) Oxygen inhibition can even prevent polymerisation occurring entirely. For this reason, a lot of EMPs in the literature are conducted within degassed reaction solutions. However, it is possible to perform EMPs in aerobic environments. One-way aerobic EMPs can be performed more efficiently is by using a high concentration of radical mediator so that propagation reactions can successfully outcompete the inhibition reactions.\(^\text{73}\) Another common solution is to use a bienzymatic system that incorporates GOx or pyranose oxidase (P2Ox) as an oxygen scavenger.\(^\text{74,75}\)

One key example from the literature even uses GOx to degas a non-EMP reaction solution, as an alternative to sparging or pressure reduction, which allowed an oxygen sensitive polymerisation to be performed in an open vessel.\(^\text{76}\) However, in the bienzymatic cascade context, both GOx and P2Ox enzymes will catalyse D-glucose and oxygen into hydrogen peroxide (\(H_2O_2\)), which can then be reduced...
by a peroxidase like HRP. The GOx/HRP biocatalytic cascade (Figure 1-5) in particular is a well
explored EMP system, as along with the oxygen scavenging capabilities of the system, it also has the
added benefit of producing hydrogen peroxidase \textit{in situ}. This mitigates the problem of HRP degradation
that occurs rapidly at high hydrogen peroxidase concentrations. For example, Liao \textit{et al.} in 2015
described an aerobic preparation protocol for a composite hydrogel that utilized a step involving a GOx-
HRP-cascade-catalysed radical polymerisation.\textsuperscript{77} Here, the authors immobilized HRP and GOx between
calcium niobate sheets which exfoliated upon instigation of the EMP, resulting in a functional
bioinorganic hydrogel capable of oxidising small molecules. The activity is due to the hydrogel’s
immobilized enzymes retaining their oxidative abilities post-fabrication for several cycles. The
incorporated nanosheets where also able to enhance the thermal stability of the enzymes.

![Figure 1-5: Reaction scheme for GOx/HRP biocatalytic cascade to produce acetylacetone radicals.](image)

\textbf{1.2.1.1 Horseradish peroxidase}

HRP is a 44 kDa predominantly $\alpha$-helical metalloenzyme (Figure 1-6) with a polypeptide chain that is
308 amino acids long that binds a heme cofactor (its reaction center and largely responsible for its redox
chemistry).\textsuperscript{78} The heme is bound \textit{via} the coordination of its iron to a histidine residue. HRP is substrate
promiscuous and is able to oxidize many different compounds.\textsuperscript{79} Its pH optimum is 6-6.5 but is stable
in the wider range of 5-9.\textsuperscript{80}
The first recorded observations of a HRP catalyzed reaction were made in 1810 by Louis Antoine Planche.\(^81\) While investigating a *Guaiacum Officinale* tree resin he noted it would turn a vibrant blue colour if a piece of horseradish root was placed in it. This was a peroxidase reaction, catalyzed by HRP, that was oxidizing a minor constituent of the resin into the pigment ‘guaiacum blue’.\(^82\) Since then HRP has gone on to become one of the most intensely studied and characterized peroxidases. In 1990 it was first successfully recombinantly expressed in *E. coli* and in 1997 its crystal structure was solved.\(^83,84\) In 2002 several of HRPs catalytic intermediates were isolated, and its final salient redox chemistry mechanisms were elucidated.\(^85\)

The general mechanism for HRP’s catalytic cycle is typical for heme catalyzed oxidations (Figure 1-7).\(^79\) Starting with the ferric resting state, water is first displaced by \(\text{H}_2\text{O}_2\) at the axial Fe-coordination site. \(\text{H}_2\text{O}_2\) is then reduced to water, \textit{via} the donation of one electron from the heme iron and another from a distal amino acid residue, which is then released from the enzyme following a heterolysis of the O-O bond to form compound I.\(^86\) Compound I is an oxyferryl species with its iron in a +4 oxidation state and a delocalised radical in its porphyrin ring.\(^87,88\) This compound has a high redox potential and goes on to oxidise two substrates before returning to the ferric resting state.
Figure 1-7: HRP catalytic cycle details. A. Heme structure in HRP. B. HRP’s catalytic cycle, typical for most mono-histidine ligated heme peroxidases. Water is first displaced from the heme’s ferric resting state by hydrogen peroxide. Water is then lost to form compound I which goes on to oxidize two equivalents in substrates to then return to its ferric species. B = distal amino acid residue.

HRP has been well established as a prominent tool in biotechnology. Most commonly, HRP is utilized as a reporter enzyme where its activity is tracked by either the formation of a chromogenic or
fluorogenic product, or electrochemically via HRP’s redox capabilities. HRP biosensors systems have been built for the detection of hydrogen peroxide, glucose, DNA and RNA, the milk allergen β-lactoglobulin, tumour markers etc.\textsuperscript{91–96} HRP has also been utilized in biocatalysis for reactions such as oxidative dehydrogenation and sulfoxidation, and in bioremediation systems for degrading synthetic dyes.\textsuperscript{97–99} Furthermore, HRP-prodrug systems have been studied for cancer treatment.\textsuperscript{100–102} Here, HRP is usually used in conjunction with plant hormone indole-3-acetic acid, a natural HRP substrate which is capable of being oxidized by HRP without addition of hydrogen peroxide.

### 1.2.1.2 Glucose oxidase

GOx is a dimeric flavoprotein consisting of two identical polypeptide chain subunits and two tightly bound flavin adenine dinucleotide (FAD) cofactors (Figure 1-8). The two subunits are joined together \textit{via} a disulfide bond.\textsuperscript{103} It was first isolated from the fungi \textit{Aspergillus niger} in 1928 and its crystal structure first resolved in 1993.\textsuperscript{104} Nakamura and Fujiki in 1968 studying GOx purified from its two most common sources, \textit{A. niger} and \textit{P. amagasakiense}, found their molecular weights to be 152 and 150 kDa and their optimum pH ranges to be 3.6-6.5 and 4.0-5.5 respectively.\textsuperscript{105}

![Tertiary structure for one of GOx’s polypeptide chain subunits and FAD cofactors. The FAD cofactor is represented in green, with phosphates in orange, nitrogens in blue and oxygens in red. A complete GOx protein is a homodimer of two of these subunits, and therefore contains two FAD cofactors, joined together via disulfide bonds. (PDB: 1CF3).](image)
Chapter 1: Introduction

GOx is a oxidoreductase enzyme that catalyzes the oxidation of glucose to D-glucono-1,5-lactone while concomitantly reducing molecular oxygen to hydrogen peroxide (Figure 1-9).\(^{104}\) The reaction initially proceeds with the enzymatic oxidation of glucose, during which the cofactor FAD is reduced to FADH\(_2\). The reduced cofactor is then reoxidized by molecular oxygen to yield H\(_2\)O\(_2\).

\[
\begin{align*}
\text{GOx} + \text{D-glucose} + \text{O}_2 & \rightarrow \text{D-glucono-1,5-lactone} + \text{H}_2\text{O}_2
\end{align*}
\]

Figure 1-9: The reaction catalysed by glucose oxidase, the oxidation of \(\beta\)-D-glucose to D-glucono-1,5-lactone utilizing molecular oxygen as an electron acceptor and therefore simultaneously producing hydrogen peroxide.

GOx is a very widely used enzyme attracting considerable commercial interest due to its relatively low cost and excellent stability.\(^{106}\) It is most widely used in glucose biosensing for monitoring applications in fermentation, soft drinks, and whole human blood etc.\(^{107-110}\) It has also been incorporated into the anode of biofuel cells and is used as a food and beverage additive to increase shelf life by removing residual oxygen.\(^{111-113}\) It is utilized in EMP reactions for its in situ production of hydrogen peroxide which is then either directly radicalised using Fenton chemistry (an oxidation reaction activated by Fe(II) salts) or fed to a peroxidase in a GOx/peroxidase bienzymatic system.\(^{114-116}\)

1.3 Hydrogels

Hydrogels are hydrophilic, three dimensional, crosslinked polymeric networks. They are categorized as soft materials and are comprised mostly of water, which they readily absorb.\(^{117}\) Hydrogels are also typically porous, biocompatible, oxygen and nutrient permeable, flexible, and structurally analogous to natural ECMs. They therefore bear many similarities with living tissues, more so than any other class of synthetic biomaterial, and are of great interest for biomedical applications, such as in drug delivery devices and tissue engineering scaffolds.\(^{118,119}\) Many different types of hydrogel exist and the materials fall into numerous sub-classes based on their structure and properties. For example, hydrogels can be neutral or ionic based on the nature of their polymer side groups and they can be built from one (homopolymeric) or more (copolymeric/monopolymeric) monomer precursors. They can also be crosslinked in a variety of different ways (for example, via ionic interactions, covalently bonded crosslinkers, physical entanglements, or supramolecular interactions) which determine whether their
gelation is classified as permanent or reversible.\textsuperscript{120-122} Furthermore, “smart” hydrogels are able to reversibly shape change dynamically in response to external stimuli such as heat, pH, electric potential, and light, by either desorbing water from their internal matrix, or adsorbing more from its environment.\textsuperscript{123}

1.3.1 Alginate

![Structure and ionic crosslinking of alginate. A. The different blocks of alginate chains. G-blocks are formed from repeating G units, M blocks are formed from repeating M units, and mixed blocks are formed from both residues. B. The eggbox model for ionic crosslinking of alginate with calcium$^{2+}$ ions. The ions rest interstitially between adjacent G-blocks. C. Schematic illustrating the calcium ion crosslinking of multiple alginate chains. Image adapted from Elsevier with permission.\textsuperscript{124}](image)

Alginate is an anionic polysaccharide, typically sourced from brown algae, comprising of $\alpha$-L-guluronic acid (G) and $\beta$-D-mannuronic acid (M) units, and is an example of a natural, ionically-crosslinkable copolymer (Figure 1-10). It is of great interest in biomedical applications due to its
biocompatibility, low toxicity, low cost, and ability to form porous hydrogel scaffolds.\textsuperscript{124} Alginate gels are easily formed via passive crosslinking with a wide variety of different divalent and trivalent cations, most commonly Ca\textsuperscript{2+} due to its high binding efficiency, water solubility, and low cytotoxicity.\textsuperscript{125,126} Ca\textsuperscript{2+} crosslinks adjacent alginate G-blocks (blocks of G-residues) in an egg-box model of ionic crosslinking.\textsuperscript{127} The M-blocks do not participate in the crosslinking and therefore different sources of alginate, which will contain different M/G ratios, will exhibit different material properties when crosslinked.

Alginate crosslinking can also be reversed. The crosslinking ions can be removed via the addition of a chelating agent, such as ethylenediaminetetraacetic acid (EDTA), which will trigger the dissolution of the hydrogel back into solubilised alginate chains.\textsuperscript{128}

\subsection{1.3.2 Poly(N-isopropylacrylamide) (PNIPAm)}

PNIPAm is an example of a synthetic smart polymer polymerised via the radical polymerisation of the vinyl monomer N-isopropylacrylamide (NIPAm) (Figure 11). If NIPAm is polymerised in the presence of a covalent crosslinker, for example N,N’-methylbisacrylamide (MBA), it will form a 3D covalently crosslinked PNIPAm/MBA network.
Figure 1-11: Schematic for the polymerisation of NIPAm monomers into PNIPAm chains. If NIPAm is polymerised in the presence of a covalent crosslinker, such as MBA, it will crosslink the PNIPAm chains to form a 3D PNIPAm/MBA hydrogel.

PNIPAM is well-studied for its ability to reversibly deswell when heated above its lower critical solution temperature (LCST; which is about 32°C in aqueous solution). This is a coil-globule transition, where the polymer in solution transitions from a soluble to insoluble state (Figure 1-12). More precisely, PNIPAm polymers contain hydrophilic and hydrophobic moieties (its amide and isopropyl groups respectively). When the temperature is below the LCST the hydrogen bonds between PNIPAm’s amide groups and the surrounding water molecules dominate the Gibbs free energy of mixing equation due to their large negative enthalpic contribution. At these temperatures, PNIPAm exists as a solubilised polymer. As the temperature increases, hydrogen bonds are weakened due to thermal agitation and the entropic penalty of organising water molecules around the hydrophobic residues increases. Eventually, at the LCST, the entropic term dominates the Gibbs free energy equation.
and the PNIPAm chains dehydrate and demix into a tightly packed globular formation.\textsuperscript{130} For PNIPAm 3D networks, concomitant with this change in hydrophobicity is a monolithic volume contraction and changes in the gels optical, mechanical and electrostatic permittivity properties.\textsuperscript{131–133} Additionally, even though PNIPAm gels are typically thermally actuated, it’s possible to trigger contraction using light by embedding gold nanoparticles and utilizing their surface plasmon resonance effect as a heat source.\textsuperscript{134}

![Figure 1-12: An aqueous solution of PNIPAm below (A) and above(B) its lower critical solution temperature (LCST). Below the LCST PNIPAm is water soluble. After being heated above its LCST the PNIPAM demixes and phase separates from the water resulting in a large increase in solution turbidity.](image)

### 1.4 Bioprinting and bioinks

Bioprinting is the additive manufacturing technique applied to cell-laden constructs i.e., the spatiotemporal deposition of biomaterials into 3D prints. Bioprinting emerged from the field of 3D printing thermoplastics, and early bioprinters were often modified thermoplastic printers.\textsuperscript{135,136} Bioprinting protocols are also identical to 3D printing. Initially, 3D digital models are built \textit{in silico} which are then converted into GCODE ("slicing"). GCODE is a universal 3D printer language that enumerates step-by-step, layer-by-layer print commands.

There are many different bioprinting methodologies, like 3D printing, and they all have their own advantages, disadvantages, and respective sub-categories. The most common methodologies are inkjet bioprinting, laser assisted bioprinting, stereolithography bioprinting, and extrusion bioprinting (Figure
Chapter 1: Introduction

1-13). The vast majority of bioprinting research focuses on printing eukaryotic cells for tissue engineering and regenerative medicine applications.\textsuperscript{136}

![Figure 1-13](image)

Figure 1-13: Overview of different bioprinting methodologies. A, Inkjet bioprinting using either thermal or piezoelectric actuation to dispense pulsed bioink droplets onto a substrate. B, Laser-assisted bioprinting where a pulsed laser source is irradiated onto a cell-rich absorbing layer causing the bioink to ablate onto a receiving substrate. C, Stereolithography bioprinting where light-initiated polymerisation of a cell-laden pre-gel is patterned via a mask or direct laser writing. D, Microextrusion bioprinting approaches using either pneumatic or mechanical (piston/screw) actuation to extrude a continuous filament of bioink. Figure adapted, with permission, from Wiley.\textsuperscript{137}

The cell-laden ink used in a bioprint is referred to as a bioink. Bioinks must balance multiple demands. They must be printable, biocompatible, processable under mild conditions, able to entrap or encapsulate cells, perfusible to gases, nutrients, and cell regulatory factors, and have good post-print shape fidelity.

Most bioinks are either based on natural structural proteins (such as silk, hyaluronic acid, or collagen), natural polysaccharides (such as agarose, alginate, or cellulose), or synthetic hydrogels.\textsuperscript{138} Often common bioink components are blended to increase mechanical performance. For example, collagen cannot be extrusion printed by itself but it can be blended with alginate to produce a printable ink.\textsuperscript{139}

Also, many bioinks are co-printed with support or sacrificial materials to expand the range of architectures achievable.\textsuperscript{140–144}
Chapter 1: Introduction

Typically, bio-inks achieve shape fidelity via a post-printing crosslinking strategy: UV crosslinking via augmentation of polymers with photocrosslinkable side groups (while cautious of cytotoxic photoinitiators and extended UV exposure), or ionic crosslinking with molecules or ions etc. Other strategies include incorporation of collagen-based materials as they form stiff gels upon cooling due to the formation of triple helices. Some utilize poloxamer triblock co-polymer additives which exhibit thermo-gelling behaviour due to their temperature dependent self-assembly into micelles. At higher temperatures they form stiff gels and at lower temperatures they thin and resolubilise into liquid.

1.4.1 Inkjet bioprinting

Inkjet printing (IP) was first developed by Hewlett-Packard as a 2D printing technology in the 70s. Then in 1992 3D printing was achieved with the addition of a z-axis adjustable elevator stage and IP became one of the first additive manufacturing processes. IP produces patterned constructs via the controlled deposition of small droplets. The main types of IP actuation for 3D printing are thermal and piezoelectric. In thermal IP the bio-ink/prepolymer solution is loaded into a cartridge connected to a computer-controlled print head. Small droplets of ink are ejected via the nucleation of air bubbles created by a heating element in the print head. The main difference with piezoelectric inkjet printers is that instead of a heating element a piezoelectric crystal is used to create acoustic waves that propel the bioink. Due to the high operating temperatures of thermally actuated IP printing, most IP bioprinters are of the piezoelectric variant. For example, Zhou et al. used IP bioprinting to pattern Matrigel encapsulated human neural stem cells into complex functional 3D neural networks with ~100µm resolution.

The main advantages of IP bioprinting is extremely high resolution, and the ability to print low viscosity bioinks that would be unprintable under other methodologies. However, piezoelectric IP is limited to low viscosity inks as high viscosity dampens the acoustic waves.

1.4.2 Laser-assisted bioprinting

Laser-assisted bioprinting (LAB) was initially a metal deposition technology and was first utilized in bioprinting in 2000 to print viable embryonic chick spinal cord cells. A standard LAB setup will
contain three main components: a pulsed laser, a donor slide and a receiver slide. The donor-slide is a layer of transparent glass coated on one side with a surface of laser-absorbing metal which has a thin layer of bioink deposited onto it. The bioink can then be transferred from the donor-slide to the receiver-slide via the laser-actuated vaporization of the metal under the bioink. LAB is therefore a nozzle-free technique and is not susceptible to the clogging problems of nozzle technologies. Very high resolution (10-50 µm) is achievable and LAB is capable of printing with very high viscosity bioinks, but it remains an uncommon bioprinting approach due to concerns over laser pulses effecting cell viability.150

1.4.3 Stereolithography bioprinting

Stereolithography (SLA) bioprinting relies on biocompatible photocurable polymers, such as gelatin methacrylate, and cytocompatible photoinitiators, such as irgacure 2959. There are two main sub-categories. The first main sub-category is laser SLA, where a photocurable bioink is cured via a rastered laser beam. The print resolution is determined by the minimum laser spot size and can be up to 25 µm. The other main sub-category is digital light processing (DLP). In DLP a projector is used to illuminate patterned light onto a photocurable bioink.151 An obvious advantage of this approach is the ability to cure entire layers of a print simultaneously. For example, Wang et al. used stereolithographic DLP bioprinting for the rapid fabrication of cm scale prints to 50 µm resolution.152 However, while rapid fabrication is true for shallow prints, this time increases for deeper structures and this can lead to long UV exposure times. Some authors have circumvented this by either using blue-light crosslinkers, or by printing acellular scaffolds which are then seeded with cells post-crosslinking.153,154 However, printing large 3D structures with blue light exposure would encounter focal plane limitations due to the hydrogel scattering light.155,156

1.4.4 Extrusion bioprinting

Extrusion bioprinting (EB) is the most utilized of all the bioprinting methodologies, in part due to its easy accessibility. This is because extrusion bioprinting evolved from fused filament fabrication (FFF), one of the most mature and predominant 3D printing technologies. Years of innovation in desktop FFF printers have dramatically increased the affordability of such systems, and the associated software
programmes are all readily adaptable for EB. As a result, academic research in extrusion bioprinting is often conducted using custom, modified 3D printers.

Extrusion bioprinting is a pressure actuated technology where bioink is extruded onto a substrate through a nozzle via pneumatic or mechanical pressure. There are some important limitations to note with EB. The continuous filament nature of deposition can lead to anisotropic character in prints due to shear alignment. Cells in bioink can also be subjected to high mechanical/shear forces due to the pressure from extrusion which can affect cell morphology or viability, although most cell lines are not significantly impacted under typical printing conditions. Also, nozzle technologies are always susceptible to clogging and the achievable resolution is slightly higher vs. other bioprinting methodologies. Finally, and perhaps most importantly, the rheological properties of extrusion based bioinks significantly affect their printability. For accurate extrusion and good shape fidelity extrusion bioinks must be shear-thinning i.e. they must become less viscous under applied shear.

The performance benefits of EB are that very high cell density and cm-scale print are achievable. This is particularly useful in tissue engineering where EB has been used to print life-sized ear-, nose-, and bone- analogous.

1.4.5 Strategies for hydrogel bioink reinforcement

Hydrogel-based bioinks are the most explored due to their excellent cell supporting potential and research maturity. However, rudimentary single-network hydrogels have limited utility due to poor mechanical performance (though this can be desirable in certain tissue engineering applications when you want a particular material to undergo a controlled rate of degradation). This mechanical weakness is because classical polymer network formation will generate hydrogels that are randomly crosslinked. This leads to heterogenous gels with areas of densely and sparsely crosslinked regions. Therefore, upon loading, the reception of stress will be localised to the weaker failure zones inside the gel. One can simply try to increase the level of crosslinking inside a gel, but this just lowers the configurational entropy of the overall network resulting in gels that are brittle and less extensible. A hydrogel can often also be made tougher by increasing its polymer mass fraction or molecular weight,
but this also tends to interfere with the microarchitecture and cytocompatibility of hydrogels by limiting nutrient diffusion or restricting space for proliferation.\textsuperscript{170,171}

Many strategies exist for the fabrication of tough hydrogels without increasing polymer or crosslinking density, but the main approaches, with consideration to hydrogel bioinks, are polymer functionalization (PF), supramolecular hydrogels, nanocomposites, and interpenetrating networks (IPNs).\textsuperscript{172} In PF, polymers are functionalised with new chemical moieties to introduce new crosslinking modalities or biological activity. Most common is for physically crosslinked gels to be augmented with stronger covalent crosslinks to create dual-crosslinked hydrogels. This is typically done with natural polymers such as alginate, gelatin, hyaluronic acid, and collagen \textit{via} methacrylate functionalisation.\textsuperscript{173,174} For example, functionalised gelatin methacryloyl (GelMA) conserves many of the biocompatible properties of gelatin, such as cell attachment. However, once photocrosslinked GelMA is stable at room temperature (whereas gelatin melts), is more degradation resistant, and can be patterned \textit{via} stereolithography.\textsuperscript{175,176} The other main polymer functionalisation utilizes click chemistry and click reactions (reactions with a high yield that are highly selective, thermodynamically favourable, and proceed under mild conditions).\textsuperscript{177,178} The thiol-ene click reaction in particular is a well-researched biomaterial augmentation due to the ubiquity of cysteine residues in natural polymers.\textsuperscript{179,180} The thiol-ene click reaction proceeds efficiently at very low radical concentrations and is not inhibited by oxygen. This rapid crosslinking functionality is extremely useful for bioprinting, solidifying bioinks within seconds of patterning. Also, as a step-growth process thiol-ene click reactions tend to create much more homogenous crosslinking in networks, which reduces stress concentrations to improve extensibility and fracture toughness.\textsuperscript{179,181}

Supramolecular hydrogels are composed of end-functionalised, short polymer chains that are able to reversibly interact non-covalently through their end groups (\textit{via} hydrogen bonding or $\pi-\pi$ interactions for example) to form large dynamic 3D networks.\textsuperscript{182,183} Under high stress these non-covalent associations can reversibly break to dissipate energy, which also helps supramolecular hydrogels have the shear-thinning properties conducive to extrusion bioprinting. DNA hybridisation represents an interesting utilization of this technology.\textsuperscript{183} Here, biomaterials are conjugated with complementary
Chapter 1: Introduction

DNA molecules to exploit their natural association. Scaffolds fabricated with DNA hybridisation have demonstrated high cytocompatibility and could be selectively degraded via proteases and nucleases. As supramolecular interactions alone can sometimes not be enough to produce a solid hydrogel, this approach is often combined with PF for post-printing additional crosslinking.

Nanoparticles (NPs) have interested the biomedical community over the past few years due to the unique properties imparted by even small concentrations of nanomaterials. NPs have been exploited for applications in imaging, cancer therapies and biosensor development and their incorporation into bioinks has been used to add new functionalities such as electrical conductivity, stimuli responsiveness, improved printability, and mechanical reinforcement. The main NPs utilized in hydrogel reinforcement are graphene, carbon nanotubes, nanoclay, magnetic NPs and polymeric NPs. Reinforcement mechanisms are thought to derive from NPs acting as reversible, sacrificial multi-polymer crosslinkers that dissipate stress by temporarily breaking their NP-polymer associations. Nanoclay is able to do this via their permanent surface charge forming electrostatic crosslinks with other nanoclay NPs and polymer strands. Other NPs such as cellulose nanofibers form sacrificial hydrogen bonds with bioink polymers due to a high specific surface area.

IPNs describe hydrogels that are made up of two, although can be more, interdigitating polymer networks. “Interdigitating” networks are not covalently linked, but mechanically interwoven in such a way that they cannot be separated unless chemical bonds are broken. Semi-IPNs describe materials where only one of the polymer networks is crosslinked, i.e., a material that is a polymer network with embedded linear polymer chains (Figure 1-14). Full-IPNs fall into two main sub-categories: (i) simultaneous IPNs, where the two networks are cured simultaneously from a homogenized mixture of their monomeric precursors, and (ii) sequential IPNs, where the networks are formed one after the other. Sequential IPNs are typically performed by curing one network and then swelling it in a pre-gel solution of the second. IPNs exhibit interesting material properties as they are the result of the synergistic concert of properties between two individual polymer networks and they have been shown to exhibit greatly enhanced toughness and fracture strength over traditional single networks. Bakarich et al. demonstrated in 2015 the 3D printing of an acrylamide/alginate IPN. The acrylamide network was
first photocrosslinked post-printing before the alginate network was then physically crosslinked via CaCl₂ immersion. The physical crosslinking step increased printed stiffness from 23 to 260 kPa, failure stress from 11 to 130 kPa, and strain at failure from around 23% to 90%. In the same year Hong et al. published a 3D printable bioink based on a PEGDA/alginate IPN. Post-physical crosslinking increased fracture strength from ~200 J/m² to ~1500 J/m², to produce a hydrogel tougher than cartilage. Printed constructs were also resistant to cyclic mechanical deformation thanks to PEGDA’s elastomeric properties and human mesenchymal stem cells demonstrated high viability in the ink over 7 days.

Figure 1-14: Structure for semi- and full-interpenetrating polymer networks (IPNs).

### 1.5 Material properties of representative hydrogels

Table 1-1 summarises several relevant material properties for some representative existing hydrogels for reference.
## Table 1-1: Material properties for several representative hydrogels.

<table>
<thead>
<tr>
<th>Hydrogel</th>
<th>Crosslinking Details</th>
<th>Printable</th>
<th>Shape-Changing</th>
<th>Compression Modulus (kPa)</th>
<th>Bacterial Compatibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5% w/v sodium alginate</td>
<td>Pre-gel printed onto an agar substrate apphcated with 50 mM CaCl2.</td>
<td>Yes</td>
<td>No</td>
<td>150 ± 25 (when crosslinked in 1 mM CaCl2 for 1 hour)</td>
<td>Printing process initially reduces bacterial viability by approximately 50%, and bacteria remain viable in the ink at least 2 days post-printing.</td>
</tr>
<tr>
<td>10% w/v NiPAm/4% w/v alginate IPN</td>
<td>NiPAm was covalently crosslinked at 4°C with 6.5 mM MBA via UV photoinitiation. Alginate was ionically crosslinked for 1 hour in 1 M CaCl2.</td>
<td>No</td>
<td>Yes</td>
<td>1000 ± 200</td>
<td>-</td>
</tr>
<tr>
<td>10-20% w/v NiPAm/3% w/v alginate IPN</td>
<td>NiPAm was covalently crosslinked at 10°C with 6.5 mM MBA via UV photoinitiation. Alginate was ionically crosslinked for 72 hours in 0.1 M CaCl2.</td>
<td>Yes</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9% wt “Flink”: 1:1:1 mixture of sodium hyaluronate, k-carrageenan and fumed silica</td>
<td>UV crosslinked via functionalisation of sodium hyaluronate into photocrosslinkable glyceryl methacrylate-hyaluronic acid.</td>
<td>Yes</td>
<td>No</td>
<td>-</td>
<td>All printing components and processes found to negligibly effect bacterial viability. UV exposure tested for 60 seconds (90 mW, 365 nm).</td>
</tr>
<tr>
<td>Acrylamide/dibenzylidene-d-sorbitol dicarboxylic acid (DBS-COOH) IPN</td>
<td>Acrylamide is covalently crosslinked with MBA with a GOx-mediated EMP. DBS-COOH self assembles into a supramolecular hydrogel in low pH.</td>
<td>No</td>
<td>No</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Polyacrylamide (PAAM)/acryloylated gelatin hybrid hydrogel</td>
<td>Acryloylated gelatin covalently crosslinked via PAAM chains. Gelation triggered via a GOx-mediated EMP.</td>
<td>Yes</td>
<td>No</td>
<td>4.6 – 25.7</td>
<td>Cell viability determined via co-culturing bacteria with hydrogel powders for 48 hours. Viability was negligibly affected.</td>
</tr>
<tr>
<td>Polydimethylacrylamide/silica nanoparticles/ acryloylated human serum albumin nanocomposite hydrogel</td>
<td>Hydrogel covalently crosslinked via an HRP-mediated EMP.</td>
<td>No</td>
<td>No</td>
<td>21.6</td>
<td>-</td>
</tr>
</tbody>
</table>
1.6 Thesis aims and objectives, structure, and major outcomes

The overall aims and objectives of this thesis are summarised here:

- To develop a bioink capable of producing shape-changing, bacteria-supporting hydrogel structures that can be fabricated under mild conditions (including room temperature, atmospheric pressure, and aerobic conditions).
- To inoculate the developed bioink with genetically modified bacteria that will augment the hydrogel structures with a functionality, or contribute to the construction/maintenance of the final material.

This thesis consists of two results chapters (Chapters 3 and 4), each with their own conclusions and future work section. All the techniques and materials used to perform the research are detailed in Chapter 2: Methods and Materials. The first results chapter describes the synthesis and printing methodology for a novel 3D ink that produces a shape-changing IPN. The ink’s initiation system incorporates an EMP and by integrating a PNIPAm network the IPN prints can contract when actuated by heat. Enzymes are utilized in the initiation step to allow material fabrication under mild conditions. Explored are some of the material properties of the IPN prints as well as the capability and elasticity of their thermosensitive contraction. The hydrogel constructs produced by the 3D printing methodology were found to have a depth dependent composition which could lead to hollow-construct 4D printing and caused non-uniform contraction regimes.

In the second results chapter the IPN ink introduced in the previous chapter is inoculated with bacteria to investigate potential biohybrid utility. The use of whole-cell derived functionality was chosen due to the advantage of bacteria being able to self-sustain themselves indefinitely, and therefore additionally any engineered utility, under relatively undemanding conditions. Two constructs are explored. In the first construct, the IPN ink is successfully inoculated with *E. coli* that have been transformed to express organophosphate-hydrolysing enzymes to produce 3D-printed bacterial detoxification microreactors. In the second construct, *E. coli* whole cells are substituted into the IPN ink’s initiation system to investigate the capacity for living *E. coli* cells to cure the IPN ink via their native redox potential and produce an
ELM. Unmodified *E. coli* cells were found to be insufficient to fully cure the ink into a robust IPN, although some curing ability was observed. This ELM experiment is then repeated with *E. coli* that have been transformed to overexpress a synthetic peroxidase (C45, structure and construction discussed in Section 2.8) to explore if this improves their ability to cure the IPN ink. This experiment was unsuccessful. However, isolated C45 was demonstrated to be able to catalyse the initiation of a polymeric material, and this is the first example of a synthetic enzyme being able to do so.

The final chapter is an overall concluding remarks/summary section.
1.7 References


27. These are the top 10 emerging technologies of 2016 | World Economic Forum. https://www.weforum.org/agenda/2016/06/top-10-emerging-technologies-2016/.


Chapter 1: Introduction


Chapter 1: Introduction


Chapter 1: Introduction


Chapter 1: Introduction


Chapter 1: Introduction


Chapter 1: Introduction


Chapter 2: Methods and Materials

All experiments performed in this work used chemicals purchased from either Sigma Aldrich or Fisher Scientific, unless otherwise stated, and were used without further purification.

Horseradish peroxidase (HRP) was purchased from Alfa Aesar (J60026.23) and glucose oxidase (GOx) was purchased from Sigma (G2133-50KU, type VII from Aspergillus niger). Deionised water (Mq), at a resistivity of 18.2 MΩ·cm, was collected from a Millipore Milli-Q Reference Water Purification System with a Quantum TIX polishing cartridge and a 0.22 µm filter attached.

2.1 General interpenetrating network bioink preparation

The optimised, standard interpenetrating network (IPN) bioink/pre-gel formulation is stated in Table 2-1. This formulation was used in all IPN printing experiments unless otherwise stated. The predominant IPN scaffold components consist of the interdigitating Ca$^{2+}$-crosslinked alginate and MBA-crosslinked PNIPAm networks. Their pre-cursor components are present in the ink in the form of sodium alginate, NIPAm monomer and MBA. Viscosifying additives, xanthan gum and calcium chloride, were added to enhance printability. Finally, the ink is also primed for the enzyme-mediated polymerisation (EMP) of the NIPAm/MBA network with the dual-peroxidase based initiation system of HRP, GOx, and AcAc (which can be triggered via the addition of glucose).\textsuperscript{1,2}
Table 2-1: Optimised IPN bioink formulation details.

<table>
<thead>
<tr>
<th>Bioink Component</th>
<th>Concentration</th>
<th>Stock Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthan gum</td>
<td>0.75 wt%</td>
<td>N/A</td>
</tr>
<tr>
<td>Sodium alginate</td>
<td>3.5 wt%</td>
<td>N/A</td>
</tr>
<tr>
<td>N-Isopropylacrylamide (NIPAm)</td>
<td>15 wt%</td>
<td>N/A</td>
</tr>
<tr>
<td>N,N'-Methylenebisacrylamide (MBA)</td>
<td>7.5 mg/mL (48.6 mM)</td>
<td>Mq</td>
</tr>
<tr>
<td>HRP</td>
<td>1.14 µM</td>
<td>Mq</td>
</tr>
<tr>
<td>GOx</td>
<td>3.12 µM</td>
<td>50 mM Sodium acetate pH 5 buffer</td>
</tr>
<tr>
<td>Acetylacetone (AcAc)</td>
<td>97 mM</td>
<td>N/A</td>
</tr>
<tr>
<td>Calcium chloride (CaCl₂)</td>
<td>25 mM</td>
<td>N/A</td>
</tr>
</tbody>
</table>

The IPN ink was typically prepped in 5 g batches. Initially, xanthan gum (37.5 mg, 0.75 wt%), sodium alginate powder (175 mg, 3.5 wt%), and NIPAm (750 mg, 15 wt%) were added to a solution of Mq water (900 µL) in a SpeedMixer™ PP10 10 mL cup. Stock solutions of MBA in Mq (1.875 mL, 20 mg/mL), HRP in Mq (125 µL, 2 mg/mL), and GOx in 50 mM NaAcetate pH 5 buffer (675 µL, 3.7 mg/mL) were then added to give final concentrations of 7.5 mg/mL, 1.125 µM, and 3.12 µM respectively. These were mixed in with gentle hand mixing, to yield a viscous, turbid, pale-yellow solution. This was then mixed in a SpeedMixer™ dual asymmetric centrifuge (DAC, Section 2.1.1) at 3500 rpm for 5 minutes before addition of AcAc (50 µL). The ink was then mixed again in the DAC at 3500 rpm for 5 minutes. Lastly, CaCl₂ (416 µL, 300 mM) was added, to lightly pre-crosslink the bioink, before a final mix in the DAC at 3500 rpm for 5 minutes yielded the final formulation.
Chapter 2: Methods and Materials

2.1.1 Dual Asymmetric Centrifuge (DAC)

The IPN ink is fairly viscous during preparation. To increase ink uniformity, and therefore reduce variability in final constructs, most mixing steps were performed in a DAC (Figure 2-1). Dual asymmetric centrifuges are able to rotate a sample around two different axes simultaneously and this combination of centrifugal forces is capable of homogenously mixing viscous solutions without air bubbles.

2.2 3D printing

3D printing, also known as additive manufacturing, is the process in which prints are constructed from computer-generated 3D digital model files. Materials are deposited layer-by-layer and then cured or sintered into a monolithic solid structure. The following protocol describes the extrusion printing of a shear-thinning pre-gel solution, which is then cured post-printing via a wash step where the print is immersed in a CaCl$_2$ and glucose solution. The CaCl$_2$ ionically crosslinks the alginate chains. Concomitantly, the glucose initiates the enzymatic cascade which produces the AcAc radicals that cure the PNIPAm/MBA network. All prints were performed in an aerobic environment and at room temperature with the Cellink INKREDIBLE+ bioprinter (Figure 2-2). Unless otherwise stated, print moves were set to 50 mm/s, extrusion pressure to 22 kPa, infill to 100%, and layer height equal to the used nozzle diameter.
3D models were designed in Tinkercad (Autodesk) and converted into STL files. STL files describe the 3D Cartesian coordinates for the vertices of a given digital model’s triangulated surface. The STL’s corresponding print commands were produced using open-source slicer software (Slic3r). Here, STL files are converted into a list of printer commands known as G-code (Section 2.2.1) which enumerates the operation of individual printer motors and sensors. The G-code was then post-processed with Cellink’s custom post processor code for printer compatibility to the INKREDIBLE+.

To begin printing, freshly prepared ink (preparation described in Section 2.1) was loaded into a 3 mL syringe barrel (Nordson). A 20-gauge (20G) tapered plastic nozzle (Metcal; Farnell) was then attached to the syringe which was then placed inside an INKREDIBLE+ pneumatic printhead. Immediately prior to printing the zero position for the printing height was manually calibrated. The desired G-code was then run on the printer and the corresponding construct was printed into a sterile petri dish. After printing, constructs were crosslinked via immersion in a 300 mM CaCl$_2$ + 5 mg/mL D-glucose solution.

Figure 2-2: Photographs of the Cellink INKREDIBLE+ bioprinter. A, The INKREDIBLE+ in a typical pre-printing set-up. B, a close up of the INKREDIBLE+ pneumatic print head with an ink loaded.
overnight. The prints were then re-immersed in 10 mM CaCl₂ solution for storage so as to keep the alginate IPN component from dissolving.³

2.2.1 G-code

G-code (Figure 2-3) is a computer numerical control programming language that enumerate instructions to a machine controller. In 3D printing this equates to printing pathway and extrusion commands. Simply, each step describes a coordinate destination, an extrusion volume (via stepper-motor commands to depress a syringe plunger), and a G-command. G commands instruct the printer on how to translate the extruder to the coordinate destination e.g., in a linear move or in a controlled arc etc. Additional typical print command lines include instructions for print bed and printhead temperature (if applicable), extruder non-infill movement and pauses, and distance unit setting. A full repository of all G-code commands is available online at https://reprap.org/wiki/G-code.

<table>
<thead>
<tr>
<th>Command</th>
<th>X</th>
<th>Y</th>
<th>Z</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1 X6.056 Y-12.056 E0.00085</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1 X5.497 Y-12.056 E0.00280</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1 X-2.056 Y-4.503 E0.05347</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1 X-2.056 Y-5.232 E0.00365</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1 X4.768 Y-12.056 E0.04831</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1 X4.040 Y-12.056 Z 1.021 E0.00365</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1 X-2.056 Y-5.960 E0.04315</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1 X-2.056 Y-6.898 E0.00365</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1 X3.311 Y-12.056 E0.03799</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1 X2.582 Y-12.056 E0.00365</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1 X-2.056 Y-7.418 Z 1.021 E0.03283</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Figure 2-3: A, An example of 3D printing G-code infill commands. The axes X, Y, and Z describe left and right, forward and backward, and up and down motion respectively. The E notation denominates extrusion. G1 is a G-command for a linear interpolation i.e., a straight-line move. B, An example of visualized G-code.
2.3 Printability assays

Printability describes the quality of a 3D inks printing performance. It is quantified via many different assay modalities examining properties such as extrudability, shape fidelity, filament uniformity, printing accuracy, etc. However, a model set of assays or test print structures doesn’t exist, and researchers tend to evaluate printability using bespoke methodologies. It is also not uncommon for researchers to only qualitatively evaluate a new inks printability, or even not evaluate printability at all.\textsuperscript{4,5} The assays, described in this section, chosen to assess the IPN inks printability are all based on multiple literature examples and are among the most ubiquitous types of quantitative printability assessment.
2.3.1 Printing uniformity assays

These assays, based from examples in the literature,\textsuperscript{6,7} analyse the performance of a 3D ink via measuring the variance in pore sizes and line widths for a standard print.

The print used in these assays was a crosshatch print designed by slicing a 30x30 mm, 1-layer high cuboid STL file to a 60% square mesh infill (Figure 2-4). The print was performed using the standard bioink formulation (preparation described in Section 2.1) extruded through an 18G plastic tapered...
needle (Metcal; Farnell). The print was then brightfield imaged on a LEICA DMI6000 inverted epifluorescence microscope.

Print pore areas were calculated using image analysis with ImageJ software. Images were initially processed via thresholding to make them binary (thresholding is a process which replaces every pixel in an image with a black or white pixel depending on whether its intensity is less or more than some fixed value). This allows ImageJ to discriminate the print from its background. After thresholding the pore areas are measured via calculating the volume and distribution of connected white pixels. Mean pore areas and standard deviations were calculated from 24 pores from each print, and 3 prints were performed.

Line widths were measured manually via ImageJ. Mean values and standard deviations were calculate from 15 measurements from each print, and 3 prints were performed. Line widths were taken from halfway between crosshatch vertices so as to measure the printed filament width as uninfluenced by the merging of perpendicular lines.
2.3.2 Filament collapse assay

This assay evaluates an ink’s ability to bridge small gaps without support. It is based on previous examples from the literature.\(^8,9\)

The filament collapse assay was conducted on a 3D printed platform (printed using a Formlabs Form 2 SLA printer loaded with a V4 clear resin cartridge) with pillar heights of 10.0 mm, widths of 5.0 mm and incrementally increasing gap distances of 1.0, 2.0, 4.0, 8.0, and 16.0 mm (Figure 2-5). Inks tested include the standard bioink formulation (preparation described in Section 2.1), Cellink’s CELLINK START (IK1900000305) and Cellink’s CELLINK Bioink (IK1020000303). Inks were printed at the lowest pressure that the ink would bridge all sections, rounded to the closest multiple of 5 kPa, and print moves were set to 50 mm/s. All inks were extruded through a 25G tapered plastic nozzle (Metcal; Farnell). The filament collapse areas were manually quantified using ImageJ software and calculated.
as the sum of the areas below the pillar heights the filament had collapsed. Mean values and standard deviation for the IPN filament collapse areas were taken from 5 repeats. Mean values and standard deviation for the CELLINK inks filament collapse areas were taken from 3 repeats.

2.4 Contractile thermosensitivity assays

PNIPAm and PNIPAm-based hydrogels are a well-studied domain due to their contractile thermosensitive (TS) properties.\textsuperscript{10} PNIPAm is a smart polymer has the ability to reversibly switch from being hydrophilic to being hydrophobic when heated above its lower critical solution temperature (32°C in aqueous solution).\textsuperscript{11} This change causes PNIPAm chains in solution to preferentially associate with themselves over the solvent. As the polymer becomes insoluble it phase separates, or demixes, from the solvent and PNIPAm solutions heated above the LCST go from being transparent to turbid (LCSTs can also defined as a cloud point). When PNIPAm chains are crosslinked this demixing behaviour translates into reversibly contractable hydrogels which can shrink many times their solvated volume when heated above their LCST.

We wanted to test how well the IPN ink prints conserved the TS of their PNIPAm single network component. To evaluate their shape changing capabilities two assays were designed. In the repeatability assay printed IPN constructs were subjected to multiple heat cycles to study how elastically the IPN would contract. Here, IPN prints were heated by being placed in an oven at 60°C for 30 minutes. Volume was measured immediately post-oven. Volume was remeasured after the print was allowed to relax at room temperature overnight. The assay was performed every day for 7 days. Mean values and standard deviation for print constructs volumes were taken from 6 repeats.

To assay how fast printed IPNs would contract a kinetics assay was designed. Here, printed IPN constructs were heated \textit{via} immersion in a water bath set to 60°C and volume measurements were taken every minute for the first 10 minutes. Two further volume measurements were then taken after 20 and 30 minutes of immersion. Mean values and standard deviation for print constructs volumes were taken from 3 repeats.
The contractile TS assays were all performed on prints from a 3x3x15 mm cuboid .STL file, sliced via a 100% infill with a rectilinear pattern (Figure 2-6). Prints were typically over-extruded to ensure the complete infill and good inter-layer adhesion of the final printed structure. This resulted in printing constructs slightly exceeding their specified dimensions. All volume measurements were calculated from measurements taken with electronic callipers. Volume changes were calculated as percentages of construct’s initial volumes (v/vi).

2.5 Ink characterisation techniques

2.5.1 Rheology

Rheology is the study of flow. Primarily it is liquids and gases that are studied but also solids when they are under stress regimes such that they plastically deform. The rheological properties of extrudable hydrogel inks are extremely relevant to their printability. For example, extrudable inks must form continuous filaments during extrusion, and they must recover quickly post-printing. Only well designed
inks will have good shape fidelity, inter-layer feature resolution, filament cross-section circularity on deposition, etc. One of the most important rheological properties of extrudable hydrogel inks is that they must be shear-thinning (i.e., their viscosity will decrease under applied shear) so they can be ejected from a nozzle. Shear thinning behaviour is characterized by rheometry via the fluid power-law model, which relates the viscosity of a fluid with the applied shear rate:

Equation 1: Power-law fluid model (or the Ostwald-de Waele relationship)

\[ \mu(\dot{\gamma}) = K\dot{\gamma}^{n-1} \]

Where \( \mu \) is viscosity, \( \dot{\gamma} \) is shear rate, \( K \) is the flow consistency index (i.e., the viscosity when \( \dot{\gamma} = 1 \)), and \( n \) is the ‘flow behaviour index’. Ordinary Newtonian fluids will have values of \( n = 1 \). Pseudoplastic (shear-thinning) materials will have values of \( n < 1 \) and dilatant (shear-thickening) materials will have values of \( n > 1 \).

A material’s flow behaviour index is solved for the fluid power-law model by collecting flow curves. These are experiments conducted on a rheometer where the shear viscosity of a sample is measured over a range of shear rates. The power-law fluid model can then be solved for \( n \). Flow curves were collected on a Malvern Kinexus Pro+ with a cone-plate geometry (2 cm diameter, 4° cone). All runs were performed at 25°C with approximately 0.3 mL of sample and a plate gap distance of 0.145 mm.

2.5.2 Cryo scanning electron microscopy

Electron microscopy (EM) is a technique to attain high resolution topographical information about a sample. An electron source produces an accelerated electron beam which is focused over a sample. The sample will then emit secondary electrons into a detector. As the beam is rastered it is possible to reconstitute surface topology via the variation in emitted electrons. As electrons, which have very short wavelengths, are used as the illuminating radiation it is possible to resolve nanometre scale feature sizes. This is a much higher resolution than conventional light microscopy which uses a much longer wavelength illuminating radiation.
To allow non-conductive samples to absorb and scatter electrons they must be coated, or stained, with heavy metals such as palladium, gold, platinum, or chromium. Typically, samples are sputter coated. In sputter coating the sample is placed within a vacuum chamber where an inert gas is ion bombarded onto a conductive target material (e.g., gold). This target material then sputters particles onto the sample and coats it in a thin conductive film (ca. 2-20 nm). This allows non-conductive samples to have detectable contrast under EM. Otherwise, non-conductive samples will simply become charged from the incident electron beam which results in anomalous image contrast, distortions, and drift.

Hydrogels present a difficulty to prepare for EM imaging as they have a very high water content and must be dehydrated before being coated. Otherwise, if left hydrated the water would evaporate under the vacuum conditions required for EM. This would significantly disturb the sample away from its in situ solvated structure. Evaporation can be attenuated by operating at very low temperatures but then samples are liable to develop image artifacts due to ice crystallisation displacing polymer chains. For authentic structural characterisation hydrogels are dehydrated via vitrifying with a cryogen that has a high cooling rate (e.g., liquid nitrogen or ethane) to produce transparent amorphous ice. The ice is then sublimated in a low-temperature vacuum. This is considered the least aggressive method of dehydrating hydrogels and best for preserving their aqueous structures for EM imaging. In Cryo-SEM samples are vitrified, sublimated, coated, and imaged in situ with a high degree of control and completely isolated from atmospheric conditions. It is considered one of the least invasive EM techniques. Its development was primarily motivated for the accurate structural determination of soft organic materials and so it is therefore perfectly suited for imaging soft hydrated materials like hydrogels. However, even in Cryo-SEM conditions must be carefully chosen to avoid ice-crystal damage or eutectic artifacts in hydrated soft samples.

Cryo-SEM imaging was performed by Judith Mantell of the Wolfson BioImaging Facility on a JSM IT300 Scanning Electron Microscope (Jeol, Japan). All samples were prepared by plunge freezing them in liquid nitrogen slurry (LNS), before gradually sublimated them over 3 minutes and then coating them in carbon prior to imaging.
2.5.3 Compression testing

Compression testing interrogates how a material behaves under compressive load. It can evaluate a material’s stiffness, modes of failure, elastic recovery etc. and is a common technique for evaluating hydrogels. Especially when interested in their strength or in how a hydrogel bioink’s mechanical properties influence cell behaviour.\(^\text{16}\) Compression testing is typically used to calculate the Young’s Modulus, which quantifies the relationship between stress and strain within a material’s linear elastic region:

Equation 2: Young's modulus

\[
E = \frac{\sigma}{\varepsilon} = \frac{(F/A)}{\Delta L}
\]

Where \(E\) is the Young’s modulus, \(\sigma\) is the stress experienced by the sample (the force \((F)\) divided by the sample cross-sectional area \((A)\)), and \(\varepsilon\) is the strain (the change in length \((\Delta L)\)). For a compression test \(E\) is referred to as the sample’s compressive stiffness.
It’s worth noting different types of compression testing exist, for example confined/unconfined and indentation, and each will typically result in a different Young’s Modulus value. Therefore, values of $E$ should always be accompanied with a clearly defined methodology.

Unconfined compression testing was performed using an Instron 3343 universal testing machine (Instron, UK) with compression platen attachments and a 10 N load cell. Samples were compressed at a rate of 1 mm/s and force and displacement readings were recorded in real time. Hydrogel samples were prepared for compression testing by being cast in 3D printed moulds (Figure 2-7), which were designed in Tinkercad and printed using a Formlabs Form 2 SLA printer loaded with a Formlabs V4 clear resin cartridge. Hydrogels with alginate components were cast into porous moulds to increase diffusion of Ca$^{2+}$ crosslinking ions into gel. PNIPAm control single networks were cast in moulds without pores. All compression samples and their crosslinking wash solutions are listed in Table 2-2.
Chapter 2: Methods and Materials

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Formulation</th>
<th>Crosslinking Wash Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPN</td>
<td>Optimised IPN ink formulation</td>
<td>300 mM CaCl₂ + 5 mg/mL glucose</td>
</tr>
<tr>
<td>Alg</td>
<td>3.5 wt% alginate, 0.75 wt% XG, 25 mM CaCl₂</td>
<td>300 mM CaCl₂</td>
</tr>
<tr>
<td>PNIPAm</td>
<td>15 wt% PNIPAm, 7.5 mg/mL MBA, 100 mM AcAc, 3.12 µM GOx, 1.14 µM HRP, 5.55 mM glucose</td>
<td>N/A</td>
</tr>
</tbody>
</table>

As hydrogels generally don’t have a defined linear-elastic region compressive stiffness calculations were calculated using data taken from the first 10% strain.

2.6 Phosphotriesterase plasmid construction, transformation, and expression

Phosphotriesterase (PTE) is a metalloenzyme that catalyses the hydrolysis of the triester linkages found in organophosphate (OP) groups, present in many insecticides and nerve agents. A number of bacteria, fungi, and cyanobacteria express it naturally and it’s also previously been successfully heterologously expressed with the intention of augmenting bacteria with bioremediating OP-degrading activity.

Described in this section is the preparation of PTE-expressing *E. coli* and their inoculation into the IPN ink. This was performed to see if the ink would successfully encapsulate the bacteria and exhibit OP-degrading activity as a rudimentary bioremediating microreactor.
2.6.1 Phosphotriesterase plasmid construction

A pET14b+ plasmid containing the gene for PTE (pET14b-PTE) was provided by Graham Day from the University of Bristol. This was amplified and then purified using a QIAprep Spin Miniprep Kit (QIAGEN) before being stored at -20°C prior to further use.

2.6.2 PTE plasmid transformation into competent cells

Approximately 100 µg of the pET14b-PTE plasmid would be transformed into a 50 µL aliquot of BL21(DE3) E. coli cells (New England Biolabs) via electroporation. This was performed in an ice cold 0.4 cm electocuvette using a Gene Pulser (Bio-Rad) set to pulse at 2.5 V every 4.2 ms. The cells would then be immediately transferred to a 14 mL conical tube that had been preheated to 37°C. This was then left in a shaking incubator at 37°C, 180 rpm for 30 minutes. 50 µL of this cell suspension (PTE-E. coli) would then be spread across an LB agar plate supplemented with 1 wt% glucose and 50 µg/L carbenicillin. Plates were then incubated overnight at 37°C. These plates could then be stored for up to a week at 4°C.

2.6.3 Lysogeny broth (LB) agar plate preparation

LB agar plates were prepared by autoclaving a 100 mL aqueous solution with 2.5 g LB and 1.5 g agar. While the solution was still warm and liquid any appropriate antibiotics were added. The solution was then decanted into multiple 9 cm petri dishes, under sterile conditions, which were left to solidify at room temperature.

2.6.4 Bioprinted organophosphate-degrading bacterial microreactor assay

For the E. coli-laden bioinks bacteria were prepared as follows: A single colony of E. coli was picked from a streaked LB agar plate and used to inoculate 30 mL of LB medium with 1 mg/mL glucose. Inoculated broth was grown in a shaking incubator at 37°C, 180 rpm. The next morning the culture was collected via centrifugation (3500 x g for 15 min) and resuspended in 5 mL of Mq water. This suspension was then used to inoculate the bioink immediately, which was then mixed in the DAC at 3500 rpm for 1 minutes. A typical 5 g preparation of bioink would contain 900 µL of this bacterial
suspension as well as 50 µg/L carbenicillin. Bioinks were then printed into gently curved rectangles (Figure 2-8) designed to tessellate with the edge of a standard 6-well plate well.
Figure 2-8: Organophosphate-degrading bacterial microreactor assay details. A, Images of 3D model and print used for OP-degrading microreactor. B, Images of 3D model and print for assay spacer. The spacer is designed to fit in a standard 6-well plate well and stop microreactors from drifting into well centres and interfering with absorbance measurements. C, Photographs of a 6-well plate well with 3D printed spacer and two microreactor prints before (left) and 30 minutes after (right) paraoxon addition. The bioink has been inoculated with *E. coli* transformed to be able to express phosphotriesterase, a paraoxon hydrolysing enzyme. The yellow colouration in the bottom photo is due to 4-nitrophenol, a yellow paraoxon hydrolysis product that adsorbs at 405 nm. Major and minor gridlines in all pictures demarcate 10 and 1 mm respectively.

*E. coli*-laden prints were crosslinked post-printing via immersion in a 100 mM CaCl$_2$ + 5 mg/mL D-glucose solution for 1 hour. Uninduced prints were then re-immersed in a 20 mM CaCl$_2$ + 5 mg/mL D-
glucose solution and incubated at 29°C overnight. Induced prints were re-immersed in a 20 mM CaCl$_2$, 5 mg/mL D-glucose + 1 mM IPTG solution.

For the assay, 2 of each print was placed per well in a 6-well plate. Prints were then washed three times with 10 mL, before being finally re-immersed in 4 mL of HEPES buffer. 80 µL of a 50 mM paraoxon, an organophosphate which has a yellow hydrolysis product (4-nitrophenol), stock solution was then added to each well (1 mM final concentration). The assay was conducted with a BioTek SYNERGY neo2 multi-mode plate reader at 22°C. Absorbance measurements for 405 nm were taken from a single point at the centre of each well every 30 seconds for 30 minutes post-paraoxon addition. 3D printed plastic spacers (designed in Tinkercad and printed using a Formlabs Form 2 SLA printer loaded with a V4 clear resin cartridge) were added to each well to ensure the hydrogels didn’t float into the centre and interfere with absorbance measurements.

The negative control *E. coli* experiment was performed using BL21(DE3) *E. coli* that had only been transformed with a plasmid encoding for carbenicillin-resistance.

### 2.6.5 Confocal microscopy of *E. coli*-laden bioink

Inoculated bioink samples (170 µL) were first washed in a sterile saline solution (1 mL, 0.9 w/v% NaCl) and then stained with SYTO 9 (10 mM) and propidium iodide (60 mM) in sterile saline solution (0.5 mL, 0.9 w/v% NaCl) stationary in a dark room for 45 minutes (the LIVE/DEAD BacLight Bacterial Viability Kit). The bioink samples were then rewashed twice with sterile saline solution (2 x 1 mL, 0.9 w/v% NaCl). The saline solution was then aspirated off the samples before imaging. All confocal imaging was performed on a Leica SP8 confocal laser scanning microscope.

To calculate cell viability, images were first thresholded to make them binary. Cells were then counted via pixel volume, and viability calculated as the volume of live cells divided by the volume of live and dead cells. Viability percentages were reported as the average from 10 images taken at random from the bioink bulk.
2.7 Casting *E. coli*-mediated single networks

This section describes the PNIPAm/MBA single network *E. coli*-mediated casting experiments. Here, the HRP in the HRP/GOx/AcAc/glucose quaternary initiation system (used in Section 2.1) is replaced with *E. coli* whole cells. We hypothesized that the endogenous redox activity of the living cells could initiate the radical polymerisation reaction and cure the network. Similar experiments have been performed recently. For example in Bennett *et al.*, where water-soluble synthetic polymers were synthesized using bacteria-mediated radical polymerisation via harnessing the cells’ native cellular redox processes. Cells remained viable post-polymerisation.

The casting experiments were all conducted in 100mM KCl, 20 mM CHES, 7.5 wt% glycerol at pH 8.58 (redox buffer) and used frozen aliquots of BL21(DE3) *E. coli* that have not been transformed with any exogenous plasmid material. BL21(DE3) *E. coli* aliquots were prepped via collecting a 1 L large culture of cells, at OD<sub>600</sub> ca. 1.4, via centrifugation (40,000 x g for 30 mins). This cell paste was then washed in 500 mL redox buffer before being re-centrifuged. The cell paste was then resuspended to 20 mL in redox buffer supplemented with 15 wt% glycerol, aliquoted, flash frozen in liquid nitrogen, and stored at -80°C. Whole cell aliquots (WC-*E. coli*) were defrosted at room temperature prior to use and used at 50% v/v concentration in the casting pre-gels.

Other reaction components were added from stock solutions: MBA in Mq (20 mg/mL), GOx in 50 mM NaAcetate pH 5 buffer (3.7 mg/mL, 23.125 µM), and glucose in Mq (10 mg/mL, 55.5 mM). The final reaction concentrations of all reactants are listed in Table 2-3. All casts were performed in 5 mL microcentrifuge tubes with the lid open to allow the influx of molecular oxygen.
Table 2-3: Whole cell-initiated casting experiment reactant concentrations.

<table>
<thead>
<tr>
<th>Reactant</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIPAm</td>
<td>16.8 wt%</td>
</tr>
<tr>
<td>MBA</td>
<td>4 mg/mL</td>
</tr>
<tr>
<td>BL21(DE3) E. coli aliquots</td>
<td>50 % v/v</td>
</tr>
<tr>
<td>GOx</td>
<td>200 nM</td>
</tr>
<tr>
<td>AcAc</td>
<td>48.7 mM</td>
</tr>
<tr>
<td>glucose</td>
<td>2.77 mM</td>
</tr>
</tbody>
</table>

2.8 C45 plasmid construction, transformation, and expression

C45 is a de novo enzyme designed by the Anderson group at the University of Bristol using the maquette approach.\textsuperscript{22,23} This is a bottom-up enzyme-design philosophy that aims at reducing enzyme complexity to elucidate the minimum requirements for catalysis, and in order to make them readily expressible in bacteria. It takes simple maquette scaffolds and develops them towards activity in iterative, tractable steps such as the coordination of functional cofactors (natural and synthetic), the mutation of important residues, or the reorientation of helices to change scaffold topology.\textsuperscript{24,25}

C45 is a maquette protein, based on a 4 α-helix bundle with a prosthetic heme cofactor, that displays peroxidase activity (Figure 2-9).\textsuperscript{26} It exhibits high catalytic efficiency, promiscuity for a variety of peroxidase substrates, and is readily expressible in E. coli. Described in this section is the preparation of pure isolated C45 enzyme and of C45-expressing E. coli to see if either can be substituted into the initiation system described in section 2.1 in lieu of HRP.
2.8.1 C45 plasmid construction

The sequence for C45 was placed on a modified pMal-p4x+ plasmid containing the sequences for the cytochrome itself, and CARB resistance, along with a C-terminus His$_6$ tag (to allow later purification via immobilized metal ion affinity) with a Tobacco Etch Virus (TEV) recognition sequence between them (to allow later removal of the purification tag). This plasmid was then transformed into E. coli T7 Express competent cells (NEB) following a standard protocol.$^{27}$

C45 was co-transformed with plasmid pEC86 which contains the sequences for the relevant maturation machinery (the cytochrome I biogenesis system I (Ccm) required for incorporation of a c-type heme into the C45 scaffold)$^{28}$ as well as for chloramphenicol resistance. To ensure both plasmids were successfully transformed into cells, carbenicillin (50 µg/L) and chloramphenicol (34 µg/L) were added to media and plates.

2.8.2 C45 expression, whole cell aliquotation, and purification.

All steps were performed under sterile conditions. Single colonies were selected from streaked plates of E. coli and used to inoculate 5 mL of LB broth. Inoculated broth was grown at 37 °C overnight in an incubator with shaking/rotation. The next morning 2.5 ml of the overnight culture would be used to
inoculate 1 L of LB broth. This would then be cultured until its optical density at 600 nm (OD\textsubscript{600}) was between 0.6 and 0.8. The culture was then induced with 1 mL of 1 M IPTG to overexpress C45. The 1 L cultures would then be returned to the incubator for a further 4.5 hours before being spun down (4000 x g for 30 mins) to a red paste.

To store whole cells with pre-expressed C45 (C45-\textit{E. coli}) the cell paste would be resuspended in 100 mM KCl, 20 mM CHES, 15 wt% glycerol at pH 8.58 (redox buffer). This would then be aliquoted and stored at -80°C.

If purifying isolated C45 the cell paste would be dissolved in a small amount of 300 mM NaCl, 50 mM sodium phosphate and 40 mM imidazole at pH 8.0 (lysis buffer) and lysed by sonication (Sonic Vibra-Cell). C45 protein purification was then achieved by following a protocol closely based on the original by Watkins \textit{et al.}\textsuperscript{22} The soluble lysate was clarified by removing the insoluble fraction via ultracentrifugation (40,000 g for 30 mins). The soluble lysate was then filtered (0.22 M syringe filter, Millipore) and loaded onto a HisTrap FF IMAC column (Cytiva) that had been equilibrated with lysis buffer. The bound protein was then washed with lysis buffer before being eluted with 300 mM NaCl, 50 mM sodium phosphate and 250 mM imidazole at pH 8.0 (elution buffer). Fractions containing the C45 protein were combined and dialyzed for 18 h against 5 L of 0.5 mM EDTA and 20 mM Tris at pH 8.0 in a 14 kDa semi-permeable dialysis membrane. Then, under anaerobic conditions, TCEP (1 mM) and TEV protease (1 \textmu M) was added to the dialyzed protein to cleave the N-terminal hexahistidine tag. This reaction was allowed to stand in anaerobic conditions overnight to ensure it progressed to completion. The protein was then filtered to remove precipitated TEV protease (0.22 \textmu M syringe filter, Millipore) and concentrated to approximately 5 mL. This was then loaded onto a Superdex 200 pg 26/600 size exclusion column (Cytiva), which had been equilibrated in redox buffer, and underwent isocratic elution at 2 mL/min. The sample eluted as 2 distinct peaks. The first peak corresponds to apo-C45 and aggregated proteins, these fractions were discarded. The second peak has two initial minor peaks likely corresponding to improperly folded C45. The major peak was pure C45 and eluted as a red solution. This was collected, concentrated to approximately 50 \textmu M and stored at 4 °C.
2.8.2.1 UV/vis characterisation of C45

UV/vis spectrophotometry is based on the principle that electrons can absorb UV-visible light to transition into higher excitation states and that only photons with equal energy to the discrete energy steps between the electronic states are absorbed. As different chemical moieties have signature electronic absorbances unique spectra can be obtained by exciting a sample with a range of monochromatic light.

Spectrophotometric analyses on C45 solutions were performed using a Cary 60 UV-vis spectrophotometer (Agilent Technologies) and a 1 cm pathlength quartz cuvette. Blanks of analyte’s buffers were read and subtracted for each sample.

2.8.3 Casting isolated-C45 initiated single networks

Isolated-C45 preparation is described in Section 2.8.2. NIPAm was added as a solid and AcAc was added neat. All other reaction components were added from stock solutions: MBA in Mq (20 mg/mL), GOx in 50 mM NaAcetate pH 5 buffer (3.7 mg/mL, 23.125 µM), and glucose in Mq (10 mg/mL, 55.5 mM). The final reaction volume was 1 mL, and the concentrations of all reactants are listed in Table 2-4. All casts were performed in 2 mL microcentrifuge tubes with the lid open to allow the influx of molecular oxygen.
Table 2-4: Isolated C45-initiated casting experiment reactant concentrations.

<table>
<thead>
<tr>
<th>Reactant</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIPAm</td>
<td>15 wt%</td>
</tr>
<tr>
<td>MBA</td>
<td>1 mg/mL</td>
</tr>
<tr>
<td>C45</td>
<td>1.14 µM</td>
</tr>
<tr>
<td>GOx</td>
<td>3.12 µM</td>
</tr>
<tr>
<td>AcAc</td>
<td>97 mM</td>
</tr>
<tr>
<td>glucose</td>
<td>5.55 mM</td>
</tr>
</tbody>
</table>

2.8.4 3D printing IPN ink with a C45-based initiation system

Preparation of the IPN ink primed with a C45-based initiation system followed the exact same preparation as outlined in Section 2.1 but with a molar equivalent of C45 substituted for HRP.

2.8.5 Casting C45-E. coli mediated single networks

Frozen C45-E. coli aliquot preparation is described in Section 2.8.2. Whole cell aliquots were defrosted at room temperature prior to use. Cast pre-gel solution’s preparation and protocol followed Section 2.7’s exactly but with C45-E. coli substituted in lieu of the non-transformed E. coli.

2.9 Statistical significance analysis

All data was analysed in Microsoft Excel using a two-tailed Student’s t-test. A value of $P < 0.05$ was considered to indicate statistical significance. Data is reported as the mean of at least three experimental repeats ± standard deviation.
2.10 References


Chapter 3: Preparation and Characterisation of a 3D-Printable Shape-Changing Enzyme-Mediated Interpenetrating Network

3.1 Introduction

The 3D printing of shape changing (or shape deforming) materials is of high interest as anisotropic actuation behaviour is often determined by heterogenous actuator structures such as laminated bilayers, crosslinking gradients, and shape or composition asymmetries.\(^1\),\(^2\) 3D printing is capable of sophisticated 3D patterning and this can allow heterogenous structuring to be built in at fabrication. Hydrogels are an auspicious target for 3D printable shape changing devices as 3D hydrogel inks are well researched and hydrogels also make unique actuators due to their biocompatibility, flexibility, and reversible macroscopic swelling/shrinking behaviour, biomimetic of plant cells.\(^3\)–\(^5\) They are also capable of actuating in response to a range of different stimuli including pH, heat, light, ionic strength and applied electric fields.\(^6\)–\(^11\) Hydrogel actuators are therefore desirable for their potential in applications such as soft robotics, artificial muscles, valves, and biomimetic devices etc.\(^12\)–\(^15\)

The earliest realization of a shape-changing 3D printed hydrogel (often called 4D printing due to the additional dimension of time) was by Gladman et al. in 2016 who, inspired by plant architectures and behaviour, extrusion printed a composite hydrogel ink in a 2D pattern that was able to fold into prescribed architectures when immersed in water.\(^16\) The ink was a combination of N,N-dimethylacrylamide, nanoclay, cellulose fibrils, glucose, glucose oxidase, and photoinitiator Irgacure 2959. The shape changes were programmed into the print structures \textit{via} the shear-alignment of the cellulose fibrils. Different alignments of the fibrils would have different swelling ratios, and shape changes were driven by patterning swelling asymmetries.

Since then, a variety of different hydrogel-based inks have been synthesized capable of printing stimuli-responsive shape-changing constructs.\(^17\) Thermally-actuated hydrogels are the most common due to the well-established thermo-sensitive properties of poly(N-isopropylacrylamide) (PNIPAm) networks but many other modalities have been explored. Chen et al. in 2019 3D printed a hydrogel octopus with
incorporated ferromagnetic Fe₃O₄ nanoparticles which was able to be actuated by a programmed magnetic field. Also, one example of an ionically conductive 4D printed hydrogel has been demonstrated. Here, authors used digital light processing (DLP) 3D printing to print electroactive hydrogel-based microstructures capable of gripping and bidirectional locomotion when placed in an electrolyte solution and an applied electric field. The migration of mobile ions creates concentration gradients in the prints which leads to osmotic pressure asymmetries and non-uniform swelling which generates shape change. Furthermore, light has also been used to reversibly actuate a 3D printed hydrogel. For example, the Bayley group in 2020 published a methodology where PNIPAm constructs, incorporated with photothermal gold nanoparticles (AuNP), were droplet printed to nanolitre resolution. Due to the surface plasmon resonance of AuNPs different print domains could be selectively and reversibly contracted via illumination with green light.

In this chapter, a novel shape-changing enzyme-mediated extrudable interpenetrating network (IPN) 3D-ink is described. The material is a printable combination of a calcium-crosslinked alginate hydrogel network and a smart synthetic PNIPAm/MBA hydrogel network. The IPN retains the reversible contractile thermosensitive (CTS) properties of PNIPAm single networks and the IPNs contractile speed and ability to repeatably contract is explored. Also investigated and discussed is the inks printability, its print dimension limitations, property tunability, internal structure, and mechanical properties.

3.2 Results and discussion

3.2.1 The IPN ink 3D printing protocol

Most IPN/DN materials require at least some form of cooling, degassing, or UV-crosslinking in their synthesis protocol. All these steps represent an additional synthetical complexity. They also limit the potential applications of the material, especially as cell-supporting inks, due to the additional stress caused by each process. The following protocol (Figure 3-1) is for a novel IPN ink which is printed and cured entirely at room temperature, without UV illumination, and under aerobic conditions.
The optimised (Appendix A) ink formulation is as follows: 3.5 wt % alginate, 0.75 wt% xanthan gum, 25 mM CaCl$_2$ pre-crosslinker, 15 wt % NIPAm, 7.5 mg/mL MBA, 0.5 mg/mL GOx, 50 ug/mL HRP, and 97 mM AcAc. The IPN network pre-gel components are present in the form of alginate chains, NIPAm monomer, and MBA crosslinker. These components were chosen due to their availability, bacterial-compatibility, established IPN cohesivity, and respective smart shape-changing properties.$^{22-25}$ The IPN gel is therefore a combination of an ionically crosslinked, with Ca$^{2+}$ ions, alginate network
and a covalently crosslinked, with MBA, PNIPAm network. This classes the IPN into a sub-genre of double network categories known as ionic-covalent entanglement (ICE) gels. Xanthan gum and a low concentration of calcium chloride, to lightly pre-crosslink the alginate, were added as viscosity modifiers to improve ink printability. The ink is primed with GOx and HRP which form the dual-peroxidase bi-enzymatic initiation system. Their enzymatic cascade, utilizing an AcAc radical mediator, will initiate the radical polymerisation of the PNIPAm/MBA network upon addition of glucose in an enzyme-mediated polymerisation (EMP) reaction (Figure 3-2).
Chapter 3: Preparation and Characterisation of a 3D-Printable Shape-Changing Enzyme-Mediated Interpenetrating Network

Figure 3-2: Schematic of GOx/HRP/AcAc bi-enzymatic initiation system which is capable of polymerising NIPAm monomers into PNIPAm polymers upon addition of glucose. If NIPAm is radically polymerised in the presence of MBA it will crosslink the PNIPAm chains to form 3D PNIPAm/MBA hydrogels.

The IPN printing protocol follows a simple two-step procedure. First, the ink is extrusion printed using a pneumatic printhead into a custom 3-dimensional (3D) construct that has been made using computer-aided design (CAD). These pre-gel constructs are then crosslinked/cured via a wash step where the prints are immersed in an aqueous solution containing CaCl$_2$ and glucose (Figure 3-3). The glucose initiates the radical EMP of the PNIPAM/MBA network as the Ca$^{2+}$ ions concomitantly crosslink the alginate ink component giving rise to a robust IPN. To promote rapid curing (and offset any diffusional
loss of pre-gel elements from out of the print during the wash step) high concentrations of CaCl$_2$ (300 mM) and glucose (5 mg/mL) were used. This glucose concentration was to ensure sufficient glucose for optimal GOx activity.$^{26}$ Moreover, as an aqueous 300 mM CaCl$_2$ solution is 1.2 wt% Ca$^{2+}$ cations and the standard ink formulation is only 3.5 wt% alginate, a crosslinking wash volume to print volume of greater than three was considered a vast excess of cations to saturate available crosslinking sites. Typically, crosslinking wash volume : print volume ratios of greater than 10 were employed. Post-crosslinking, the final material was tough enough to handle, flexible, and self-supporting. Printing dimensions of the millimetre/centimetre scale were readily achievable, and prints maintained structural integrity for multiple weeks.

3.2.1.1 The effect of the wash crosslinking step on shape fidelity

As 3D printing is a patterning technology, shape fidelity is a key parameter to describe the performance of any 3D ink. Alginate is known to contract when crosslinked with a high enough concentration of anions (due to the elastic forces that resist swelling created in the gel network by ionic crosslinking),$^{27}$ and covalently-crosslinked hydrogels in general are known to expand when being placed in water (due to their hydrophilic nature). Therefore, to quantify how well cured IPNs conserved their pre-crosslinked dimensions, print dimensions were measured before and after crosslinking (Figure 3-4). It was found that, converse to anionically crosslinked alginate single networks, the IPN ink expanded slightly upon curing in a CaCl$_2$ + glucose solution (4% along each axis as measured by electronic callipers (Figure
3-4B)). However, this expansion was consistent for all axes and feature dimensions. Therefore, the original print aspect ratios were conserved, and the shape change was very predictable. It was also observed that the crosslinked IPN conserved the reversible CTS properties of PNIPAm single networks. The contracted construct length over the original \((L_T/L_{RT})\) was 0.67 ± 0.03 for \(n = 3\) (see Section 3.2.4 for further details).

Two control conditions were also performed where prints were immersed in an aqueous solution containing either \(\text{CaCl}_2\) (300 mM) or glucose (5 mg/mL) to selectively crosslink the alginate or drive PNIPAm/MBA network formation (Figure 3-4C and D) respectively. As expected, the calcium-crosslinked control behaved like a low-weight percentage (3.5 wt%) alginate solution should when crosslinked with a very high anion concentration (300 mM). It contracted slightly upon curing, ca. 10% along each axis, and demonstrated no observable thermosensitive response. This lack of TS was anticipated as the PNIPAm/MBA pre-gel elements are only present in the ink as small monomers/molecules and without a glucose initiator they would be expected to simply wash out leaving behind an alginate scaffold supplemented with XG.

The glucose-crosslinked control demonstrated very different behaviour. The print expanded greatly during the wash step, ca. 44% along each axis, but (like the fully crosslinked IPN) expansion was consistent for all axes and feature aspect ratios were conserved. This expansion disparity between the glucose-crosslinked print and the fully crosslinked print, and the contracting nature of the calcium-crosslinked print, suggests that the calcium crosslinking plays an important role in containing the PNIPAm/MBA network formation within the original print confines. The thermosensitive contraction of the glucose-crosslinked prints was also attenuated \((L_T/L_{RT} = 0.77 ± 0.04, n = 3)\) as compared to the fully crosslinked prints which also suggests some of the NIPAm monomers were fully evacuated during the wash step before they could be incorporated into the propagating PNIPAm network (as a higher NIPAm wt% is related to improved CTS)\(^2\).
Chapter 3: Preparation and Characterisation of a 3D-Printable Shape-Changing Enzyme-Mediated Interpenetrating Network

Figure 3.4: IPN wash crosslinking step experiments. A, STL file (top-left), visualized G-code (top-right), and photographs of the dumbbell print used in the experiments (bottom). B, Photographs of a dumbbell print post-curing in a CaCl₂ and glucose solution before and after heating (60°C, 30 min). Average swelling ca. 4% along each axis during crosslinking. \( L_T/L_{RT} = 0.67 \pm 0.03 \) for \( n = 3 \). C, Photographs of a dumbbell print post-curing in a CaCl₂ solution before and after heating (60°C, 30 min). Print has contracted ca. 10% along each axis during crosslinking. No significant contraction of the crosslinked network was measured after heating. D, Photographs of a dumbbell print post-curing in a glucose solution before and after heating (60°C, 30 min). Print has swollen ca. 44% along each axis. \( L_T/L_{RT} = 0.77 \pm 0.04 \) for \( n = 3 \). Major and minor gridlines in all images demarcate 10 and 1 mm respectively.
3.2.1.2 Dimension limitations of the IPN ink wash crosslinking protocol

Due to the nature of the IPN wash curing step, where crosslinking elements/initiators diffuse into the surfaces of printed structures from an immersing solution, the IPN material was expected to have a depth-dependent composition. In other words, the final IPN structure should be heavily influenced by the rate of travel of each network's propagation into the print. If one of the alginate or PNIPAm networks formed significantly faster, then its presence would template/restrict the other i.e. in order for early-stage interlocking, network formation must outcompete phase separation. Also important during the wash step would be the overall rate of IPN formation vs. the diffusion rate of uncured pre-gel elements exiting the print. In summary, it was predicted that certain small or thin print features wouldn’t be able to contain the PNIPAm pre-gel elements during the wash crosslinking step and that certain large features would struggle to crosslink homogenously. These size boundaries would then represent the dimension limitations of the IPN wash crosslinking step for homogenous structures.

For an initial experiment, a dumbbell print (identical to those printed in Figure 3-4) was bisected (Figure 3-5). The cut face displayed no observable cavities, inhomogeneities, or lamination artifacts and the print appeared solid and monolithic. This suggests the IPN synthesis is unproblematic at these size regimes/aspect ratios. The absence of cavities and delamination also implies excellent inter-layer adhesion post-crosslinking. This inter-layer adhesion is likely due to the alginate network only being lightly crosslinked pre-printing and that PNIPAm’s monomeric pre-gel elements are too small to be affected by shear phenomena. Therefore, you would expect good inter-layer curing.
Chapter 3: Preparation and Characterisation of a 3D-Printable Shape-Changing Enzyme-Mediated Interpenetrating Network

Figure 3-5: Photographs of dumbbell prints being bisected. The cut face (bottom-right) appears solid and monolithic and displays no observable cavities, inhomogeneities, or lamination artifacts.

To test thinner print dimensions than those presented in the dumbbell print, a crosshatch print was designed (Figure 3-6) where the individual lines were the width of the ink as extruded through a 20G blunt needle under typical printing conditions (ca. 1 mm diameter). Initially, the standard IPN ink was printed under standard conditions. The resultant crosslinked construct only demonstrated negligible CTS. It did however demonstrate clear shape integrity and an observable increase in opacity which is indicative of calcium crosslinking in alginate gels. It therefore appears that an alginate network formed successfully, but the print features were too thin to successfully encapsulate the PNIPAm pre-gel components long enough for them to form a complete, robust PNIPAm network capable of augmenting the IPN with observable shape-changing properties. It is possible small domains or oligomers of PNIPAm/MBA gel formed, but that they were not interconnected or were simply too small to interdigitate with the alginate. As a follow up experiment, the crosshatch print was repeated with an ink containing 8x the HRP concentration of the standard formulation (Figure 3-6). This was to confirm if this would increase radical production rate enough to successfully cure the PNIPAm/MBA network within these size regimes. However, no observable differences were noted.
Chapter 3: Preparation and Characterisation of a 3D-Printable Shape-Changing Enzyme-Mediated Interpenetrating Network

Figure 3-6: Photographs of crosshatch prints printed at room temperature using a 20G blunt needle with the standard IPN ink (above) and an alternate ink with 8x [HRP] (below) before and after heating (60°C, 30 min). Both prints were crosslinked in 300 mM CaCl₂ + 5 mg/mL glucose. Measurements were rounded to the nearest 0.5 mm and taken from opposite corners of the print. Both inks demonstrated negligible CTS. Gridlines in all photos demarcate 10 mm.

To observe the consequences of wash-crosslinking larger prints, a 12x12x10 mm cuboid was printed (Figure 3-7). Post-crosslinking there were observable distortions in the print shape as various faces in the print had either slightly puckered or bulged. Upon bisecting the print and observing the cut face, clear differences were notable compared to the dumbbell print in Figure 3-5. The cuboid print displayed inhomogeneities in density and opacity, as well as a central cavity. The crosslinking methodology clearly struggled to homogenously cure the IPN ink at these print depths, to the extent where regions too far from the surface failed to cure completely.
Chapter 3: Preparation and Characterisation of a 3D-Printable Shape-Changing Enzyme-Mediated Interpenetrating Network

Figure 3-7: Photographs of a 12x12x10 mm cuboid print printed using the standard IPN ink under standard conditions: A, Immediately post-printing. B, Post-wash crosslinking step. Subtle variations in shape are observable compared to A, various cube faces have either bulged or puckered. C, Being bisected. The cut face of the print (D) displays clear inhomogeneities in density and opacity. Also visible is a central cavity. Major and minor gridlines in all images demarcate 10 and 1 mm respectively.

The optimum print feature dimension regime was therefore assumed to be between 2-8 mm in size and all prints that were performed within this regime cured homogenously. This limitation is not a restriction on overall print size however, as a large library of shapes can be made by printing a shell structure with an infill pattern. This is in fact the archetypal printing methodology for plastic extrusion-based 3D-printing. Figure 3-8 demonstrates two different centimetre (cm) scale prints designed to demonstrate this hypothesis. The first was a 32x32x5 mm square printed using a shell with a crosshatch infill pattern with 2mm wide struts. The final crosslinked construct matches the shape fidelity, and CTS performances, of the smaller dumbbell prints. To explore taller prints a second construct was printed as a buttressed 16x16x17 mm tower with a central channel. This central channel supports optimal crosslinking wash feature dimensions and struts are again kept at 2 mm wide. Once again shape fidelity
was excellent. Both cm scale prints were also again robust enough to handle, and self-supporting post-crosslinking.

Figure 3-8: STL files and photographs of cm scale IPN prints. A, A 32x32x5 mm square printed via a shell and crosshatch infill structure. Shape fidelity, and construct CTS performance match the dumbbell prints. B, A 16x16x17 mm buttressed tower print, designed with a central channel to support optimal crosslinking wash feature dimensions. Print confirms structures over 1 cm tall can be crosslinked and demonstrate excellent height fidelity. Once crosslinked IPNs of this size are still self-supporting (bottom-right). Major and minor gridlines in all images demarcate 10 and 1 mm respectively.
Chapter 3: Preparation and Characterisation of a 3D-Printable Shape-Changing Enzyme-Mediated Interpenetrating Network

It is important to note that the larger prints, that contained central channels due to containing an infill pattern, required gentle agitation during crosslinking. Specifically, slow stirring every 10 minutes for the first hour post-immersion. Large prints that were crosslinked unagitated demonstrated non-uniform CTS performance (Figure 3-9). The struts appeared to bulge during the heating phase and defined print features like Figure 3-9’s support buttresses became more amorphous upon contraction and its central pore fully closed. However, prints crosslinked with gentle agitation contracted very uniformly and constructs like the buttressed tower retained its print feature dimensions, such as it’s open central pore, after heating. This is probably due to the higher concentration of surfaces inside the print as well as them being less available to the crosslinking solution. Without agitation these surfaces would absorb a lower concentration of crosslinking elements vs. the outer print surfaces leading to a less homogenous IPN composition and therefore non-uniform CTS performance.

Figure 3-9: Photographs of the buttressed tower print (shown in Figure 3-8) before and after heating (60°C, 30 min) post-crosslinking (300 mM CaCl₂, 5 mg/mL glucose). The above photographs display a print that was crosslinked unagitated in solution, after heating the struts appear to have contract non-uniformly forcing the strut walls to bulge and the central channel to close. In the below photographs, where crosslinking occurred with gentle agitation, contraction is more uniform. The central channel has remained open and print features in general, such as the buttress struts, stay defined and have retained their dimension ratios. Major and minor gridlines in all images demarcate 10 and 1 mm respectively.

3.2.1.3 Printing hollow IPN constructs

To discover the effects of accelerating the formation for one of the IPN’s networks relative to the other an ink was printed with 4 mg/mL HRP (8x concentration in standard formulation, Figure 3-10). All other ink components remained the same and the standard printing protocol and crosslinking wash was used. To allow a direct comparison, the same dumbbell construct used in Figure 3-4 was printed. Unsurprisingly, shape fidelity post-printing was unaffected as HRP concentration shouldn’t affect
printability due to it being neither a viscosifier nor at a high-enough weight percentage to be considered a support material. However, post-crosslinking the print demonstrated much larger swelling vs. the standard ink (24% increase along each axis as compared to 4%). Like the standard ink this expansion was repeatable and consistent for all axes so specific print geometries were still designable by anticipating the swelling. Also noticeable, upon bisecting the print post-crosslinking, was a hollow cavity. This can be thought of as an additional printing dimension, where a bespoke shape is printed, and which then permanently evolves during crosslinking into a larger hollow version of itself; identical conceptually to irreversible 4D printing.  

Chapter 3: Preparation and Characterisation of a 3D-Printable Shape-Changing Enzyme-Mediated Interpenetrating Network

Figure 3-10: 3D printing hollow IPNs using the standard ink formulation and preparation except for 0.4 mg/mL HRP. A, STL file and photographs of dumbbell print pre- (above) and post-crosslinking (below). Clear swelling is observable post-crosslinking; however, it is consistent for all axes (ca. 24% increase as compared to original length). B, Photographs of print being bisected, and of cut face. Print is robust enough to handle and contains a hollow uncured core. C, Print CTS performance confirms the presence of a PNIPAm/MBA network, $L_T/L_{RT} = 0.58 \pm 0.04$ for $n = 3$. Major and minor gridlines in all images demarcate 10 and 1 mm respectively.
Chapter 3: Preparation and Characterisation of a 3D-Printable Shape-Changing Enzyme-Mediated Interpenetrating Network

It’s possible that the shallow curing depth of the ink is due to the increase in HRP concentration causing a reduction in glucose depth penetration (due to its increased consumption). This would lead to radical production, and therefore also PNIPAm/MBA gel precursor consumption, being monopolised near print surfaces. This rapid PNIPAm gel corona formation would decelerate calcium ion diffusion into the gel attenuating alginate crosslinking as well, and because PNIPAm gels are hydrophilic in nature the print would also begin to swell. This would prevent the ink’s alginate component from crosslinking all the way through the ink, leading to a hollow construct. As the print’s CTS performance is also significantly improved vs. the standard ink (Figure 3-10 C, \( L_T / L_{RT} = 0.58 \pm 0.04, n = 3 \) [vs. \( L_T / L_{RT} = 0.67 \pm 0.03 \)]) it would also seem this ink is better at encapsulating the EMP components and substrates before they diffuse away. It could also be that the hollow nature of the constructs simply allows for more contraction to occur, or that the attenuation of alginate crosslinking has caused some of it to diffuse out of the print.

This ink augmentation unlocks categories of construct shapes that would be unprintable under the standard preparation due to their bridging requirements and inner surface unavailability for the wash crosslinking step. However, the post-crosslinking swelling, although predictable, does restrict the design of certain print features and their sizes. This could be overcome by co-printing the ink with the standard formulation. As the crosslinking wash is the same as in the standard IPN printing protocol it should be possible crosslink both inks together in the same step. It’s also possible that other simple variations in the standard IPN ink’s concentrations would result in similarly advantageous printing capabilities and that by using a library of related inks much more post-crosslinked construct geometries become printable.

### 3.2.2 IPN pre-gel rheology

Extrudable 3D inks must be shear-thinning so that they flow uniformly and retain their shape post-deposition. To find out if the IPN ink was shear-thinning, and to confirm some of the ink component functions, the ink and some variations of it were characterized by rheometry (Figure 3-11). The IPN ink was indeed found to have shear thinning behaviour, with viscosity decreasing exponentially with applied shear. The flow index was calculated from Figure 3-11A’s curve using the power-law fluid
model (Section 2.5.1) and was calculated as 0.7103, far below a Newtonian fluid value of 1 and characterized as shear-thinning/pseudoplastic. Not unsurprising as calcium-crosslinked alginate gels in general are established shear-thinning materials.\(^{31}\)

![Rheology](image)

**Figure 3-11**: Rheology of IPN ink and various controls. Blue, orange, green, red, purple, and brown respectively indicate data for the standard IPN ink, the standard ink without XG, the standard ink without CaCl\(_2\) pre-crosslinking, the standard ink without XG or CaCl\(_2\) pre-crosslinking, the standard ink without NIPAm monomer, and the standard ink solvated in minimal media. 

### A

- Shear viscosity (Pa s) vs. shear rate (s\(^{-1}\))

### B

- Graph plotting \(\log (\mu/K)\) vs. \(\log (\dot{\gamma})\) to solve for gradient \(n\) (the flow behaviour index) where \(K\) is the flow consistency (i.e., the viscosity when \(\dot{\gamma} = 1\)), \(\mu\) is the viscosity, and \(\dot{\gamma}\) is the shear rate.

To explore how the ink behaved with and without certain components, rheological characterisation was carried out on 5 other inks. The IPN ink without XG, without CaCl\(_2\), without XG and CaCl\(_2\), without NIPAm, and solvated with minimal media (M9 minimal media, Sigma Aldrich, M9959) were all
characterised. The later experiment was performed due to the basic requirements of needing to print cells with growth/supporting media which typically contain monovalent cations. If the IPN ink was to have potential cell printing applications, it was important to ascertain if typical cell growth media components would affect printability. Monovalent cations were anticipated to possibly effect printability due to occupying, and therefore interrupting, alginate pre-crosslinking sites.\textsuperscript{32,33}

Table 3-1: Power-law constants values for IPN ink and various controls. $K$ is the flow consistency index (i.e., the viscosity when shear rate ($\dot{\gamma}$) = 1) and $n$ is the flow behaviour index. Also tabulated are the $R^2$-squared values for $n$.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>$K$</th>
<th>$n$</th>
<th>$R^2$ for $n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPN ink</td>
<td>68.487</td>
<td>0.7103</td>
<td>0.9966</td>
</tr>
<tr>
<td>IPN ink w/o XG</td>
<td>45.158</td>
<td>0.465</td>
<td>0.9691</td>
</tr>
<tr>
<td>IPN ink w/o CaCl$_2$</td>
<td>4.2319</td>
<td>0.5005</td>
<td>0.9947</td>
</tr>
<tr>
<td>IPN ink w/o XG or CaCl$_2$</td>
<td>2.0769</td>
<td>0.4328</td>
<td>0.9959</td>
</tr>
<tr>
<td>IPN ink w/o NIPAm</td>
<td>101.19</td>
<td>0.7303</td>
<td>0.9985</td>
</tr>
<tr>
<td>IPN ink with minimal media</td>
<td>10.812</td>
<td>0.6212</td>
<td>0.9969</td>
</tr>
</tbody>
</table>

All the inks power-law constants are tabulated in Table 3-1. The flow behaviour index ($n$) value is of interest to printability because the further $n$’s deviation from 1 the more non-Newtonian a material behaves i.e., the more its viscosity is affected by strain. Values of $n$ are below 1 describe pseudoplastic (shear-thinning) materials, values of $n$ are greater than 1 describe dilatant (shear-thickening) materials. In the case of pseudoplastic materials ($n < 1$), the greater the deviation from 1 means the more liquid the material behaves under applied shear and so the slower it recovers to a solid post-printing. This affects shape fidelity. Out of all the inks characterised the standard ink has the second highest $n$ value, only the ink with NIPAm removed is higher. This is because solvated NIPAm monomers are not polar molecules and reducing a polar component of the ink by 15 wt% interrupts the strong intermolecular forces between the remaining water molecules, and between the remaining water molecules and the solvated alginate chains (which are naturally anionic). This reduces viscosity, reflected in the much lower $K$ value. Predictably, removing either or both of the ink viscosity modifiers, XG and CaCl$_2$,
resulted in lower $K$ and $n$ values. Removing CaCl$_2$ had a bigger impact on viscosity and removing XG having a bigger impact on $n$. Also, the monovalent ions in minimal media did appear to severely impact viscosity causing it to be unprintable ($K$ was reduced from 68.487 to 10.812, Table 3-1; Figure 3-12).

![IPN ink](image1) ![IPN ink with minimal media](image2)

*Figure 3-12: Photographs of IPN ink with and without minimal media after being inverted for 1 minute. The ink with minimal media is much reduced in viscosity causing it to flow under gravity, whereas the IPN ink does not.*

### 3.2.3 Printability assays

To explore the printability of the IPN-producing ink, two assays were performed. They specifically looked at the uniformity of filament deposition and print features, and how well the ink supported loads before crosslinking. Both tests are based on archetypal ink performance assessments. The printing uniformity assay is based on typical model crosshatch/lattice prints, often used to quantify print feature fidelity but here utilized to assay uniformity.$^{34,35}$ The filament collapse assay is replicated from an experiment by Lewis et al. It involves printing filaments over gaps of increasing distances and is used in multiple papers on printability.$^{36-38}$

#### 3.2.3.1 Printing uniformity assay

Uniformity was assessed by printing a 1-layer regular crosshatch structure and analysing the uniformity of its pore areas and line widths (Figure 3-13). Multiple prints demonstrated excellent uniformity with narrow pore area distributions, the average relative standard deviation within all the samples was 8%. All samples also display a bimodal distribution of pore areas. This is an artifact of the printing pathway.
All the y-axis lines were printed before the x-axis ones. As the needle prints the x-axis lines it is rastered through the previously printed y-axis lines. This leads to some minor “castling” of the ink at alternating crosshatch nodes causing fluctuating filament widths along the print’s x-axis lines. This is visible in the right image of Figure 3-13B, as is the two populations of pore area sizes in their alternating columns (the narrower pore regime is highlighted in yellow). The black line in Figure 3-13C indicates the pore area for the crosshatch’s STL file (2.36 mm²). All the measured pore areas are below this due to the ink spreading into the pores slightly post-printing, this is a common shortcoming of hydrogel-based 3D inks. 

Figure 3-13: Ink uniformity assay. A, Photograph of a printed crosshatch. Printed with a blunt 18G needle. Background gridlines demarcate 10 mm. B, A composite image of a printed crosshatch made up of a tile-scan of optical microscopy images. Alternate columns are highlighted that display the narrower pore regime in the bimodal distribution of pore area sizes. Scale bar = 5 mm. C, Histogram of pore areas for three repeats of the crosshatch print. The black line indicates the crosshatch STL pore area = 2.36 mm². D, Histogram of filament widths for three repeats of the crosshatch print. The black line indicates the diameter of an 18G needle = 0.838 mm.
Filament widths also displayed narrow distributions (Figure 3-13D), with an average relative standard deviation within all the samples of 4%. Some of the samples displayed bimodal distributions due to the exact same influences as discussed for the bimodal pore area distributions. The black line on Figure 3-13D indicates the diameter of the 18G needle used to print the crosshatch (0.838 mm). The average increase in line width from this diameter (the spreading ratio) was 0.13 mm (16%). Although this number is in part due to the intrinsic material properties of the ink, the spreading ratio is also largely a function of the printing pressure (i.e., the extrusion rate) and is also greatly affected by printing substrate hydrophilicity/hydrophobicity. It is also true that printing pressure can be used as a printing parameter to tailor filament width and as this assay isn’t optimized for intrinsic spreading ratio quantification this is not a reliable number. This is generally understood in 3D printing hydrogels that filament widths aren’t equal to needle gauge, although they are an important factor.

3.2.3.2 Filament collapse test

The filament collapse test results are shown in Figure 3-14. The test platform pillars, displayed in Figure 3-14A, had heights of 10.0 mm and increasing gap distances of 1.0, 2.0, 4.0, 8.0, and 16.0 mm left to right. Alongside the IPN ink, two other commercial bioink formulations were tested for comparison (CELLINK START and CELLINK Bioink). All the inks were capable of bridging the final 16.0 mm gap. CELLINK START possessed the lowest overall collapse area of the three inks. However, this ink
Chapter 3: Preparation and Characterisation of a 3D-Printable Shape-Changing Enzyme-Mediated Interpenetrating Network

is designed purely to be a structural material, and not perform any other type of function (e.g. biocompatibility). It is therefore optimised purely for printability and was expected to have the best performance. However, the CELLINK Bioink is CELLINK’s universal bioink formulation and is designed with the caveat of having to support cells. This therefore represents a more applicable comparison for the IPN ink, and their filament collapse performances were not statistically significantly different. In fact, the IPN ink outperforms CELLINK Bioink in total collapse area up to the 8 mm gap ($3.66 \pm 0.29 \text{ mm}^2$ vs. $4.92 \pm 0.39 \text{ mm}^2$ [n=3 for both]). All the inks also display visually unobservable collapse in pillar distances up to 4.0 mm. This demonstrates the bridging capabilities of the IPN ink are closely comparable to modern state-of-the-art bioink formulations i.e., it is as good at supporting its own weight pre-crosslinking and should be able to achieve analogously complicated print structures without the need for support materials.

3.2.4 IPN thermosensitive contractile properties

To investigate how well the printed IPN conserves the CTS smart properties of PNIPAm single networks a series of assays were developed. To ensure the results would be commensurate with typical print structures, all CTS assays were performed with 15x3x3 mm prints (Figure 3-15A) as this construct was well within the known optimal print dimensions for efficient crosslinking.

3.2.4.1 TS repeatability assay

Initially, the IPN prints were tested to see how they would elastically contract under repeated heat cycles. Prints were heated by being placed in a 60°C oven for 30 minutes, their volume was measured immediately upon removal, and they were then allowed to relax overnight to be re-measured the next day. The first heating cycle led to a contraction to $38 \pm 5\%$ of the prints’ original volumes (Figure 3-15). However, these then recovered to only $90 \pm 10\%$ of the original volume. This decrease in recovered volume continued by an average of 6% per cycle until after the third temperature cycle where the prints settled into fluctuating between $76 \pm 2\%$ and $31 \pm 2\%$ the original printed volume.
This decrease in recovered volume for the first 3 cycles wasn’t expected. PNIPAm single networks possess completely repeatable shape-changing properties and PNIPAm-based IPNs exist in the literature that emulate this behaviour.\textsuperscript{23,41} It’s indicative of an inhomogeneous or non-monolithic PNIPAm network formation. Possibly due to the room temperature curing step, as it’s well established for single network PNIPAm networks that reaction temperatures higher than 24.5°C lead to inhomogeneous networks.\textsuperscript{42} Also, double networks in general are synthesized at low temperatures as it better preserves network precursor uniformity during the curing steps.\textsuperscript{43} However, low temperatures
would be incompatible with the EMP in the IPN ink as HRP’s efficiency is much reduced at low
temperature.\textsuperscript{44} Although, as the reduction in the IPN’s recovered volume seems to plateau after the third
cycle it may just be that the material needs to mature over the first few cycles into a stable network
configuration (polymer chain networks are known to be susceptible to contraction/expansion-induced
realignment).\textsuperscript{45,46} The contraction of the alginate network in a CaCl$_2$ solution may also be introducing
new ionic crosslinking sites, which would inhibit full recovery.

3.2.4.2 Alginate concentration’s effect on TS

![Figure 3-16: Plot showing the contractile thermosensitivity performance for 3 different types of IPN prints over 1 heat cycle (60°C oven for 30 minutes). The inks used were the standard IPN ink (with 3.5 wt% alginate) and identical inks but with 2 and 5 wt % alginate. Values represent mean averages ± standard deviation for n=6 experimental repeats.]

To confirm the reported effects of PNIPAm : alginate ratio on IPN CTS performance,\textsuperscript{22} a single heating
cycle was performed on IPN inks containing 1.5 wt% higher and lower alginate as compared to the
standard formulation (Figure 3-16). As expected, the CTS performance was improved by reducing the
alginate wt% of the ink and vice versa, demonstrating that the presence of the alginate network
attenuates PNIPAm’s CTS. It is again possible that these inks are co-printable due to their common
crosslinking step and by varying the alginate components wt% you could pattern asymmetric
Chapter 3: Preparation and Characterisation of a 3D-Printable Shape-Changing Enzyme-Mediated Interpenetrating Network

contraction into IPN prints. One caveat would be that lowering the alginate wt% of the ink would impact printability as it’s the major structural component pre-crosslinking (Figure 3-17).

3.2.4.3 IPN Print thermosensitivity kinetics assay

To measure the kinetics of the contraction, the IPN prints were placed in a 60°C water bath and had their volumes measured at regular time intervals (Figure 3-18). Over 30 minutes the IPN prints contracted dramatically to ca. 22 ± 3% their original volume. The initial contraction rate was fast, with a contraction to 73 ± 4% their initial volume in the first minute and a contraction to 33 ± 2% their initial volume by the first 5. 50% contraction was achieved in 3 minutes. The data fits an exponential decay with rate constant -0.01 and a pre-exponential factor of 75.89. This overall contraction profile behaviour is typical of PNIPAm-based IPNs and is a function of their thermal conductivity (as the transfer of heat

Figure 3-17: Printing with the standard IPN ink formulation (A, 3.5 wt% alginate) and a lower wt% alginate formulation (B, 2 wt% alginate). The lower alginate ink displays poorer corner/feature definition, increased slumping, and a higher spreading ratio. Major and minor gridlines in all images demarcate 10 and 1 mm respectively.
from the gel surface to its bulk is a determinative factor).\textsuperscript{47} However, the specific function constants will be particular to the IPN print shape as surface-to-volume ratio, and the distance interstitial water would have to travel to evacuate, is a key parameter in a gels contraction speed.

It should be noted that the contracted volume, after 30 minutes of heating, in the kinetics assay is higher vs. the repeatability assay as directly submerging the print in a pre-heated water bath is a more efficient heating method vs. simply placing the immersed prints in an oven.

![Figure 3-18](image)

*Figure 3-18: Plot for volume contraction of a printed construct vs. time spent in a 60°C water bath. Values represent mean averages ± standard deviation for n=3 experimental repeats. The data fits an exponential decay with rate constant -0.01 and a pre-exponential factor of 75.89.*

### 3.2.5 Cryo-scanning electron microscopy of IPN prints

To get some insight into the internal structure of the IPN prints, samples were prepared and imaged using cryo-scanning electron microscopy (CryoSEM). Hydrogels can be challenging to image however, as they must be dehydrated without disturbing their “true” solvated structure (introducing eutectic artifacts from ice nucleation).\textsuperscript{48} Initially, prints were incrementally dehydrated in ethanol over several hours before being critically point dried, sputter coated and then imaged via SEM (Figure 3-19A) but
the images taken were featureless (amorphous textured solids with no evidence of a porous network). This is because of the high weight percentage of the hydrogel, 19.25 wt%, which is unconducive to ethanol dehydration and samples must be free of water for critical point drying to be effective. The next print samples were imaged using CryoSEM, considered the gold standard in imaging hydrogels and soft biological samples, and much improved micrographs were resolved (Figure 3-19B). The imaged structures resembled those in the literature of IPNs and displayed a tightly crosslinked continuous porous network.

Figure 3-19: Electron micrographs of standard IPN ink prints. A. Print was prepped for imaging via an ethanol wash before being critically point dried, sputter coated, then imaged with SEM. B. CryoSEM.

To investigate how homogenous the IPN prints were, they were imaged at a fracture face near their core (Figure 3-20A) and near their surface (Figure 3-20B). These images confirm that the IPN prints do have depth-dependent structures. Immediately noticeable when comparing A and B is the seemingly higher...
degree of crosslinking/smaller pore structure the print core seems to have vs. the surface. This could be due to better indigitation between the two networks at the surface templating a more open structure. Then, as the PNIPAm formation is expected to penetrate faster than the alginate crosslinking (the kinetics of propagating radicals vs. the diffusion of calcium ions into alginate), towards the print’s cores the networks become more segregated resulting in tighter crosslinking.

Analogous single network gels were prepared to compare to the IPN print images. Figure 3-20C and D images are of prints where the ink contained no HRP or GOx and were crosslinked with CaCl$_2$ only. They will therefore be 3.5 wt% alginate single networks, the only difference between the two is that D was printed with 0.75 wt% XG and C was not. Image E is of a casted PNIPAm/MBA single network cured via HRP-GOx EMP, using the exact same network precursor and initiation system weight percentages and molar ratios as used in the standard IPN ink. Image F is of a standard IPN print that has then been treated with multiple washes of 200 mM ethylenediaminetetraacetic acid (EDTA) over several days. EDTA is able to chelate the calcium cations that are crosslinking the alginate, and therefore selectively dissolve that network and leave the PNIPAm single network behind.$^{51}$

Interesting to notice between images C and D is the observation of aggregates within C’s network (this is looked at in closer detail in Figure 3-21). Image E shows that absent of the alginate network the PNIPAm forms a much more closely crosslinked gel. Therefore, the alginate is likely templating these larger pore formations in the IPN. Which would explain why the IPN displays an unusually strong CTS for a PNIPAm network with high crosslinking concentration, as larger pores allow increased contraction. Image F seems to confirm this as dissolving the alginate network from the IPN post-crosslinking reveals a much more open network as compared to E.
Further images of the alginate networks without and with XG (Figure 3-21A and B respectively) confirm previous observations. The alginate, when printed without XG, forms these aggregates of highly concentrated material (highlighted and enlarged in the image [Figure 3-21A, left and right respectively]). This could be due to lightly pre-crosslinking the alginate (final concentration 25 mM) via the addition of a highly concentrated CaCl$_2$ solution (300 mM). As CaCl$_2$ is a highly soluble form of calcium and will readily dissolve into its component ions, it crosslinks alginate very fast. $^{52}$ Therefore, areas of the alginate that were immediately in contact with the 300 mM CaCl$_2$ solution when it was added will crosslink disproportionately higher before the ink was mixed in the dual asymmetric centrifuge (DAC) moments later. $^{53}$ The DAC could then just be mixing in domains of highly crosslinked alginate with domains of less crosslinked alginate. Overall, the ink would form a viscous pre-crosslinked solution but with non-uniform calcium crosslinking. As XG is a viscosifying agent, its presence could be slowing down the diffusion of calcium ions into the ink during pre-crosslinking, allowing more time for the CaCl$_2$ solution to be mixed in more homogenously before its absorbed and resulting in much the less pronounced aggregating of the final material as seen in Figure 3-21B.
Additionally, Figure 3.21B images display a more open pore structure vs. A, so it’s possible XG is also acting as a porogen.

![Figure 3.21](image)

Figure 3.21: CryoSEM of different alginate single network prints. A, Standard IPN ink print without XG, HRP or GOX that has only been crosslinked in 300 mM CaCl$_2$. Therefore a 3.5 wt% alginate print. Right image is an enlargement of the area in left image highlighted with a white square. B, Standard IPN ink print without HRP or GOX that has only been crosslinked in 300 mM CaCl$_2$. Therefore a 3.5 wt% alginate + 0.75 wt% XG print.

### 3.2.6 Compression testing of the IPN gel

One of the main advantages of IPN hydrogels is the additional mechanical reinforcement provided by the concert of two networks. As such, compression testing was performed to explore how the IPN performs under compressive loads. For comparison alongside the standard IPN, 15 wt% PNIPAm single networks crosslinked with 7.5 mg/mL MBA, and 3.5 wt% alginate single networks supplemented with 0.75 wt% XG, were also tested. As calcium-crosslinked alginate gels mechanical properties are highly
dependent on crosslinking concentration and time, alginate single network samples were treated identically to IPN samples.\textsuperscript{54}

Samples were prepared as 12 x 7 mm (d x h) cylinders. This size regime was chosen to ensure the results would be relevant for optimal print dimensions, but they were cast to avoid the inhomogeneities produced by printing. Also, as hydrogels have a complex stress-strain curve, strain moduli were taken using the first 10% strain as standard.

The Youngs moduli for all 3 hydrogels are shown in Figure 3-22. As anticipated, combining the two networks yielded a higher compressive stiffness. The IPN gave a Young’s modulus of 63 ± 15 kPa, which was significantly higher than both the PNIPAm and alginate single networks (26 ± 6 kPa and 40 ± 6 kPa, P<0.001 and P<0.01 respectively). However, this Young’s modulus is far below what’s achievable with certain literature examples of PNIPAm/alginate IPNs, which have moduli in the low MPa, but these examples tend to be cured at low temperature to promote network interdigitation, as well as being non-printable formulations.\textsuperscript{47} This is further evidence that the room temperature crosslinking wash step doesn’t produce perfectly homogenous IPNs.
Chapter 3: Preparation and Characterisation of a 3D-Printable Shape-Changing Enzyme-Mediated Interpenetrating Network

Observing the samples post-compression suggests the IPN retains some of the elastic recovery of PNIPAm single networks (Figure 3-23). All the PNIPAm samples were able to be compressed by 56% of their height multiple times (at this strain the 8N load cell failsafe would be activated) with no perceptible loss in height (i.e., > 0.1 mm, as measured by electronic callipers). The IPN samples observably recovered from 40% strain but mechanical compression to failure experiments performed on the IPN samples found an average ultimate compressive strength of 54 ± 3.6 kPa at an average strain of 46 ± 3% (Figure 3-23F) indicating there was some loss in elastic limit. Comparing stress vs. strain graphs for compression cycles on PNIPAm and IPN samples up to these limits (56 and 40% strain respectively) shows closely repeatable behaviour, reinforcing the theory that both the materials suspected elastic limits lie above these strains. However, proper assessment of traditional materials (e.g., metals) typically perform many magnitudes more compression cycles to confirm this. Conversely to both the IPN and PNIPAm samples, the alginate samples were always irreversibly deformed. Also notable in the stress-strain curves of all the hydrogels is a stiffening effect at higher strains. This

Figure 3-22: Youngs moduli for different hydrogels as measured by unconfined compression testing: a 15 wt% PNIPAm single network, a 3.5 wt% alginate single network supplemented with 0.75 wt% XG, and the standard IPN formulation. All data was analysed using a two-tailed Student’s t-test.
nonlinear stress-strain behaviour is typical of viscoelastic materials such as soft biological tissues and hydrogels. As the compression test is being performed and the hydrogels are reduced in volume they deform gradually, and their densification returns a greater force on the load cell. The IPN and alginate samples also slightly exude water during compression, so their polymer density will increase as load is applied. This increase in polymer density will also increase strain hardening.
Chapter 3: Preparation and Characterisation of a 3D-Printable Shape-Changing Enzyme-Mediated Interpenetrating Network

Figure 3-23: Post compression behaviour and stress vs. strain graphs for different hydrogels. A, Photographs of 15 wt% PNIPAm single network compression testing sample before, during and after testing. The far-right picture is taken after compressing the sample by 40% 3 times. B, Photographs of a 3.5 wt% alginate single network, supplemented with 0.75 wt% XG, compression testing sample before, during and after testing. The far-right picture is taken after compressing the sample by 56% once. C, Photographs of a standard IPN formulation compression testing sample before, during and after testing. The far-right picture is taken after compressing the sample by 56% 3 times. D, 3 compression cycles for the same 15 wt% PNIPAm single network sample. E, 3 compression cycles for the same IPN sample. F, Compression to failure for 3 different IPN samples.
3.3 Conclusions and further work

Outlined in this chapter was the printing protocol for, and characterisation of, a novel bienzymatic IPN-producing 3D ink that can pattern a material that is the combination of a natural polysaccharide network and a synthetic smart polymer scaffold. Post-crosslinking yielded robust constructs that were robust and flexible. Investigations into the optimal printing dimensions revealed high and low feature dimension boundaries. Printed features smaller than 1 mm could not crosslink the PNIPAm network efficiently and print features larger than 10 mm failed to cure homogenously. However, within this print feature size regime multiple cm scale prints were achievable using shell with infill structuring. Also demonstrated was the tunability of various bioink functions, with alginate wt% effecting CTS performance and high HRP concentration prints forming hollow constructs. This suggests the potential of co-printing related inks to pattern asymmetric smart and material properties into the same construct. These experiments also demonstrate how the ink components in the standard formulation are optimized for a functional compromise in printability, CTS performance, and shape fidelity. Network ratios favouring alginate will promote printability but attenuate CTS, and network crosslinking kinetics are balanced to promote solid curing while still being fast enough to retain original print dimensions.

The printability of the ink was explored in crosshatch print uniformity and a filament collapse assay. The ink was found to deposit very regular filaments and histograms for crosshatch line width and pore area were closely grouped. In bridging capabilities, the ink was found to match those of state-of-the-art cell-supporting bioinks.

Assays on the material’s CTS capabilities reveal it retained the smart properties of PNIPAm single networks, although a maturation period of 3 cycles had to be performed before volume cycles stabilized. Within the first minute prints could contract to \(73 \pm 4\%\) their initial volume, and 50% contraction was achieved in 3 minutes. This incomplete reversibility of the ink is unanalogous with PNIPAm single networks, and some PNIPAm-based IPNs, suggesting that the crosslinking wash protocol doesn’t produce perfectly homogenous or monolithic interdigitated networks. This hypothesis seems to be confirmed by some of the non-uniformity imaged in SEM and the sub-optimal compression testing.
performance. Regardless, the IPN demonstrated elastic recovery up to 40% strain within the mm print size regime dimensions.

Due to the known bacteria-friendly compatibility of alginate hydrogels, and the mild material synthesis protocol, the IPN ink developed here was consequently taken forward to experiments in the following chapter to explore its potential as a functional bacterial bioink.\textsuperscript{61}

For future work, it would be interesting to see if the IPN prints’ CTS performance continues to plateau after the third cycle as suggested in Section 3.2.4.1. There should also be multiple thermal cycles performed on the other alginate wt% inks to see if they contract more elastically or not from the first cycle vs. the standard formulation. Also, PNIPAm’s homogeneity, and CTS performance, is known to improve at synthesis temperatures lower than optimal for HRP and GOx.\textsuperscript{22,42} Therefore, it could be interesting to explore the material and smart properties of constructs printed and crosslinked at temperatures slightly lower than room temperature. It may even be simple enough to compensate for the reduced enzyme efficiency at low temperature by using a much higher enzyme concentration. These experiments would require the addition of an environmental chamber for the 3D printer, but this would be worthwhile regardless as precise regulation of environmental temperature and humidity would greatly increase print uniformity and control. Using other PNIPAm crosslinkers may also potentially improve CTS performance. For example, using larger crosslinkers, such as poly(ethylene glycol) diacrylate (PEGDA), is known to increase the mesh size of PNIPAm single networks and lead to an increase in the deswelling ratio.\textsuperscript{62}

Beyond an alginate/PNIPAm IPN network, it is worth considering some alternative compositions to explore what other materials can be printed and what their respective properties would be. As the HRP/GOx/AcAc/Glucose quaternary initiation system simply produces free radical species, it’s compatible with many radically-initiated polymerisations.\textsuperscript{63-65} It would be interesting to explore the materials produced by an ink incorporated with other water soluble monomers such as acrylamide, (hydroxyethyl)methacrylate (HEMA), or PEGDA. The incorporation of oligomers or dendrimers instead of monomers might also improve material synthesis simplicity as these elements would be
slower to diffuse out of the print during the wash crosslinking step. It would also be worth investigating how other ionic crosslinkers would change the nature of the IPN prints. Alginate is known to be crosslinked by many different types of cations, including trivalent, and some authors report the synthesis of IPN hydrogels that containing alginate networks crosslinked by $\text{Fe}^{3+}$ and $\text{Al}^{3+}$ cations.\textsuperscript{23,66} There are also other strategies to crosslink alginate with calcium alternative to $\text{CaCl}_2$ immersion that produce more homogenously crosslinked alginate networks. Two of the most common is the use of calcium sulphate slurry, which retards gelation rate due to its low solubility, or calcium carbonate.\textsuperscript{67,68} Calcium carbonate is not soluble in water at neutral pH. It is used in combination with glucono-δ-lactone (GDL) which gradually hydrolyses into gluconic acid upon on addition to an aqueous solution at a rate dependent on temperature. This gradually lowers the pH and disassociates $\text{CaCO}_3$ in a controlled manner. There may be some interesting material property improvements to be made from incorporating one of these techniques into the IPN printing protocol.

3.4 Acknowledgement of collaboration

Work in this chapter was completed by the author unless otherwise mentioned here. The author would like to thank Rafael Moreno, for his help in performing the rheometry, and Judith Mantel, who performed all the EM imaging with samples prepared by the author.
Chapter 3: Preparation and Characterisation of a 3D-Printable Shape-Change
ning Enzyme-Mediated Interpenetrating Network

3.5 References


Chapter 3: Preparation and Characterisation of a 3D-Printable Shape-Changing Enzyme-Mediated Interpenetrating Network


Chapter 3: Preparation and Characterisation of a 3D-Printable Shape-Changing Enzyme-Mediated Interpenetrating Network


Chapter 3: Preparation and Characterisation of a 3D-Printable Shape-Changing Enzyme-Mediated Interpenetrating Network


4.1 Introduction

The field of 3D bacterial bioprinting is also only a few years old. This is mostly due to the prioritization of eukaryotic cell printing because of the obvious medical applications in tissue engineering and regenerative medicine. An early example of whole-cell bacterial patterning is a 2011 PLOS One paper by Xu et al. which utilizes *E. coli* as a living sacrificial porogen. The authors cultured the bacteria inside agarose gels which they would then decellularize after a period of time to leave behind an interconnected microporous hydrogel scaffold. By extruding the bacteria through a nozzle into a viscous agarose solution, they were able to pattern microchannels. The level and nature of porosity was tunable through controlling the initial seeding density and culture time of the bacteria. Although this methodology utilizes extrusion of whole-cell bacteria, it is not strictly 3D additive manufacturing as the bacteria are not being deposited layer-by-layer in a constructive process. They are instead being extruded into a viscous agarose bath. This limits the range of construct shapes producible by this fabrication method.

There is then a long gap in the literature until 2017 when authors began developing simple 3D bacterial bioprinting platforms. One of the earliest systems that attempted to manufacture 3D printed bacterially augmented materials utilized an alginate bioink to print *E. coli*. The authors modified a commercial 3D printer with a syringe pump and demonstrated reproducible patterning ability. The printed bacteria also remained viable for at least 48 hours post-printing and retained their original spatial patterning for at least 24 hours. However, shape retention of the alginate bioink was accomplished by printing onto a solid agar substrate with dissolved CaCl$_2$. This means successive print layers had less accessibility to the Ca$^{2+}$ ionic crosslinker, as it would have needed to diffuse through the bulk structure. In practice, this limits the height of print structures achievable with this method and in their paper the authors only demonstrate structures with low heights.
Since then, bacterial bioprinting has matured and further progress is being made quickly, especially as it’s a nascent field. In 2017 again, Schaffner et al. published work on the 3D bioprinting of two types of bacteria-laden functional materials. They demonstrated the versatility possible with harnessing bacteria’s natural metabolic pathways by inoculating bioinks with either P. putida or A. xylinum. This produced constructs capable of degrading phenol pollutants or producing biomedically-relevant cellulose materials (utilized in artificial skin and medical patches) respectively. Direct ink writing of the bacteria was performed using a light-curable hydrogel bioink composed of glycidyl methacrylate-functionalised hyaluronic acid, k-carrageenan, and fumed silica to produce constructs that were resilient, self-sustaining, and patternable on the millimeter length scale. Liu et al. in 2018 printed logic gates by 3D printing programmed bacterial cells in a F127-DA based bioink. By spatially patterning genetically modified bacteria, and due to cell induction via chemical diffusion, they also demonstrate the spatiotemporal evolution of system outputs possible with 3D printed bacterial-logic systems. Living networks were fabricable on the cm scale with up to 30 µm resolution. As a prototype, the authors 3D bioprinted a living network onto a thin elastomer layer which could adhere to human skin and act as a “living tattoo”. This “living tattoo” was capable of fluorescing in different displays dependent on the sensing of different chemical triggers. In 2019 Freyman et al. published on the first bioprinted living bacterial electrode. Here, the authors utilized an bioink composed of alginate and cellulose, cured by dropping on a CaCl₂ solution, to bioprint S. Oneidensis MR-1, an exoelectrogenic bacteria that can oxidize organic matter and display the produced electrons on its outer surface. When used in a microbial fuel cell the device showed stable current for up to 93 h. The Voight lab published a paper in 2020 bioprinting functional bacterial biohybrid materials via printing initially with spores and then germinating the spores in situ. The advantage being that spores are more resilient to materials processing and don’t require a continuous supply of water or nutrients. This allowed the bioprinted constructs to survive greater bioprinting stresses and to be generally more robust, until such a time they are activated on demand by rehydration. As a final representative example, Joshi et al. in 2018 published research on a bionic mushroom. Here, authors interlaced 3D-printed cyanobacterial cells and graphene nanoribbons onto the caps of mushrooms to augment the non-photosynthetic fungi with photocurrent generation. The mushroom substrate could nourish the bacteria via water transport from its underground...
mycelium network and the conductive graphene ribbons allow any produced electricity to be harvested. This interesting research exhibits the potential utility of patterning artificial mutual symbiosis that occurs between different microbiological kingdoms.

In Chapter 3, a novel interpenetrating network (IPN)-producing 3D ink was developed and characterized. Printed constructs were robust, flexible and demonstrated the contractile thermosensitive properties of its assimilated PNIPAm single network. In this chapter, the focus is on innoculating the ink with bacterial cells (E. coli) to augment prints with biologically-derived utility. Specifically, two prototypes are explored. In the first, the ink is innoculated with phosphotriesterase (PTE)-expressing E. coli in order produce a 3D printable bacterial microreactor capable of detoxifying organophosphates. In the second, the HRP in the initiation system of the ink is substituted for the native redox activity of living E. coli whole cells (E. coli endogenously express many redox-active enzymes, including peroxidases). Attempts to improve this system were made utilizing E. coli that had been transformed to overexpress a de novo peroxidase (C45). C45 is a highly efficient synthetic de novo peroxidase, developed recently by the Anderson group at the University of Bristol, that is readily expressible in E. coli systems. For more information on C45’s structure and its construction see Section 2.8. Both prototypes are classed as biohybrid materials, but the later prototype would also qualify as an ELM. The utilization of living systems in radical polymerisations is a very novel and unexplored area of research, and it has never been used before in conjunction with 3D bioprinting. Radical polymerisation has been done inside living cells; Geng et al. for example performed light-mediated polymerisation of new-to-nature polymers inside HeLa cells without any significant impact on viability. Radical polymerisation has also been initiated from engineered yeast and mammalian cell surfaces to grow grafted polymer coatings on live cells, again without compromising viability. But most of the research is interested in manipulating cell phenotype and not in initiating material synthesis. The utilization of living cell-initiated polymerisations to encapsulate cells in a monolithic macromolecular material remains a very niche research sub-genre.
4.2 Results and discussion

4.2.1 Printing an organophosphate-degrading biohybrid material via an E. coli laden IPN ink

Organophosphate (OP) detoxification was selected as a valuable utilization of the IPN ink due to the pervasiveness of OP insecticides. Synthetic OP compounds (chemicals that can be reduced to a central phosphate moiety with alkyl or aromatic substituents) have been extensively used as pesticides since the end of the Second World War and thousands of tonnes are still used worldwide every year today. As a result of their excessive and continuous use, and exacerbated by their high aqueous solubility, OPs have contaminated many terrestrial and aquatic ecosystems. OPs are also the basis for many nerve agents, most famously VX (discovered in the 1950s) and the ‘Novichok’ class of agents developed in the late 1980s. They operate as a neurotoxin by inhibiting acetylcholinesterase, which is responsible for the hydrolysis of neurotransmitter acetylcholine. The overaccumulation of acetylcholine leads to overstimulation of the nervous system, causing a cholinergic crisis and seizures. Typically, OP decontamination is done via incineration, sorbent materials, or chemical reagents. All these methods are either very expensive, or require the use of corrosive or potentially toxic materials. The use of microbes and purified enzymes for detoxification (bioremediation) has attracted a lot of research as an alternative because many enzymes have been isolated from soil bacteria that are capable of hydrolysing OPs under mild, physiological conditions. It is even hypothesized that this family of enzymes has evolved in soil bacteria due to the extensive use of OP-pesticides in agriculture. Among these enzymes, PTE is considered a particularly strong candidate as a bioremediation catalyst due to its wide substrate specificity and high catalytic efficiency. Paraaxon is used as the OP substrate in this chapter’s bioremediation assay as it has a hydrolysis product which absorbs strongly at 405 nm. Therefore, its breakdown can be easily tracked colorimetrically (Figure 4-1).
Figure 4-1: Enzymatic hydrolysis of paraoxon nerve agent by phosphotriesterase (PTE) into 4-nitrophenol and diethyl hydrogen phosphate. The hydrolysed bond is highlighted in red. 4-nitrophenol’s conjugate base, 4-nitropholate, has an absorbance maximum at 405 nm and its concentration can therefore be tracked colorimetrically.

Described in this Section is the 3D printing of a biohybrid material (BM) via the inoculation of the IPN ink with *E. coli* (acellular ink formulation and development described in Section 2.1 and Chapter 3). The *E. coli* have been transformed to express PTE (PTE-*E. coli*, construction described in Section 2.6) to augment the IPN prints with OP bioremediating activity. Prior to inoculation, bacteria were resuspended in deionised water (Mq) from lysogeny broth (LB) as the monovalent cations present in LB would impair ink printability (see Section 3.2.2). Inoculation of the IPN ink was performed immediately before a final mix in the DAC at 3500 rpm for 1 minute and then printing. Confocal microscopy of the bioink was performed to assess *E. coli* viability (69 ± 4%) and distribution (Figure 4-2). One minor variation from the standard acellular IPN ink printing protocol was performed to mitigate any additional stress to the inoculated bacteria: *E. coli*-laden prints were crosslinked post-printing with a 100 mM CaCl$_2$ + 5 mg/mL D-glucose solution for 1 hour rather than with a 300 mM CaCl$_2$ + 5 mg/mL D-glucose solution (hypertonic for *E. coli*) overnight.
Chapter 4: Printing the Interpenetrating Network Ink with Living Cells

4.2.1.1 E. coli laden IPN print integrity

Initially, E. coli-laden prints appeared to yield constructs very similar to their acellular versions. For example, dumbbell-shaped print structures were true to model parameters and could be readily handled (Figure 4-3). To explore further, bioprinted samples were bisected to evaluate their integrity (Figure 4-4A). Immediately apparent was an uncured central channel running through the print. Previous experiments demonstrate that these print dimensions produce solid constructs when using the acellular ink (Figure 3-5). This suggests that the incorporation of E. coli is interfering with the curing process.

It is unlikely that the bacteria were disrupting the alginate network, especially as there are multiple E. coli-laden alginate bioinks in the literature that rely on calcium crosslinking.\footnote{1,30,31} However, it is very possible that the cells could be interrupting the PNIPAm/MBA network. This would be due to their known endogenous mechanisms for dealing with oxidative stress (for example, the upregulation of radical quenching superoxide dismutases and catalases), which would attenuate radical polymerisation propagation steps.\footnote{32} It is also known that polymer radicals can chain transfer to available protein...
cysteine residues, which would be present in the native *E. coli* proteome. These mechanisms would quench radical propagation centres as they progressed inwards from print surfaces and may explain the observed cavities.

To validate that the cavity was due to the presence of *E. coli* cells and not to the reduced crosslinking time and ionic crosslinker concentration used in the bacteria laden IPN print preparation, an acellular print was prepared under identical crosslinking conditions (Figure 4-4B). No cavity was observed in this acellular print.

![Image](image1.png)  
*Figure 4-4: Photographs of a PTE-* *E. coli* laden (A) and an acellular (B) IPN print being bisected and having cut face observed. A clear central cavity channel is observable in the cut face of the PTE-* *E. coli* laden print. Major and minor gridlines in images demarcate 10 and 1 mm respectively.*

**4.2.1.2 *E. coli* laden IPN print thermosensitivity**

To explore how the *E. coli*-laden prints thermosensitivity (TS) performance compared to their acellular analogues, bioprinted samples were tested using the exact same assay as used in Section 3.2.4.1 (Figure 4-5). Over one heat and recovery cycle the *E. coli* laden prints contracted to \( V/V_1 = 52 \pm 9\% \) and recovered to \( V/V_1 = 104 \pm 2\% \). The standard acellular IPN prints contracted to \( V/V_1 = 38 \pm 5\% \) and recovered to \( V/V_1 = 90 \pm 10\% \) on their first contraction cycle. The IPN print’s TS performance is therefore significantly affected by the addition of *E. coli* (two-tailed Student’s t-test, \( P<0.01 \)). Not only has the initial contraction cycle diminished, the print recovers to slightly larger than its original volume. However, this still represents a large volume change and further heat/recovery cycles would have to be performed to see how this behaviour would evolve over multiple iterations. Also, this change in TS performance supports the hypothesis that PNIPAm formation is affected by the inclusion of *E. coli*.
made in Section 4.2.1.1. The cavities observed in the bisected prints and the TS performance reduction are likely both due to the living cells attenuating radical polymerisation propagation steps.

Figure 4-5: E. coli laden IPN print thermosensitivity assay. A. Photographs of the E. coli laden printed structure used in the thermosensitivity assay above and below the IPN’s lower critical solution temperature. Major and minor gridlines in images demarcate 10 and 1 mm respectively. B. Plot showing the contractile thermosensitivity performance for acellular and E. coli laden IPN prints over 1 heat cycle (60°C oven for 30 minutes). Values represent mean averages ± standard deviation for n=3 experimental repeats. The contracted volume for the acellular and E. coli-laden prints are statistically significantly different (two-tailed Student’s t-test, P<0.01).

4.2.1.3 Paraoxon assay of bioprinted constructs

To assay the bioremediating activity of the PTE-E. coli laden prints, microreactors were printed and presented with paraoxon (Figure 4-6). Microreactors were printed as gently curved rectangles (4mm width, 3 mm height) designed to tessellate with the edge of a standard 6-well plate well. Microreactors were tested with and without isopropylthio-β-galactoside (IPTG) induction (PTE-E. coli (i) and PTE-
*E. coli* respectively). An acellular control and a microreactor that had been inoculated with *E. coli* that had not been transformed to express PTE were used as controls. All *E. coli* used in the essay were grown and incubated in the presence of glucose to suppress PTE expression prior to inoculation (low basal expression levels of pET systems can be maintained by glucose supplementation). Printed microreactors were also washed three times with sterile HEPES buffer prior to the assay to remove any non-encapsulated cells. Microreactors were restricted to the peripheries of 6-well plate wells using 3D printed spacers, and absorbance measurements for 4-nitrophenol were taken from well centres. Therefore, absorbance readings accounted for substrate/product transport in and out of the IPN microreactors, and beam scatter was avoided from the hydrogels.
Figure 4-6: 3D bioprinting organophosphate degrading bacterial IPN microreactors. A, Photographs of a 6-well plate well with two 3D printed bacterial microreactors in HEPES buffer before (top) and 30 minutes after (bottom) paraoxon addition (80 µL of a 50 mM stock for a 1 mM final concentration). The bioink has been inoculated with E. coli transformed to be able to express phosphotriesterase, an organophosphate hydrolysing enzyme. The yellow colouration in the bottom photo is due to 4-nitrophenol, a yellow paraoxon hydrolysis product that absorbs at 405 nm. The visible clear plastic construct is a 3D printed porous plastic scaffold designed to keep the microreactors away from the centre of the well where the absorbance is being measured. B, Paraoxon degradation was observed over time via absorbance at 405 nm. Plots are for uninduced PTE-E. coli (blue), induced PTE-E. coli (yellow), acellular (green), and E. coli (orange) microreactor wells. Paraoxon addition occurred at T=0. Complete remediation for both induced and uninduced PTE-E. coli occurred within 20 minutes. Values for induced and uninduced PTE-E. coli data represent mean averages ± maximum/minimum values for n=3 experimental repeats. C, microreactor activity in Au/s for the first 600 seconds. No significant difference between the induced and uninduced PTE-E. coli laden microreactors. Both the E. coli and acellular controls displayed negligible activity. Statistical significance analysed using a two-tailed Student’s t-test.

Both the PTE-E. coli laden microreactors degraded paraoxon at a similar rate (Figure 4-6C) and complete hydrolysis of 1 mM paraoxon to 4-nitrophenol was observed in less than 15 minutes. This suggests that the uninduced microreactor could still produce enough PTE, via basal level expression, indicating that its concentration was not rate limiting. This was perhaps not surprising as PTE is remarkably efficient at hydrolysing paraoxon, with turnover numbers (k_{cat}) and catalytic efficiency (k_{cat}/K_{M}) values close to the diffusion-controlled limit (2280 s^{-1} and 6.2 \times 10^{7} M^{-1} s^{-1} respectively).^{35}
vectors are also known to have problems of expression instability, certain pET vectors have even been shown to express higher levels of protein when uninduced. Furthermore, the detoxifying activity of the PTE-E. coli laden microreactors confirm the capability of paraoxon to translocate to the intracellular-PTE. This was to be expected however, as OPs are typically small lipophilic molecules and most are known to penetrate cell membranes.

These results also demonstrate that encapsulated E. coli survived the room temperature bioprinting process and remained viable in the microreactors overnight (incubated at 29°C). This was expected however, as most of the bioink components are known to be compatible with bacteria, and E. coli are known to be robust organisms capable of surviving in environments of non-physiological toxicities, including sterile, distilled water. Both the control experiments, the acellular and non-PTE expressing E. coli, demonstrated negligible absorbance and displayed no yellow colouration from paraoxon hydrolysis at any time after the addition of paraoxon. Therefore, confirming that the production of 4-nitrophenol in the other experiments was catalysed by the heterologous expressed PTE.

4.2.2 Utilizing E. coli as a source of redox potential

To explore if the role of the bacteria could be further integrated into the BM, the isolated HRP utilized in the standard IPN ink preparation was substituted with the native endogenous redox activity of living E. coli whole cells. Here, the whole cells would play a role in the formation of the material itself, which would qualify the construct as an ELM. Some complications were anticipated due to the competing redox attenuation systems that would also be present in E. coli (the oxidative stress responses etc. mentioned in Section 4.2.1.1) but as a control, non-genetically modified E. coli whole cells were inoculated into the ink initially (WC-E. coli).

Preliminary experiments utilizing a WC-E. coli/GOx/AcAc/H2O2 quaternary initiation system demonstrated that the living cells could cure PNIPAm/MBA single network pre-gels that were structurally sound and cured without cavities (Figure 4-7A). These experiments were performed with 50% v/v WC-E. coli aliquot (whole-cell E. coli aliquot, preparation described in Section 2.7). However, these solutions would take overnight to fully polymerise to the point where they had appreciably
solidified and could be inverted. This would be incompatible with the IPN material printing methodology as it requires a much faster synthetic network formation because the crosslinking of the alginate and the crosslinking and polymerisation of the PNIPAm network occur simultaneously during the wash step. If the PNIPAm network forms too slowly it is simply washed out of the ink as NIPAm monomer (Figure 3-6). Thus, it appears as if the non-genetically modified \textit{E. coli} cells alone wouldn’t produce enough redox activity to cure IPN prints effectively. To ensure the cells were responsible for the polymerisation initiation, an acellular control was performed (Figure 4-7B); no gel formation was observed.

The curing experiments in Figure 4-7A represent the utilisation of a relatively high concentration of whole cells (the WC-\textit{E. coli} aliquots are roughly estimated to be around 5.6\% v/v whole cells [using OD\text{600} and assuming the volume of a single \textit{E. coli} cell = 1 \mu m^3]^{41,42}$, therefore using 50\% v/v of the aliquots equates to 2.8\% v/v whole cells). Lower cell densities led to poor network curing (Figure 4-8A). Using 15\% v/v WC-\textit{E. coli} aliquot resulted in partial curing of the pre-gel to a viscous sludge and using 5\% v/v WC-\textit{E. coli} aliquot failed to cure the pre-gel at all.

To test how the IPN ink would even print with high cell content, a dumbbell print was performed loaded with 50\% v/v WC-\textit{E. coli} aliquot (Figure 4-8B). The cells clearly effected the ink’s printability at these concentrations. The ink suffered reduced viscosity leading to poor print feature definition and slumping.
as compared to acellular prints (Figure 4-8C). It could be that the cells are behaving like non-interactive porogens in the ink, disrupting the crosslinking concentration and homogeneity. Therefore, at high enough *E. coli* concentrations the ink’s structure becomes too compromised. As a comparative reference, one of the earliest 3D bacterial bioprinting examples used a 2.5% w/v sodium alginate bioink and inoculated its ink to a final *E. coli* cell content of roughly only 0.2% v/v.¹

![Figure 4-8: Photographs of: A, PNIPAm/MBA single network curing experiments performed with 50, 15 and 5 % v/v WC-*E. coli* aliquot. B, Bioprinting the IPN ink with high cell loading concentrations (50 % v/v WC-*E. coli* aliquot). Printability/viscosity is clearly affected with observable slumping of the ink pre-crosslinking. C, Acellular prints of the IPN ink for comparison to (B). Print features such as edges and corners are notably more defined, as is a much-reduced spreading ratio. All gridlines demarcate 10 mm.](image)
4.2.2.1 Utilizing C45-expressing E. coli as a source of redox potential

In order to augment the E. coli’s endogenous redox activity to speed up the curing time and reduce the concentration of whole cells required to print, E. coli were transformed to be able to overexpress a de-novo peroxidase (C45) that had recently been developed by the Anderson group at the University of Bristol.\textsuperscript{11} C45 was chosen as it is readily expressed by E. coli and has one of the best reported catalytic efficiencies among de novo enzymes (The turnover number and specificity constant of C45 is even comparable to HRP [Table 4-1]). Successful expression and purification of C45 in E. coli was confirmed with identical size exclusion chromatography traces and UV/vis spectra (Appendix B) to the original paper.\textsuperscript{11}

Table 4-1: HRP’s and C45’s turnover numbers ($k_{\text{cat}}$) and ABTS specificity constants ($k_{\text{cat}}/K_m$). Values taken from Watkins et al.\textsuperscript{11}

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{\text{cat}}$</th>
<th>$k_{\text{cat}}/K_m$ (ABTS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRP</td>
<td>4100 s\textsuperscript{-1}</td>
<td>5.13 x 10\textsuperscript{6} M\textsuperscript{-1} s\textsuperscript{-1}</td>
</tr>
<tr>
<td>C45</td>
<td>1200 s\textsuperscript{-1}</td>
<td>3.2 x 10\textsuperscript{6} M\textsuperscript{-1} s\textsuperscript{-1}</td>
</tr>
</tbody>
</table>

Initial experiments demonstrated that isolated C45 could successfully cure simple NIPAm/MBA single network pre-gels when utilized in a C45/GOx/AcAc/Glucose quaternary initiation system (Figure 4-9A). However, when using molar equivalents of C45 and HRP, the curing times were significantly different. The HRP-crosslinked PNIPAm/MBA single network in Figure 4-9A solidified within 15 minutes whereas the analogous C45 experiment took over one hour. Note, these times do not reflect the reaction inside the standard IPN ink as the reactant concentrations are different. The C45-based initiation system was also successfully incorporated into an acellular PNIPAm/Alginate IPN ink print (Figure 4-9B and C) with a wash crosslinking step (here, the standard IPN formulation is used with a molar equivalent of C45 for HRP). Printability was clearly unaffected, but this was expected as neither HRP nor C45 is a structural component of the ink. The resultant construct was handleable like its HRP-mediated analogue but had a poorer contractile thermosensitivity (CTS) performance ($L_T/L_{RT}$[C45] = 0.86 ± 0.02 vs. $L_T/L_{RT}$[HRP] = 0.67 ± 0.03, n = 3, P<0.001, Figure 4-9D). This poorer CTS performance
is probably due to the slower enzyme kinetics of C45 vs. HRP noted in the PNIPAm/MBA single network curing experiments and quantified in the literature values in Table 4-1.

![Figure 4-9: Photographs of experiments utilizing a C45/GOx/AcAc/Glucose quaternary initiation system. A, Casted PNIPAm/MBA single network pre-gels cured via a C45-, sans-peroxidase, and HRP-based quaternary initiation system respectively. B, Dumbbell shaped print printed with the C45-incorporated IPN ink. Printability is unaffected by the C45/HRP substitution. C, The dumbbell print in (B) post-crosslinking with a 300 mM CaCl₂ + 5 mg/mL glucose solution. D, Demonstrating the CTS of the C45-incorporated IPN print, $LT/L_{RT} =$ length contraction $= 0.86 \pm 0.02$ for $n = 3$. Major and minor gridlines in images demarcate 10 and 1 mm respectively.

As a further demonstration of C45’s slower initiation of the PNIPAm/MBA network within the ink, both an HRP- and a C45-incorporated IPN ink dumbbell print was crosslinked via submersion in a 5 mg/mL glucose solution (Figure 4-10). This would selectively cure only the PNIPAm network and not the alginate, which has been demonstrated to be required to template the PNIPAm formation within the
original print confines (see Section 3.2.1.1). The HRP-initiated print swelled greatly post-crosslinking (new length post-crosslinking = 38 mm), but the network was not sufficiently diluted to sacrifice print-dimension ratios, definition, or final self-supportability/rigidity. The C45-initiated print, due to the slower kinetics allowing more of the PNIPAm network to dissolve/dilute, had markedly different material properties. Shape definition was much poorer and print swelling had significantly increased (new length post-crosslinking > 52 mm). The final construct was also markedly more transparent, especially when solvated in water (Figure 4-10C), due to the increased dilution of the pre-crosslinked alginate network. However, the construct was still structurally stable and could be handled without damage.

Figure 4-10: Curing HRP- (A), and C45- (B), incorporated dumbbell-shaped IPN prints with 5 mg/mL glucose solutions (thereby only crosslinking [and propagating] the PNIPAm/MBA network). The leftmost image in both (A) and (B) is the print pre-crosslinking. Although both constructs had swollen post-crosslinking, the C45 print had lost much more of its original shape and definition. Major and minor gridlines in images demarcate 10 and 1 mm respectively. The scale bar in the central (B) image is 30 mm. C, Photographs of the HRP-incorporated print (left) and the C45-incorporated print (right) post glucose-initiated crosslinking and aqueously solvated. The C45-incorporated print displays a clear increase in transparency.
Early experiments with C45-expressing whole cells (C45- \textit{E. coli}) were inauspicious (Figure 4-11). Curing times were not markedly increased and solutions still took many hours to fully cure. Casted PNIPAm/MBA single networks also appeared to be much softer compared to their WC-\textit{E. coli} cured analogues. Upon bisecting the casts, it was clear that only an outer shell had cured while the core had remained unsolidified under the surface (Figure 4-11A). Unexpectedly, it appeared that by transforming the bacterial cells to overexpress the C45 peroxidase their redox activity had been impaired rather than improved. It is possible that during C45 overexpression C45-\textit{E. coli} prioritization of the de-novo enzyme’s production monopolised the available heme.\textsuperscript{43} This could detract from the production of endogenous heme enzymes that have better redox performance, or at least better performance/more stable within the environment of the IPN ink and the cellular milieu (C45 was optimized for performance as an isolated enzyme in a favourable buffer solvent). This might explain why C45-\textit{E. coli} demonstrates a poorer curing ability vs. the untransformed whole cells. It is also known that overexpression of a recombinant protein in \textit{E. coli} can lead to the formation of inclusion bodies or increase the metabolic burden of the cell, both of these phenomena would effect overall viability.\textsuperscript{44,45} Metabolic burden in particular, the energetic and precursor consumption caused by the production of non-essential proteins, has several knock-on effects to cells such as growth rate and metabolic pathway downregulation. It may be that the overexpression of C45 over-compromised the cells and caused the attenuation of their redox potential. However, these explanations would not describe why C45-\textit{E. coli} mediated PNIPAm/MBA gels selectively cure an outer shell. Perhaps \textit{E. coli} cells at an interface express a phenotype that improves native redox activity or dampens radical quenching. \textit{E. coli} are known to recognise interfaces and change their behaviour. For example, forming biofilms after adhering to a surface, or pellicles at air-liquid interfaces.\textsuperscript{46,47}

The surface network of the casts was confirmed to be PNIPAm \textit{via} the demonstration of reversible CTS performance (Figure 4-11B). Clear contraction is observed but due to the uncured core the surface network “balloons” against the resisting internal liquid.
A PNIPAm/MBA network cured inside a 5 mL Eppendorf tube from 1 mL of pre-gel solution with a C45-E. coli/GOx/2,4-diacetoxyl glucouronic quaternary initiation system. Upon removing the hydrogel from the Eppendorf tube its shape noticeably relaxed. Bisecting the hydrogel revealed an uncured core, only an outer shell had solidified. B, Testing the CTS of a PNIPAm/MBA casted hydrogel prepped identically to (A) but with 2 mL of pre-gel solution. Hydrogel cast was heated in an oven at 60°C for 30 min. Contraction is observable but clearly the uncured core is resisting the contraction leading to a spherical “ballooning” of the PNIPAm skin. Scale bars = 10 mm

4.3 Conclusions and further work

Presented in this chapter is an augmentation of the IPN bioink to make a bioremediation bacterial microreactor via the inoculation of genetically modified E. coli. The E. coli appeared to survive the printing process and remain viable in the IPN prints, evidenced by their ability to hydrolyse paraoxon. The CTS apparent in the acellular IPN constructs was conserved in the cell-laden prints, although attenuated (likely due to the native mechanisms in bacteria for dealing with oxidative stress quenching some of the radicals generated during the wash crosslinking step). These results represent a novel 3D printable bacteria-laden biohybrid material, that contains a smart polymer scaffold, which is performing a utility due to inoculated genetically modified organisms. This demonstrates the potential for a wide range of applications as E. coli, and other unicellular organisms, can be transformed to express many other
desirable exogenous proteins. Beyond this, synthetic biology is aiming to improve and expand the functions of natural proteins. The scope of bacterial bioprinted devices is analogous to the potential for synthetic gene circuits. 3D spatially designed co-cultures of various bacterial strains working in sequence absorbing molecular inputs and metabolising them via multiple cascading enzymatic reactions.

To further explore how the bacteria behave in the IPN prints, time-lapse microscopy should be used. It would be interesting to know how the bacteria proliferate within the IPN, how motile they are, and how long they remain encapsulated. Bacterial compatibility/encapsulation may also be improved via biofilm formation. This could be done by the addition of quorum-sensing factors known to lead to mature biofilm formation, or by transforming bacteria to overexpress artificial biofilm components. This could add very interesting material properties to prints as it would essentially represent the addition of a tertiary network. Another methodology for improving bacterial incorporation would be to transform cells to express a cell surface bioink-binding protein. For example, in Guo et al. where E. coli, printed in a dextran-based hydrogel, were transformed to express a sugar-binding adhesin. Alginate itself can also be chemically modified to improve cell adhesion by displaying known adhesion ligands. Also, some discovered bacteria are capable of expressing alginate lyases which would compromise one of the IPN networks. E. coli are not known to produce any, but it would be cautious to ensure they did not catalyse any breakdown of the alginate network over time. Further experiments with the bioremediation microreactor might also assay the effect on the detoxification rate of heating the microreactor above its LCST. Deswelling the microreactor would likely affect the reactant diffusion coefficient within the IPN so it may be possible to control reaction rate via the microreactor CTS.

Also demonstrated in this chapter is the ability for living E. coli cells and a de novo peroxidase (C45) to cure synthetic pre-gel solutions and mediate IPN bioinks respectively. The C45-mediated polymerisation of PNIPAm networks is the first example of a synthetic enzyme catalysing the formation of a polymeric material and it was also successfully substituted into the IPN ink, as an isolated enzyme, in place of HRP. High-concentration bacterial bioprinting with the IPN ink proved to be problematic, with printability significantly affected. However, due to the rapid growth of bacteria it may be better
for future experiments to incubate lower concentration prints before crosslinking them. Alternatively, the IPN ink could be printed cell-free and then be inoculated post-crosslinking. However, this is likely to effect cell distribution. It may also be productive to test printing other unicellular organisms such as alternate bacterial strains or fungi to explore if they have the same effect on printability. Freeze dried yeast, for example, is known to enhance the printability of some bioinks. Other unicellular organisms may also interfere with the radical propagation of the PNIPAm network with less of the severity as *E. coli*. Alternatively, the NIPAm monomers in the pre-gel could be substituted for oligomers/dendrimers such as poly(ethylene glycol) diacrylate, which is readily available for purchase as an oligomer, as these would require less crosslinking reactions to achieve equivalent weight networks. Oligomers would also be less in danger of diffusing out of the gel during the wash crosslinking step as opposed to much smaller monomers. The contractile thermosensitivity of the smart PNIPAm network would be lost, but other synthetic networks might form more robust, tougher materials with other interesting properties. Furthermore, it would also be interesting to stain biopsies of both the *E. coli* and *C45-E. coli* mediated networks to discover more about what the cells are doing to cure the networks. It would be useful to know if the cells remain viable after the living-cell initiated radical polymerisation. The structure of the *E. coli* mediated PNIPAm networks should also be looked at with electron microscopy. The localization of redox-initiating activity at cells may have potentially templated the network, especially in its relation to the bacteria, in new and interesting ways.

4.4 Acknowledgement of collaboration

Work in this chapter was completed by the author unless otherwise mentioned here. The author would like to thank Mark Shannon, for his help in performing the confocal imaging.
Chapter 4: Printing the Interpenetrating Network Ink with Living Cells

4.5 References


Presented in this thesis was the development of a novel IPN-producing ink that could be 3D printed to produce shape changing hydrogels. Also demonstrated was the ink’s bacteria-supporting properties and augmentation into a biohybrid material. The ink is processed and printed entirely at room temperature under mild and aerobic conditions. Results chapter 1 outlined the printing protocol for the acellular ink and the characterisation of some of its material (pre- and post- crosslinking) and printability properties. Also explored was ink property tunability and the depth-dependent composition the crosslinking methodology produced in the hydrogels. Due to the limitations of the crosslinking method, only certain print regimes could be cured robustly but large structures could be fabricated by using infill patterning conventions. Certain ink printability metrics matched state-of-the-art commercial formulations, but IPN CTS performance was not completely elastic (likely due to non-homogenous IPN structure or force-induced network maturation). Results chapter 2 explored inoculating the ink with genetically modified bacteria to produce a functional biohybrid material or engineered living material. Described here is the production of a functional organophosphate degrading bioreactor and attempts to utilize the redox potential of living cells to cure the ink. Living cell-mediated polymerisation of single networks was achieved with unmodified E. coli cells but the slow kinetics were not compatible with the IPN ink printing protocol. E. coli that had been transformed to overexpress a synthetic peroxidase (C45) were then utilized but no redox activity improvements were observed. However, EMP of PNIPAm was achieved with isolated C45, and this is the first example of a synthetic enzyme being able to initiate polymeric material synthesis. Within the context of existing hydrogel systems, stiffer, tougher IPNs have been synthesized but none that are 3D printable with an incorporated EMP initiation step to enable mild condition fabrication. Post-printing viability also demonstrates the potential for this technology to be a platform to produce many types of biohybrid materials.
Chapter 6: Appendix A

Appendix A contains photographs from some of the experiments optimising the IPN-producing ink. The ink was optimised for post-crosslinking shape retention (shape fidelity) and thermosensitive contraction magnitude. Most of the prints in the following photographs are of the below dumbbell model (24x12x3 mm). Some of the following inks perform better than the optimized formulation in some respects but worse in others and can be considered alternative formulations. All major and minor gridlines in the following images demarcate 10 and 1 mm respectively.

The optimised ink formulation (also tabulated in Section 2.1):

<table>
<thead>
<tr>
<th>Bioink Component</th>
<th>Concentration</th>
<th>Stock Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthan gum</td>
<td>0.75 wt%</td>
<td>N/A</td>
</tr>
<tr>
<td>Sodium alginate</td>
<td>3.5 wt%</td>
<td>N/A</td>
</tr>
<tr>
<td>N-Isopropylacrylamide (NIPAm)</td>
<td>15 wt%</td>
<td>N/A</td>
</tr>
<tr>
<td>N,N'-Methylenebisacrylamide (MBA)</td>
<td>7.5 mg/mL (48.6 mM)</td>
<td>Mq</td>
</tr>
<tr>
<td>HRP</td>
<td>1.14 µM</td>
<td>Mq</td>
</tr>
<tr>
<td>GOx</td>
<td>3.12 µM</td>
<td>50 mM Sodium acetate pH 5 buffer</td>
</tr>
<tr>
<td>Acetylacetone (AcAc)</td>
<td>97 mM</td>
<td>N/A</td>
</tr>
<tr>
<td>Calcium chloride (CaCl₂)</td>
<td>25 mM</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Increasing the NIPAm pre-gel component to 20 wt\% led to an increase in post-crosslinking swelling (16\% along each axis as measured by electronic callipers vs. 4\% for the optimized formulation).

Increasing the CaCl$_2$ pre-crosslinking concentration to 50 mM resulted in the ink phase separating during mixing in the dual asymmetric centrifuge.
Increasing the HRP concentration to 4 mg/mL led to a substantial increase in post-crosslinking swelling and hollow constructs (further explored in Section 3.2.1.3).
Increasing the XG to 1.5 wt% led to no change in post-crosslinking swelling vs. the optimized formulation but did cause a slight decrease in thermosensitive contraction magnitude ($\frac{L_T}{L_{RT}} = 0.73 \pm 0.01$ vs. $0.67 \pm 0.03$, n = 3). Furthermore, no obvious increase in printability was observed.
Increasing the alginate component of the ink to 4 wt% improved post-crosslinking shape fidelity (2% swelling along each axis) but decreased thermosensitive contraction magnitude ($L_T/L_{RT} = 0.75 \pm 0.02$ vs. $0.67 \pm 0.03$, n = 3). It also seemed to slightly improve printability.
Photographs demonstrating ink printability with and without xanthan gum (XG). A, STL file for print constructs of a cube (17x17x8 mm) with 4 internal pores (4x4x8 mm). B, construct printed with ink with XG. C, construct printed with ink without XG.

Ink formulation for B:

<table>
<thead>
<tr>
<th>Ink Component</th>
<th>Concentration</th>
<th>Stock Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthan gum</td>
<td>1 wt%</td>
<td>N/A</td>
</tr>
<tr>
<td>Sodium alginate</td>
<td>3 wt%</td>
<td>N/A</td>
</tr>
<tr>
<td>Calcium chloride (CaCl₂)</td>
<td>15 mM</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Ink formulation for C:

<table>
<thead>
<tr>
<th>Ink Component</th>
<th>Concentration</th>
<th>Stock Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium alginate</td>
<td>3 wt%</td>
<td>N/A</td>
</tr>
<tr>
<td>Calcium chloride (CaCl₂)</td>
<td>15 mM</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Chapter 7: Appendix B

C45 details:

**C45 nucleotide sequence:**

CAGGTGACGCATCCTCGCATTATCCGCATTAACGACGATGATGTTTTCCGCCTCGGCTC
TCGCCAACATCATCATATCATATGCTAGAGCGGCGA AAAACCTGTA TTTCAGGGCA
TGACGCGGAAACAAATATGGAAGCAATTTGAGGATGCACTGCAAGAAGTTTGAGGAGGCA
TTGAATCAATTTGAAGACTTAAAGCAGCTTGGCGGATCAGGTCCGGGAGCGGCGGTGA
AATCTGGAAGCAGTTTGAAGATGCGTTCACAGAAATTTGAGGAAAGCTTAAAACCAATTG
AGATTTAAACAGCTAGCCGGGAGTGGGGCCTCTGGAAGAGCAGCGGGAGAGATATG
GAAGCAGTTTGAAGATGCACTTACAGAAATTTGGAAGAGGCTTTGAACCAATTTGAAGACC
TTAAGCAACTAGGTGGGGAGCGGCAGTGGGCTCTGGAAGAGTGTATCGCCTGTCACGAA
GACGCTTGCAGAAATTTGGAAAGCGCTTCAATTTGAGGACCTTAAGCAGCTGTA
A

**C45 protein sequence:**

GMTPEQIWKQFEDALQKFEEALNQFEDLKQLGGSGSGGGEIWKQFEDALQKFEEALNQFE
DLKQLGGSGSGSGGGEIWKQFEDALQKFEEALNQFEDLKQLGGSGSGGGEIC1ACEDAL
QKFEEALNQFEDLKQL
Size exclusion chromatography traces (280 nm, blue; 420 nm, yellow) for C45. The first peak at 52 minutes corresponds to apo-CTMs, aggregated proteins and high molecular weight contaminants. The second peak at 108 minutes corresponds to pure holo-CTM. Fractions within the grey box were pooled for experimentation.
UV/visible spectrum for C45. The characteristic Soret band for the $\pi-\pi^*$ transition of bound heme is visible at 405 nm.

Photograph of purified C45 aliquot.