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PHYSICOCHEMICAL AND NANOMECHANICAL BEHAVIOUR OF  
3D PRINTED PEGDA HYDROGEL STRUCTURES FOR TISSUE  
ENGINEERING APPLICATIONS

SCHOOL OF AEROSPACE TRANSPORT AND MANUFACTURING  
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Supervisor: Dr Susan A. Impey

Associate Supervisor: Dr Adrianus Indrat Aria and Prof Saurav Goel

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PEGDA Hydrogel Structures for Tissue Engineering Applications

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## **ABSTRACT**

Poly(ethylene glycol) diacrylate (PEGDA) hydrogels are well established in tissue engineering and organ-on-chip applications as scaffolds for 3D templates in aqueous environments due to their high water content, biocompatibility and low toxicity. The versatility of PEGDA hydrogels as a platform for cell encapsulation and tissue engineering is attributed to their ability to be modified in various ways, including concentration, molecular weight, and polymerisation technique. Since properties of the PEGDA host material will affect the functionality of the cells and tissues, and vice versa, a key missing feature of the currently developed screening solutions is the lack of proper understanding of the behaviour of the 3D printed PEGDA soft support structures holding living tissues in a dynamic human-like tissue microenvironment. Thus, the aim of this research is to demonstrate repeatability and reliability in the measurement of physicochemical and nanomechanical properties of multilayer 3D printed UV crosslinked PEGDA hydrogels for use in organ-on-chip devices. The research offers insights into long term stability of hydrogels through studying how changes in both environmental and printing parameters can be extrapolated to other biomaterials for benefit of other tissue engineering applications.

Recent advancements in the use of PEGDA hydrogels for tissue engineering are reviewed, with a focus on bulk cross-linking and 3D printing synthesis methods. Characterisation methods for 3D printed PEGDA hydrogels are also discussed. The current state of development of biomedical applications, particularly in organ-on-chip devices, is highlighted. The thermal response of multilayer PEGDA hydrogels made using in-house projection lithography was compared to

monolithic hydrogels created through bulk photo-cross-linking. The results indicated that the volume of multilayer PEGDA hydrogels changes in response to the temperature with dimensional change between +10% and -11.5%, and also displaying an anisotropic characteristic where the axial dimensional change was higher than the lateral dimension. The results also confirmed the swelling behaviour to be reversible between 8 and 45 °C. The nanomechanical properties of monolithic and multilayer PEGDA hydrogels fabricated through bulk cross-linking and layer-by-layer projection lithography were studied. The findings showed that an increase in the number of layers results variation in axial elastic modulus between 1.69 and 0.67 MPa. Additionally, the research examines the structural heterogeneity of 3D printed hydrogels which is linked to the degree of cross-linking of the printed layers and showed variations in lateral elastic modulus between 2.8 and 11.9 kPa. The results suggest that by controlling the cross-linking throughout the 3D printed structure, the surface nanomechanical properties of the hydrogels can be manipulated to direct cell attachment and adhesion in specific regions within the structure, offering potential for future improvement in the reproducibility and reliability of 3D printed hydrogels for tissue engineering and organ-on-chip applications.

Keywords:

Poly(ethylene glycol) diacrylate, Photo-cross-linkable hydrogels, Tissue engineering, 3D printing, Nanoindentation, AFM, Cross-linking density

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## LIST OF ABBREVIATIONS

|       |                                                  |
|-------|--------------------------------------------------|
| 3D    | Three-dimensional                                |
| AFM   | Atomic Force Microscopy                          |
| AR    | Aspect Ratio                                     |
| CCM   | Cell Culture Medium                              |
| CF    | Contractile Force                                |
| CGTT  | Critical Gel Transition Temperature              |
| CLIP  | Continuous liquid interface production           |
| DEX   | Dexamethasone                                    |
| DIW   | Deionised Water                                  |
| DF    | Deflection Force                                 |
| DLP   | Digital Light Processing                         |
| DLW   | Direct Laser Writing                             |
| DMD   | Digital Micromirror Device                       |
| DOC   | Degree of Cross-linking                          |
| DSC   | Differential Scanning Calorimetry                |
| ECM   | Extracellular Matrix                             |
| EWC   | Equilibrium Water Content                        |
| FEP   | Fluorinated Ethylene Propylene                   |
| FLOAT | Fast hydrogel stereolithography printing         |
| FMS   | Force Measurement System                         |
| GelMa | Gelatin Methacryloyl                             |
| HOD   | Deuterium hydrogen monoxide                      |
| LAP   | Lithium phenyl-2,4,6-trimethylbenzoylphosphinate |
| LbL   | Layer-by-Layer                                   |
| LED   | Light Emitting Diode                             |
| LDV   | Laser Doppler Vibrometry                         |
| MW    | Molecular Weight                                 |
| MTS   | Muscle Tissue Strip                              |
| NBW   | Non-bound Water                                  |
| NDW   | Normalised Dried Weight                          |
| NMR   | Nuclear Magnetic Resonance                       |

|         |                                                 |
|---------|-------------------------------------------------|
| NVF     | Normalised Volume Fraction                      |
| NWF     | Normalised Weight Fraction                      |
| OCT     | Optical Coherence Tomography                    |
| P407    | Poloxamer 407                                   |
| PBS     | Phosphate-Buffered Saline                       |
| PCL     | Poly-( $\epsilon$ -caprolactone)                |
| PDMS    | Polydimethylsiloxane                            |
| PEDOT   | Poly(3,4-ethylene dioxythiophene)               |
| PEG     | Poly(ethylene glycol)                           |
| PEGDA   | Poly(ethylene glycol) diacrylate                |
| PEGMEMA | Poly(ethylene glycol) Methyl Ethyl Methacrylate |
| P-h     | Load-Displacement                               |
| PNIPAM  | Poly(N-isopropylacrylamide)                     |
| QY      | Quinoline Yellow                                |
| RI      | Refractive Index                                |
| SBW     | Strongly Bound Water                            |
| SEM     | Scanning Electron Microscopy                    |
| SI      | Supporting Information                          |
| SLA     | Stereolithography                               |
| SMT     | Skeletal Muscle Mircotissue                     |
| TEMPO   | 2,2,6,6-tetramethylpiperidin-1-yl)oxyl          |
| TMS     | Tetramethylsilane                               |
| UV      | Ultraviolet                                     |
| WBW     | Weakly Bound Water                              |

# **Chapter 1**

## **General Introduction, Aims and Objectives and Thesis Outline**





## **1.1 Introduction**

Poly(ethylene glycol) diacrylate (PEGDA) hydrogels are well established in tissue engineering and organ-on-chip applications as scaffolds for 3D templates in aqueous environments due to their high water content, biocompatibility, low toxicity, and the ability to tune their physical, chemical and biological properties to control cell-material interactions [1–3]. PEGDA hydrogel characteristics cover a broad range and this contributes to their versatility as a platform for cell encapsulation [4–6], drug delivery [7–10] and tissue engineering [11–15]. A great advantage of using PEGDA hydrogels is that their mechanical properties can be modified and tuned for a specific application by changing their concentration, molecular weight, residual monomer, polymerisation techniques and curing methods [16–21].

When a specific biological response is required for an application, bioactive molecules can be introduced into the PEGDA hydrogel to control cell interactions and promote cell integration and tissue generation [22–24]. The mechanical environment of the hydrogel is another important factor that heavily influences cell adhesion, migration, and phenotype [25–27]. Therefore, to get the desired biological response from the cells, the physical, chemical and mechanical properties of the PEGDA hydrogel should be known and tuned precisely [28].

This research focuses on 3D multilayer PEGDA hydrogels, specifically focusing on their application in contractile tissue studies [16,29–35]. These hydrogels play a pivotal role in the formation of contractile muscle tissue strips [36–42]. The physicochemical and nanomechanical properties of these hydrogels are of

importance, not only for the specific biological tissue research at hand but also for broader implications in tissue engineering.

Using a 3D multilayer PEGDA hydrogel matrix with embedded anchoring cantilevers, created through 3D printing, allows for confinement and immobilisation of suspended cells, ultimately fostering growth of contractile muscle tissue strips [31,43–48]. To comprehend the mechanical intricacies and the impact of changing parameters, tests have been conducted to explore how alterations in PEGDA concentration and the diameter of the cantilevers can affect the mechanical behaviour of the multilayer cantilevers and, consequently, the contractile movements of the tissue strips (Figure 1-1) [31,49,50].

In the realm of contractile tissue studies, using beam bending theory is crucial. This theory allows for the calculation of the force exerted on living tissue based on cantilever horizontal deflection ( $\delta$ , unit: mm), and deflection force (DF, unit: N) using the following equations [31]:

$$DF = \frac{6EI}{(3Lx^2 - x^3)} \delta \quad (1-1)$$

$$I = \frac{\pi d^4}{64} \quad (1-2)$$

where ( $x$ , unit: mm) is the distance from the fixed end to the point of force application, ( $d$ , unit: mm) is the diameter of the cantilever, ( $L$ , unit: mm) denote the length of the cantilever, ( $I$ , unit: mm<sup>4</sup>) is the second moment of inertia for a cylinder shape cantilever, and ( $E$ , unit: MPa) is the elastic modulus.

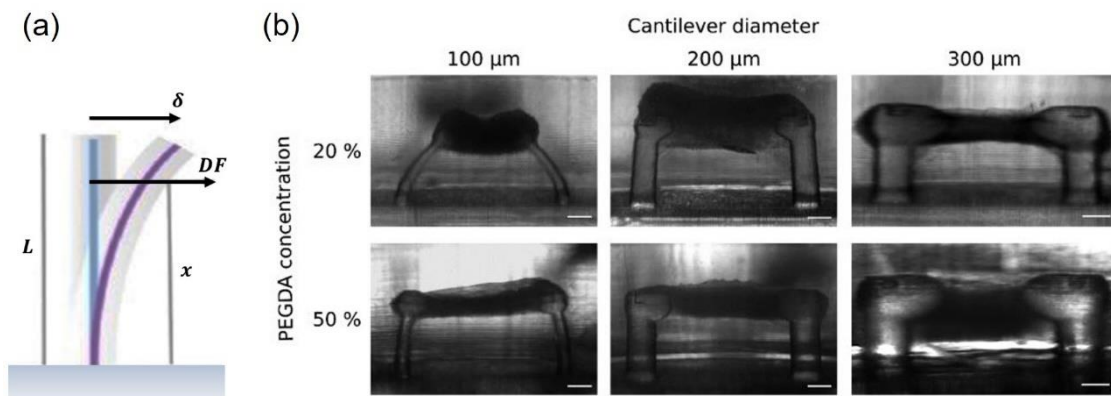


**Figure 1-1 (a) Illustration of deflection of a cantilever due to applied force. (b) Deflection of cantilevers with varying diameter and PEGDA concentration. All cantilevers are of equal height and the formed tissues consist of an equal number of cells. Scale bars: 200  $\mu\text{m}$ . [31]**

Figure 1-1b shows a strong correlation between cantilever stiffness and the ability of the tissue to deflect these cantilevers. Furthermore, the concentration of PEGDA seems to exert a significant influence on the contractile force exhibited by the formed tissue. In support Figure 1-2 shows that the stiffness of the cantilever increases almost linearly as both the diameter and PEGDA concentration increase.

These findings underscore the complex nature of 3D multilayer PEGDA hydrogel systems. They exhibit dynamic changes over multiple time-scales, often featuring local property variations that differ significantly from the bulk material [51]. To gain a comprehensive understanding of these intricate systems, it is vital to investigate their physical, chemical, and mechanical behaviour within a relevant physiological environment and to characterise their stability over the duration of intended cell and tissue studies.

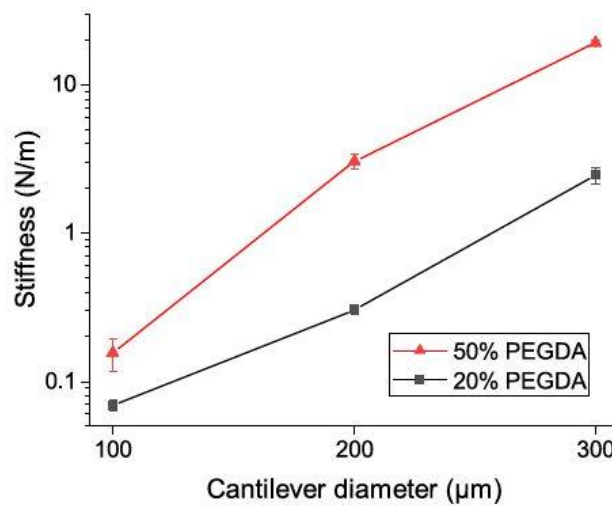


**Figure 1-2 The change in cantilever stiffness is dependent on the cantilever diameter and the concentration of PEGDA in the prepolymer solution. Error bars represent standard deviation of n=5. [31]**

This research also addresses several challenges in the investigation of PEGDA hydrogels. These include the need for hydration, the small sample size (often in the range of hundreds of microns, as depicted in Figure 1-3), and the specific environmental conditions they are subjected to [52]. Notably, one of the unexplored dimensions of PEGDA hydrogel behaviour is its aging effects, which introduce an element of uncertainty in predicting its mechanical behaviour and functionality over a defined timeframe.

The dimensional stability of 3D multilayer PEGDA hydrogels holds significance, primarily due to growing use as extracellular matrix (ECM) and support scaffolds in organ-on-chip devices. Achieving long-term dimensional and mechanical stability for the intended applications hinges upon the careful selection and optimisation of printing parameters. This encompasses considerations such as layer thickness, UV exposure time, and the choice of molecular weight, monomer

concentration, photoinitiator/photoabsorber in the prepolymer solution, and other crucial factors. These parameters must be refined not only for the precision and accuracy of the printing process but also for ensuring the enduring stability of the structures under real-world conditions.

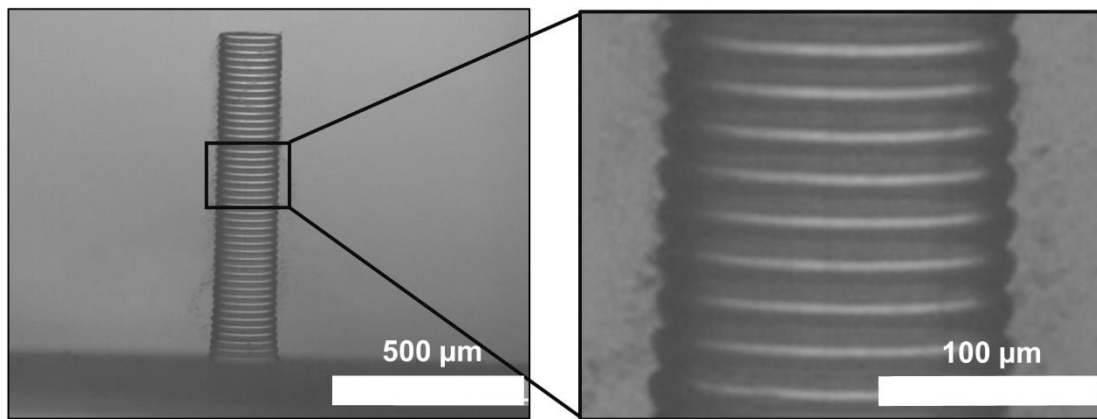


**Figure 1-3 Microscopic image of 3D printed PEGDA hydrogel cantilever using digital light processing (DLP) with layer thickness of 20  $\mu\text{m}$ .**

## **1.2 Scope of Research**

Based on the literature review, the following research gaps that are intended to be addressed are described:

1. Effect of external temperature on the long-term dimensional stability of multilayer UV cross-linked PEGDA hydrogels to address their thermal response in various environmental conditions that differ to the fabrication conditions has not been investigated.
2. An understanding of the basic nanomechanical behaviour and characteristics of multilayer UV cross-linked PEGDA hydrogels. The influence of layer by layer deposition and printing parameters such as UV exposure time,

number of layers, layer thickness on the elastic properties has yet to be elucidated.

3. Time dependent behaviour (aging) of hydrogel has not been investigated. The relationship between the environments such as temperature and cell culture medium has yet to be studied.

## **1.3 Aims and Objectives**

### **1.3.1 Aims**

The aim of this research is to measure physicochemical and nanomechanical properties of multilayer 3D printed UV crosslinked PEGDA hydrogels for use in organ-on-chip devices. The research may offer insight into stability of hydrogels through studying changes in both environmental and printing parameters, and this can be extrapolated to other biomaterials for benefit of other tissue engineering applications.

### **1.3.2 Objectives**

**One:** Develop in-house capability for repeatable and reproducible fabrication of 3D multilayer PEGDA hydrogels.

**Two:** Explain the effect of changing fabrication parameters, i.e., number of layers and UV exposure time, on nanomechanical properties of multilayer PEGDA hydrogels using nanoindentation. Additionally, examine the effect of photoabsorber concentration on degree of cross-linking of multilayer PEGDA hydrogels.

**Three:** Describe the stability of nanomechanical behaviour of multilayer PEGDA hydrogels stored in deionised water and cell culture medium for a duration of 30 days.

**Four:** Explore the nanomechanical properties of multilayer PEGDA hydrogels along the printing axis using atomic force microscopy (AFM) by probing the interface interlayers and examining the homogeneity of the mechanical properties across the entire length of the structure.

**Five:** Describe the effect of temperature variation between 8-45 °C on dimensional and weight change of multilayer hydrogels fabricated using projection lithography and monolithic hydrogels fabricated using bulk cross-linking. Investigating the anisotropic behaviour of multilayer and monolithic PEGDA hydrogel structures when temperature varies between 8 °C and 45 °C.

## 1.4 Thesis Structure

The thesis is constructed from research presented over 4 papers which are detailed in Table 1-1. The papers are numbered from 1 - 4 for reference with details of their status and their designation. The contributions of the PhD candidate towards the papers are outlined in Appendix A.

**Table 1-1 List of all research papers**

| Paper number | Chapter number | Title                                                                                            | Status    | Journal         | DOI                      |
|--------------|----------------|--------------------------------------------------------------------------------------------------|-----------|-----------------|--------------------------|
| 1            | 2              | Additive Manufacturing and Physicomechanical Characteristics of PEGDA Hydrogels: Recent Advances | Published | Polymers (2023) | 0.3390/poly<br>m15102341 |

|   |   |                                                                                                                                                 |           |                                          |                                      |
|---|---|-------------------------------------------------------------------------------------------------------------------------------------------------|-----------|------------------------------------------|--------------------------------------|
|   |   | and Perspective for Tissue Engineering                                                                                                          |           |                                          |                                      |
| 2 | 3 | Thermal response of multilayer UV cross-linked PEGDA hydrogels                                                                                  | Published | Polymer Degradation and Stability.(2022) | 10.1016/j.polydegradstab.2021.109805 |
| 3 | 4 | Nanoindentation response of 3D printed PEGDA hydrogels in hydrated environment                                                                  | Published | ACS Applied Polymer Materials (2023)     | 10.1021/acsapm.2c01700               |
| 4 | 5 | Modulus Heterogeneity in 3D Printed Poly(ethylene glycol) diacrylate Hydrogels: Atomic Force Microscopy Investigation under Hydrated Conditions | Published | ACS Applied Polymer Materials (2023)     | 10.1021/acsapm.3c00197               |

Three main specimen types were used throughout the project:

1. Multi-20  $\mu\text{m}$ : Multilayer hydrogels with layer thickness of 20  $\mu\text{m}$
2. Multi-150  $\mu\text{m}$ : Multilayer hydrogel with layer thickness of 150  $\mu\text{m}$
3. Mono-1200  $\mu\text{m}$ / 5mm: Monolithic hydrogel samples with 1.2 mm or 5 mm thicknesses

A review of literature and a separate detailed introduction to the topics specific to each paper is given within the papers themselves. A summary overview of each paper highlighting the specific questions it sets to answer, and its contributions to knowledge is given in Appendix B.



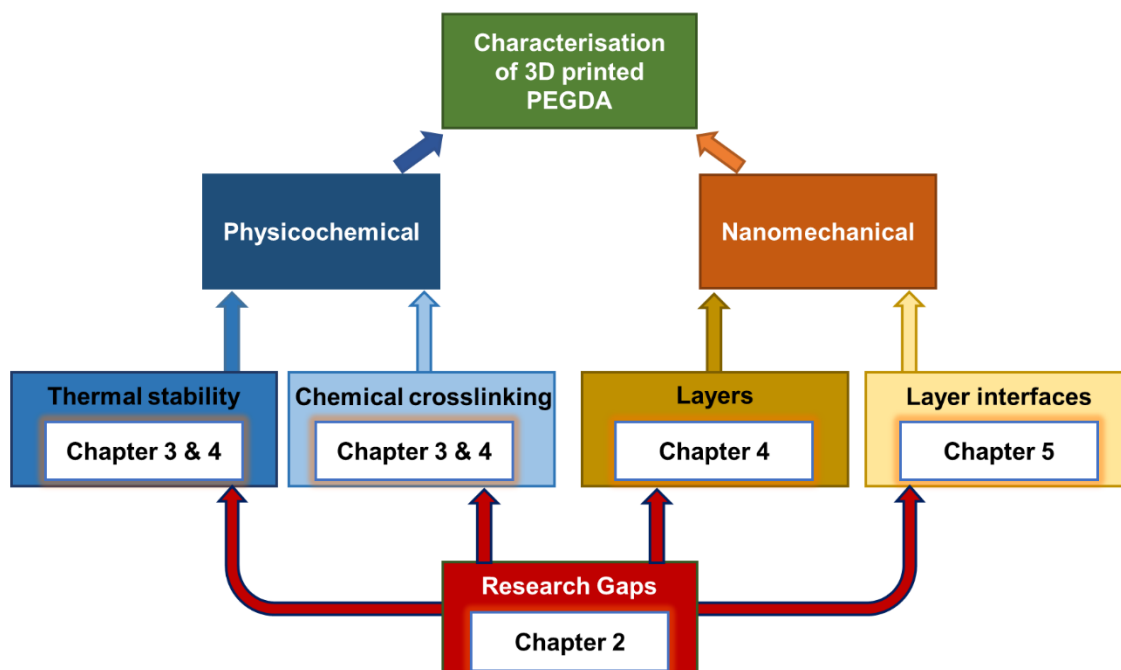


**Figure 1-4 Links between the papers and the topics covered in the thesis.**

Each chapter addresses specific objectives of the research project, and these contributions are indicated in Table 1-2. Collectively the papers add to the current understanding of 3D printed PEGDA hydrogels and help inform future research in the field of tissue engineering and additive manufacturing. The topics are interlinked, and they are covered in each paper as presented in Figure 1-4. Chapter 2 focuses on reviewing the literature on PEGDA hydrogel and its application within tissue engineering and organ-on-chip devices. Chapter 3 aims to address objectives related to physicochemical characteristics of 3D PEGDA hydrogels and their response to environmental changes. Chapter 4 looks at the effect of layered structure on the surface nanomechanical behaviour of 3D PEGDA hydrogels and extends the physicochemical characterisation with time. It reviews additional printing parameters and their effect on the physicochemical and mechanical behaviour of PEGDA hydrogels. Chapter 5 investigate the

heterogeneity in the mechanical characteristics of multilayer PEGDA hydrogels and maps the modulus of the multiple layers in a hydrated environment. Chapter 6 provides a general discussion of the findings from the research papers, their implications to the wider tissue engineering community. Chapter 7 draws conclusions from the research conducted and outlines the future work with impact for industry and recommendations to the sponsoring company.

**Table 1-2 Objectives covered in each chapter**

| Chapter # | Objectives covered |
|-----------|--------------------|
| 3         | 1,2,5              |
| 4         | 2,3                |
| 5         | 4                  |

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# **Chapter 2**

## **Additive Manufacturing and Physicomechanical Characteristics of PEGDA Hydrogels: Recent Advances and Perspective for Tissue Engineering**

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## **Abstract**

This short review discusses the recent advancements in the use of poly(ethylene glycol) diacrylate (PEGDA) hydrogels for tissue engineering applications. PEGDA hydrogels have attracted significant interest in the biomedical and biotechnology community due to their soft and hydrated properties, which closely mimic those of natural tissue. They can be manipulated through exposure to light, heat and the introduction of cross-linkers to achieve desirable functionalities. The review compares and examines the two main synthesis methods of bulk cross-linking and three-dimensional (3D) printing of PEGDA hydrogels. Characterisation methods are also reviewed, with a focus on 3D printed PEGDA hydrogels, to understand the physical, chemical and mechanical characteristics of these hydrogels. The review also highlights the current state of development of biomedical applications, particularly in tissue engineering and organ-on-chip devices. Lastly, the review discusses the existing challenges and future prospects of engineering 3D PEGDA hydrogels for tissue engineering and organ-on-chip devices.

## 2.1 Introduction

Hydrogels have been extensively studied in recent years as potential solutions for biomedical applications such as tissue engineering and organ-on-chip devices. This is due to their biocompatibility and ability to be tailored for specific physical, chemical and mechanical properties [1–5]. Additionally, the structure and properties of hydrogels mimic the micro-environment of various human organ tissues [6–13] and thus, hydrogels are widely used in a diverse range of tissue engineering and organ-on-chip devices [14–16]. Among the hydrogels, poly(ethylene glycol) diacrylate (PEGDA) hydrogels have received particular attention as three-dimensional (3D) tissue-engineered constructs, bio-sensing medium, and drug-controlled release matrices due to their excellent properties such as hydrophilicity [17–20].

Due to its biocompatibility and ability to mimic the extracellular matrix (ECM) of living tissues, PEGDA hydrogel has found extensive use in various biomedical applications [21,22]. PEGDA hydrogels are synthesised by the cross-linking of PEG diacrylate monomers, which results in a three-dimensional network of polymer chains that can retain large amounts of water [23]. The first reported use of PEGDA hydrogel in biomedical applications was in the 1970s, when it was used as a scaffold for in vitro cell culture. PEGDA hydrogels were found to be biocompatible and non-toxic, making them suitable for use in cell culture studies (Figure 2-1) [24,25]. In the 1980s, PEGDA hydrogels were further developed for use in drug delivery applications. The hydrogels were found to be able to retain drugs in their pores, and release them in a controlled manner over time [26]. This property made PEGDA hydrogels suitable for use in the sustained release of

drugs for the treatment of chronic diseases [27]. In the 1990s, PEGDA hydrogels were also used in injectable wound healing applications [28]. These hydrogels were found to be able to promote the growth of new blood vessels, and to promote the growth of new tissue in wounds. In the 2000s, PEGDA hydrogels were further developed for use in tissue engineering applications [29,30]. The compatibility of hydrogels with diverse cell types, including stem cells, has been demonstrated, and they facilitate the development of new tissue [31,32]. The capacity of PEGDA hydrogels to imitate the extracellular matrix (ECM) of living tissue enhanced their appeal for tissue engineering applications [33–38]. This property made PEGDA hydrogels suitable for use in the regeneration of various types of tissues, such as cartilage and bone [39–42]. In recent years, PEGDA hydrogels have been further developed for use beyond tissue engineering into other biomedical applications, such as in vivo drug delivery and wound healing [43–46]. The hydrogels have also been used in various other applications, such as in the fabrication of micro- and nano-devices [47–49].

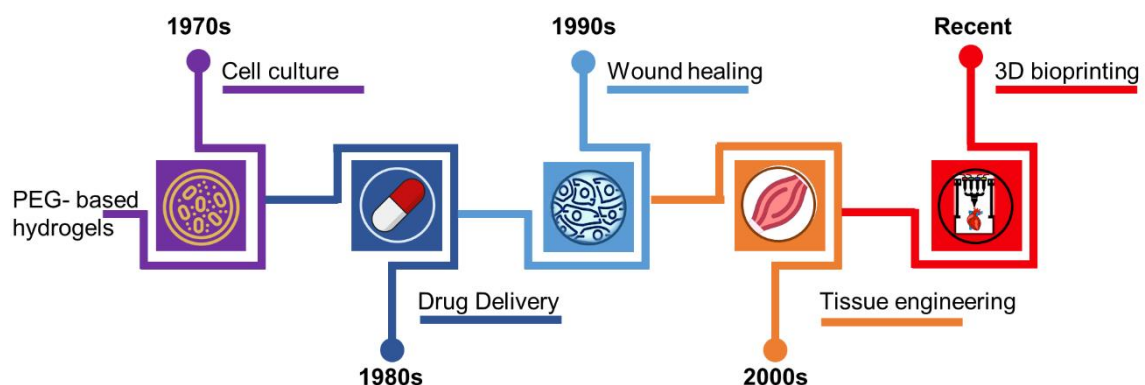


**Figure 2-1 An overview of use of PEG-based hydrogels in biomedical and biological applications.**

Moving away from moulding and bulk cross-linking processes, 3D printing using stereolithography (SLA) and digital light processing (DLP) among other advanced bioprinting techniques offer a faster and more dependable manufacturing approach for creating complex scaffold shapes with reliable mechanical properties [50–53]. The use of 3D printed PEGDA hydrogels have been studied extensively for developing skeletal muscle microtissues and contractile cardiac tissue as the demand for PEGDA hydrogels continues to grow (Figure 2-2). However, there are still critical gaps in knowledge regarding the behaviour and properties of 3D printed PEGDA hydrogels. Further research is needed to understand the impact of layer-by-layer (LbL) fabrication, isotropic behaviour, homogeneity, and stability of the 3D printed PEGDA hydrogels. In addition, more investigation is required to determine the basic nanomechanical behaviour of 3D PEGDA hydrogels, and how different fabrication parameters such as layer thickness, ultraviolet (UV) dosage can affect their surface behaviour. Techniques such as nanoindentation and atomic force microscopy (AFM) are suitable for evaluating the surface properties, including the spatial variation in mechanical properties that are crucial for cell adhesion and tissue formation, as well as assessing the orientation between the applied force and layers in multilayer 3D printed structures fabricated using LbL techniques [54–56]. A large body of literature reports that mechanical cues at the substrate interface on which the cells are cultured strongly influence cell behaviours, including growth, migration, proliferation, differentiation, and tissue formation [57–62]. The surface elastic modulus, topography, and adhesion of hydrogel substrates can direct the fate of





## **2.2 Manufacture of 3D PEGDA Biostructures**

The scalability of 3D printing is a crucial factor in the rapid fabrication of 3D biomimetic structures for tissue engineering and regenerative medicine. This technology allows for precise control of structure and size, which can be customised to fit specific needs, and the cost-effective production of complex structures in large quantities. Therefore, the combination of additive manufacturing techniques, bio-materials, cells, and the scalability of 3D printing offers a powerful approach for the development of functional tissues and organs that can meet the clinical demands of tissue engineering and regenerative medicine. Various 3D bioprinting strategies have been developed over the past decade, each with its own unique advantages and limitations. In this section, we will discuss their working principles, and their suitability for different applications (Figure 2-3b).

### **2.2.1 Fabrication Techniques for PEGDA Hydrogels**

Different techniques have been developed to perform photo-cross-linking on PEGDA, which include the utilisation of bulk cross-linking, 3D printing, and microfluidic methods. Each of these techniques offers unique advantages and limitations and can be chosen based on the specific requirements of the application.

Bulk cross-linking of PEGDA hydrogel is the most straightforward process among the other two techniques, it involves mixing the monomers and initiators together in a container (mould) and then cross-linking them under controlled conditions (Figure 2-3a) [67–69]. The key to this process is controlling the conditions under which the monomers are cross-linked. This includes factors such as temperature,

time, and the concentration of photoinitiator used. However, due to the nature of bulk cross-linking, it can lead to inhomogeneity in cross-linking, which can affect the mechanical properties of the hydrogel [70]. This inhomogeneity is caused by the distance of light source to the hydrogel and the direction of light, which results in variations in the intensity of light reaching different parts of the hydrogel, leading to non-uniform cross-linking [71,72]. By carefully controlling these conditions and the distance and direction of the light source, it may be possible to minimize the inhomogeneity in cross-linking and mechanical properties, but it can't be eliminated.

Extrusion-based 3D printing is a technique which involves creating structures made from melted materials (filament) using rollers and a heating system. However, the technique has undergone rapid development and expansion into bioprinting, becoming the most popular technique in the field. Filaments are replaced with hydrogels and bioinks containing living organisms, which are extruded through a shear-thinning process rather than being melted through the application of heat [73,74]. The main working principles that have been developed for extrusion-based bioprinting include pneumatic- and mechanical-driven fluid dispensing systems. Pneumatic systems can be valve-free or valve-based, while mechanical systems are mainly controlled by a piston or a screw (Figure 2-3c). Extrusion-based bioprinting has several advantages, including the capacity to deposit high-viscosity bioinks and large cell densities, and the ability to continuously extrude bioinks without interruptions [75,76]. However, their bioprinting speed is relatively slow and cell viability is usually moderate due to high shear stresses experienced by cells during the extrusion process [77,78].

Despite these limitations, extrusion-based bioprinting has been widely adopted in bioprinting due to its simplicity in instrumentation [79]. In an experiment, a combination of PEGDA hydrogel and charged monomers such as 3-sulfopropyl acrylate, along with Poloxamer 407 (P407), were used for extrusion printing. The material was successfully printed in a pyramidal shape but its use for direct cell encapsulation might be limited due to P407 cytotoxicity [46]. Another study used to produce human-scale constructs such as nose and ear using a combination of Gellan gum and PEGDA. Murine bone marrow stromal cells (BMSCs) and a mouse osteoblastic cell line (MC3T3-E1) were encapsulated in the constructs, which showed structural stability, biocompatibility, and non-toxicity [80]. The technique was further developed to create 3D printed tracheal utilizing PEGDA, sodium alginate and calcium sulphate using a home-made extrusion bioprinter with assisted UV laser. The results showed that by optimizing the printing parameters such as laser power intensity the stiffness of the printed biological tissue can be altered to match that of the biological tissue or organ [81]. Extrusion based printing technologies are developed further to include multi-functional, multi-material 3D printing that can be utilized to print PEGDA hydrogels with fabrication strategies for living tissues and soft robotics [82].

Inkjet printing enables precise and non-contact deposition of small quantities of materials on diverse surfaces. It offers two types of printing: continuous and drop-on-demand (DOD) [83], in which cells and biomaterials can be patterned into desired substrates by repeatedly depositing droplets at pre-designed locations to form a structure (Figure 2-3d) [84]. Inkjet bioprinting provides higher resolution but may have limitations in terms of achievable cell densities and printing speed,

as compared to extrusion-based bioprinting [85]. A biocompatible ink was created by combining Pluronic F127 (PEO-PPO) and PEGDA. The resulting gel structures were stable in an aqueous environment and were able to be seeded with fibroblast cells delivered by an inkjet printer [86]. Another biocompatible ink was developed by synthesizing a photo-cross-linkable polycaprolactone dimethylacrylate (PCLDMA) and mixing it with PEGDA. They optimized the ink properties under a nitrogen atmosphere during printing, demonstrating its potential for 3D structure production with good biocompatibility [87]. In another study, PEGDA hydrogel and Irgacure 2959 photoinitiator were combined to create micropatterns on the surface of a thin PEGDA layer that was deposited on a polycarbonate substrate using inkjet printing. The micropatterns (wrinkles) were created through plasma etching and were intended to enhance cell adhesion and differentiation [88].

Manufacture of PEGDA hydrogels using microfluidics is a cutting-edge technique that utilizes microfluidics channels to produce hydrogels with a high degree of control over their properties (Figure 2-3e) [89]. Microfluidics is a field of research that deals with the manipulation of fluids at a microscale, and microfluidic devices are designed to control the flow of fluids through tiny channels. In the case of PEGDA hydrogels, microfluidic devices are used to control the flow of the monomers and initiators that are used to create the hydrogel [90]. This allows for precise control over the concentration and ratio of the monomers and initiators, which in turn allows for precise control over the properties of the resulting hydrogel [91]. For example, by controlling the flow of the initiator, it's possible to achieve different degree of cross-linking in the hydrogel, which will affect the

mechanical properties of the hydrogel [92]. One of the major advantages of microfluidic fabrication is the ability to produce PEGDA hydrogels with highly defined microstructures. For example, by using microfluidic devices, it's possible to create hydrogels as microspheres and micromoulds [93]. These microstructures are particularly useful in cell encapsulation and drug delivery applications, as they can be used to create hydrogels that mimic the natural microenvironment of cells and allow for controlled release of drugs [94–97].

Another way to create 3D PEGDA scaffold architectures is through use of SLA and DLP which are vat photo-cross-linking technologies that share similarities but also exhibit significant differences (Figure 2-3f) [98–102]. In SLA, a vat of photopolymer resin is subjected to an ultraviolet (UV) laser. The UV laser projects a predetermined shape onto the photopolymer vat, which reacts to the UV light and forms a single layer of the desired 3D object. The build platform then lowers one layer, repeating the process for each layer of the design until the 3D object is finished [103]. DLP is a variation of SLA that utilizes a digital projector to flash a single image of the layer across the entire resin at once using ultraviolet (UV) or visible light [104]. DLP incorporates a digital micromirror device (DMD) chip, composed of an array of reflective aluminium micromirrors that redirect incoming light from the UV source to project an image of a designed pattern or a layer of a 3D CAD model onto the resin [105]. The primary difference between the two 3D printing technologies is their light sources. SLA uses a UV laser beam, while DLP uses UV light from a projector. In DLP, the UV light source remains stationary and cures the complete layer of resin at once, making it faster but with a fixed resolution that ranges from 20 to 100 microns [106]. On the other hand, SLA's

laser beam moves from point to point, allowing for higher accuracy and better-quality prints with intricate details with smaller than 25-micron XY resolution [107,108]. Additionally, SLA 3D printers have a more scalable build volume, whereas DLP printers are optimized for specific use cases. These two techniques and their application in creating 3D PEGDA hydrogels for tissue engineering are discussed further in the following sections.

3D printing has gained popularity as a method of fabricating PEGDA hydrogels due to its ability to provide controlled homogeneity in cross-linking and mechanical properties, which is superior to that of bulk cross-linking [109,110]. Techniques such as extrusion-based printing [111], SLA and DLP printing offer precise control over the hydrogel's shape and properties and allows for rapid creation of complex shapes [73,112–114]. SLA is a highly advanced technique for 3D printing hydrogels, using a LbL approach and UV to control photo-cross-linking [101,115]. DLP of PEGDA hydrogel compositions with varying water content and concentrations of a photoinitiator and light absorber showed promising potential for patient-specific implant solutions [116]. Another study investigated DLP 3D printing of PEGDA-based photopolymers with Dexamethasone (DEX) drug-loading, finding that drug release is prone to burst-release and DEX degrades significantly in the 3D-printed samples over time [117].

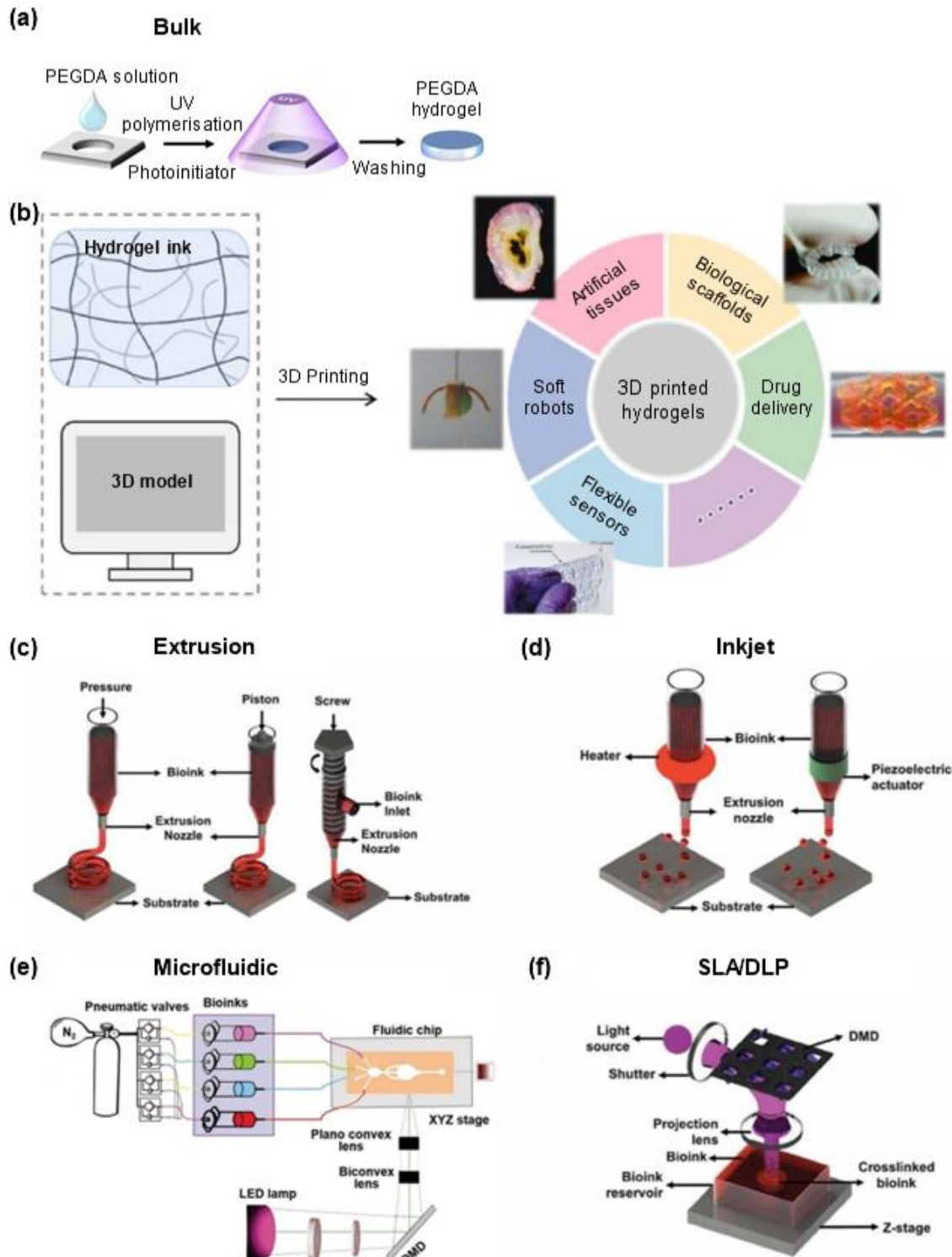


Figure 2-3 (a) Schematic illustration of synthesis of PEGDA hydrogels using a mould technique for bulk cross-linking [118]. Reproduced with permission from ref [118]. Copyright 2016 Elsevier. (b) 3D printing of hydrogels for various biomedical applications [112]. Reproduced with permission from ref [112].

**Copyright 2022 Elsevier. Schematic of 4 different 3D printing techniques used for 3D PEGDA hydrogel fabrication, (c) Extrusion [119], (d) Inkjet [119], (e) Microfluidic-based 3D printing [99], (f) SLA/DLP [119]. (c,d,f) reproduced with permission from ref [119]. Copyright 2019 Wiley. (e) reproduced with permission from ref [99]. Copyright 2018 Wiley.**

Bulk fabrication offers a wide range of material options and is more suitable for products with simple geometries that are cross-linked at once. The process is relatively simple and requires less complex systems and processes. However, creating the physical mould of a part or product requires significant upfront capital expenditure (CAPEX) for equipment such as CNC machines, making it more suitable for large-scale production [120,121]. Additionally, the size of the structural features that can be produced is limited to millimetre size, which may not be suitable for applications requiring micro- or nano-scale features (Figure 2-4).

On the other hand, 3D LbL printing offers design agility and the ability to produce complex geometries with high precision, with the size of the structural features that can be produced in micrometre size. Scalability can be an issue but could be more cost-effective for small-scale production. The choice of materials is also limited due to its reliance on specialized monomers and photoinitiators with specific viscosity, transparency, and requirement of additives like photoabsorbers. The process is also slower due to the layer-by-layer printing process, and the available material options are generally more limited compared to bulk fabrication methods.



Higher defects in 3D printing may be a result of the LbL fabrication process and the additional interfaces that are created within the printed structure. This can lead to defects such as delamination, voids, and warping. The design flexibility of 3D LbL printing, which can lead to more complex geometries, can make the printing process more difficult, resulting in higher defects (Figure 2-4).

In terms of operating expenditure (OPEX), for bulk fabrication once the mould is created, the OPEX may be relatively low, particularly if the process is automated and requires minimal labour [122]. Bulk fabrication can be considered a highly scalable process, meaning that it is possible to produce many identical or similar parts quickly and efficiently (Figure 2-4).

The choice between bulk fabrication and 3D LbL printing depends on the specific needs of the production process, including the scale of production, complexity of the design, required material properties, and budget constraints. Manufacturers must carefully consider all these factors before deciding on a manufacturing method. In evaluating the capital and operational costs of each process, manufacturers can make an informed decision that meets their production needs and aligns with their budget constraints.

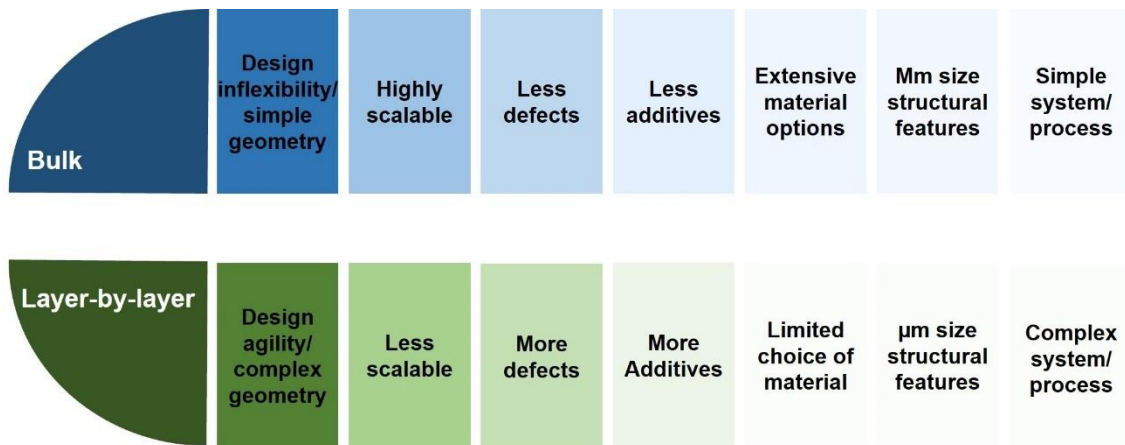


**Figure 2-4 Key features of bulk and 3D LbL fabrication processes.**

### **2.2.2 Photo-Cross-Linking Mechanism**

PEG, or polyethylene glycol, is a hydrophilic, water-soluble and biocompatible polymer. Its molecule is neutral, highly mobile and hydrated in aqueous solution [105,106]. PEGDA is a derivative of PEG in which the hydroxyl groups at the ends of the PEG chains are replaced with acrylates. PEGDA hydrogels are considered synthetic hydrogels that are chemically cross-linked through the photo-cross-linking technique, in which a UV or visible light source is used to induce the cross-linking [107]. This rapid and easy synthesis technique allows for the creation of a soft polymer with tailored physical, chemical, and mechanical properties [108,109]. When PEGDA hydrogels are exposed to UV or visible light, the photoinitiator molecules absorb the light energy and generate free radicals. These free radicals then react with the double-bond acrylate groups present at the end of every PEG chain, leading to the formation of covalent bonds between the PEG chains (Figure 2-5a,b). This results in the formation of a 3D cross-linked polymer network, which gives the hydrogel its mechanical strength and shape

[110,111]. In brief, PEGDA-based hydrogels are synthesised by dissolving PEGDA powder in deionised water at a specific ratio. A photoinitiator is added to the solution and then exposed to UV light for a set amount of time to initiate photo-cross-linking [112]. The mechanical properties of the hydrogel can be adjusted by changing the molecular weight, pre-polymer concentration, and photo-cross-linking conditions. For example, increasing the pre-polymer concentration can increase the compressive modulus of the hydrogel but also decrease its hydrolytic degradation rate [113].

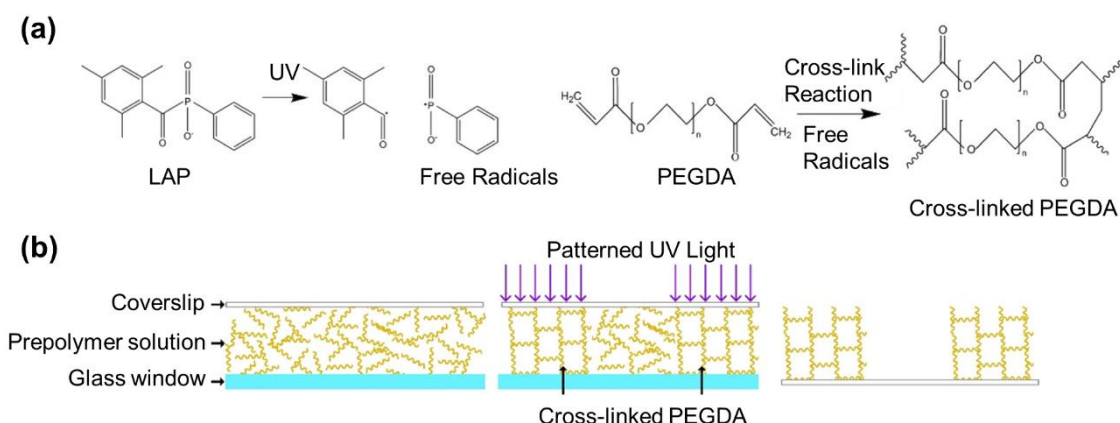


**Figure 2-5 (a) A schematic illustrating the production of free radicals from a photoinitiator, Lithium phenyl-2,4,6-trimethylbenzoylphosphine oxide (LAP), and the resulting cross-linking of PEGDA monomers by these free radicals. (b) A diagram depicting a typical photo-cross-linking process [114]. Reproduced with permission from ref [114]; Copyright 2018 America Chemical Society.**

## 2.3 Properties of Photo-Cross-Linked PEGDA Hydrogels

### 2.3.1 Swelling Behaviour and the Effect of the Environment

The ability to control the elasticity, swelling, and degradation of hydrogels is crucial in biomedical applications, particularly in tissue engineering. Hydrogels are a type of polymer that have the ability to absorb and retain large amounts of

water, which leads to the formation of a gel-like structure (Figure2-6a). They are widely used in biomedical applications due to their biocompatibility and ability to mimic the mechanical properties of natural tissue (Figure2-6b). The physical properties of hydrogels can vary depending on the type of polymer and the conditions under which they are prepared. Varying the molecular weight and concentration of the PEGDA in the prepolymer solution can significantly affect the structural and physical properties of the hydrogels (Table 2-1) [134].

Increasing the molecular weight of PEGDA from 3.4 kDa to 20 kDa in the prepolymer solution while maintaining a 10% precursor concentration, led to a 101% increase in swelling ratio and 190% increase in mesh size resulting in higher cell proliferation [135]. To the contrary, an increase in PEGDA concentration resulted in a decrease in hydrogel swelling, mesh size and limited vessel invasion which may be required for neovascularisation [136]. UV irradiation of PEGDA solution with different UV dosage resulted in hydrogels with varying cross-link density. Increased UV exposure time led to a decrease in swelling capacity due to enhanced cross-linking and higher elastic response in the polymer chains [137]. The PEGDA hydrogel's swelling ratio is reduced with increasing ionic strength in the physiological range (0-300 mM). The hydrogel's swelling ratio and mesh size gradually increase as the pH is raised. This was observed across the entire pH range tested (2.5-11) with an increase of about 10% [137]. PEG hydrogels are often used in short-term, in vitro studies as non-degradable controls due to the hydrolytic stability of the poly(ether) backbone. A 6-week study of storage of PEGDA hydrogels in PBS showed a slight increase in

swelling ratio of 5-10% but it was within the standard deviation, indicating no degradation [138].

Fabrication techniques used for making the hydrogel can also affect the swelling kinetics of hydrogels. Research has shown that a 3D printed GelMA/PEGDA hydrogel produced using extrusion based printing can exhibit a nearly 10% increase in swelling ratio when compared to bulk cross-linked hydrogels due to the presence of pores within the 3D printed hydrogel matrix, which increases the surface area exposed to the PBS solution, resulting in increased water absorption [139]. In an extrusion based printed Poly(N-isopropylacrylamide) (PNIPAM)/PEGDA bilayer the PEGDA composite fiber was reported to have a swelling ratio in the range of 0.1-3 [124]. In SLA printing, a variety of 3D printed PEGDA hydrogels with different molecular weights and concentrations have been synthesised in combination with other polymers such as Poly-( $\epsilon$ -caprolactone) (PCL), Poly(ethylene glycol) Methyl Ethyl Methacrylate (PEGMEMMA), and poly(3,4-ethylenedioxythiophene) (PEDOT) for biomedical applications. The swelling ratios of these hydrogels range from 0.03 to 73.00, as reported in studies [67,140,141]. The values of swelling ratio reported in literature may vary due to the different methods used to calculate it (Table 2-1); some use percentage increase from dried hydrogel [142–144], others do not multiply the results by 100 [145–147] and others calculate by dividing the swollen state hydrogel by the dried hydrogel [124,148].

Examining the volumetric change as a result of swelling and water uptake is crucial when it comes to printing resolution and fidelity of the printed parts (Figure2-6c). A study found that dog-bone shaped 3D printed PEGDA (575 Da)

had a swelling ratio of approximately 35%, but there was a dimensional change of 15% in length/width and 40% in thickness [144]. When measuring the swelling ratio of bulk PEGDA hydrogel cylinders with molecular weights of 10 kDa and 20 kDa, it was found that the volumetric swelling ratio calculated by measuring the physical dimensions of the hydrogel with optical microscopy was not proportional to that of the gravimetric swelling ratio measured by weighing the samples [149]. This means that while the swelling ratio indicates a change in volume, the volumetric percentage change may be different from the swelling, as the dimensional changes in length/width and thickness are also considered. A study that focused on microfluidic channel fabrication found that the mixture of water-miscible acrylate (HBA), PEGMEMA, and PEGDA resulted in internal cross-sections as small as 71  $\mu\text{m}$ , which is smaller than the intended internal diameter of 108  $\mu\text{m}$ . This was due to the swelling and volume change of the hydrogel material during the fabrication process [150]. To optimise the dimensional accuracy of 3D printed PEGDA hydrogels, a study varied UV exposure time and PEGDA concentration. Results showed that increasing UV exposure decreased accuracy and as PEGDA concentration increased from 2% to 80%, the percentage change in lateral dimensions while swelling also increased from 1% to 11% [151]. The volumetric degree of swelling can also be calculated indirectly by measuring the swelling ratio while taking into account the solvent and polymer density. However, that approach might not be as accurate because it does not directly measure the volume change but assumes constant solvent density and the density of the initial prepolymer solution which may change due to incomplete cross-linking after the initial photo-cross-linking process [149,152].

It is also important to understand the swelling kinetics of PEGDA hydrogels in cell culture media within the relevant physiological environment [153]. In many studies, the use of phosphate-buffered saline (PBS) at a pH level of 7.4 was implemented to replicate the necessary physiological conditions for cell culture [154–159]. It was reported that a combination of Poly( $\epsilon$ -caprolactone) maleic acid (PGCL-Ma) & PEGDA produced by bulk cross-linking has resulted in no degradation when stored for 20 days [142]. Introduction of PEGDA in a PEGDA/GelMA hydrogel also has shown to reduce the enzymatic degradation profile of the hydrogel photo-cross-linked with UV light in PBS [139,146]. However, a bulk photo-cross-linked PEGDA hydrogel on its own has shown to undergo a very slow degradation when stored in BPS at 37 °C for a duration of 8 weeks [160]. The slow degradation of ester linkages in acrylated PEG diols in vivo can take months or even years [138]. However, higher molecular weights and lower concentrations could significantly accelerate the degradation of PEGDA hydrogel due to higher ratios of hydrolysable esters and lower cross-link density [156].

Temperature can affect the swelling kinetics of PEGDA hydrogels. While a study reported insensitivity to changes in temperature and solvent composition, it was found that bulk cross-linked PEGDA hydrogels had slightly higher swelling ratios when incubated in deionised water at 37°C compared to room temperature [124,137]. However, a recent study that looked reversible swelling/deswelling mechanism using environmental scanning electron microscopy (SEM) showed that the depending on degree of cross-linking the 3D printed PEGDA fabricated by SLA can revert back close to its original volume when the temperature varies

between 4-15 °C [161]. The reason for this is that when temperature is low, the volume of the hydrogel increases due to the absorption of condensed water, and when the temperature increases, the water is desorbed from the hydrogel and the volume reduces again. This phenomena can occurs when the samples are not fully submerged in the storage solution.

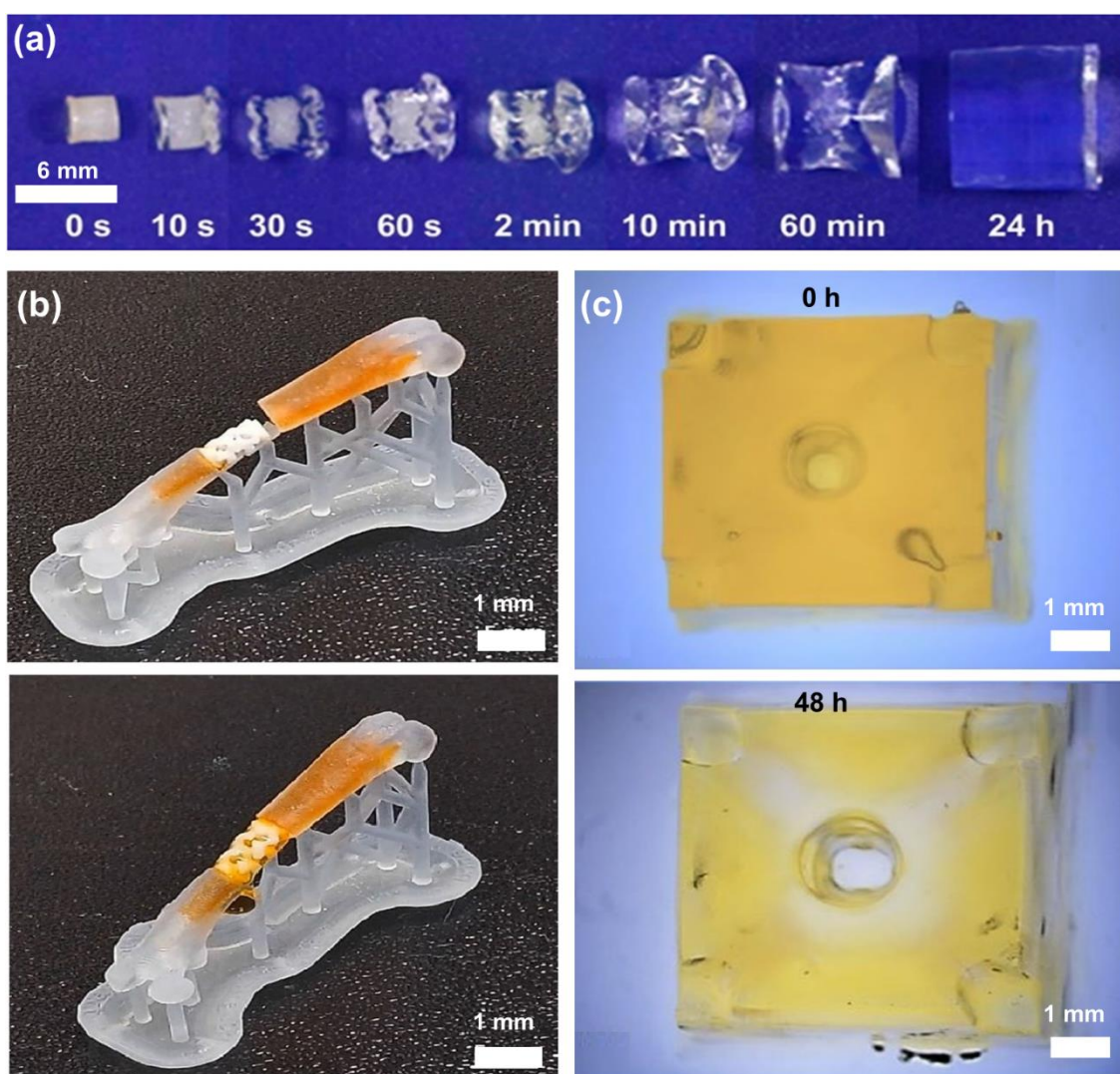


**Figure 2-6 Swelling behaviour of PEGDA hydrogels at different scale and shape complexity. (a) Side view of swelling of bulk polymerised PEGDA hydrogel in at 37 °C for first 24 hrs [162]. Reproduced with permission from ref [162]. Copyright 2017 American Chemical Society. (b) Images of a complex brushite/PEGDA hydrogel**



biocomposite implant that can be implanted into a bone defect and fixed through swelling. The biocomposite is initially placed freely in the dry state (top), but swells and tightly fills the defect within 30 mins by absorbing a red-colored aqueous solution containing an azo dye (bottom) [163]. Reproduced with permission from ref [163]. Copyright 2020 Elsevier. (c) 3D-printed samples in water stored for 48 hrs, showing an increase in inner channel and overall structural dimensions [164]. Reproduced with permission from ref [164]. Copyright 2021 Springer Nature.

The properties of hydrogels depend on the type of polymer and the chemical and physical bonds between polymer chains. Ideally cross-linked hydrogels have better mechanical properties than non-ideally cross-linked ones, but the effect of these cross-linking sites on water and polymer interaction is not well understood [165]. Photo-cross-linking process can be hindered by factors such as oxygen inhibition, which can lower the conversion rate of the prepolymer solution. Acrylates are particularly sensitive to oxygen inhibition, and the dissolved oxygen in the solution must be completely consumed before cross-linking can begin, leading to a slower reaction rate [161]. PEGDA ( $\text{CH}_2=\text{CHCO}(\text{OCH}_2\text{CH}_2)_n\text{OCOCH}=\text{CH}_2$ ) is a type of PEG that has repeated ethylene oxide units and active end groups which can be cross-linked by exposing it to a light source and a photoinitiator [166]. FTIR spectroscopy was used to study the surface chemical composition of bulk PEGDA membranes with varying PEGDA monomer concentrations. The spectra for all cross-linked samples were mostly identical, indicating similar chemical composition [166]. The gel fraction was used as a complementary technique to qualitatively evaluate the efficiency of the network formation or degree of cross-linking [167]. In another

study, the chemical structure was characterised by proton nuclear magnetic resonance ( $^1\text{H}$  NMR) spectroscopy where generated spectra indicated that carbon–carbon double bonds of PEGDA monomer were photo-cross-linked in presence of photoinitiator [168]. An indirect way to qualitatively examine the degree of cross-linking or conversion of the double bonds in PEGDA hydrogel is through measuring the peak areas of  $\text{C}=\text{C}$  versus  $\text{C}-\text{O}-\text{C}$  bonds to indicate the degree of conversion of acrylate groups [153]. Understanding the degree of cross-linking can aid in understanding how it affects the microstructure, porosity, and mechanical properties of PEGDA hydrogels. The following section discusses the different characterisation tools used for understanding the mechanical properties of PEGDA hydrogels with more focus on SLA fabricated samples.

**Table 2-1 Swelling characteristics of PEGDA containing copolymer**

| PEGDA containing copolymer                                      | Molecular weight (kDa) | Conc (%)   | Bulk/3 D              | Sample type                                                        | Storage solution | pH  | Temp (°C) | Volumetric change Yes/No         | Duration (day) | Degradation                | Swelling ratio | Ref   |
|-----------------------------------------------------------------|------------------------|------------|-----------------------|--------------------------------------------------------------------|------------------|-----|-----------|----------------------------------|----------------|----------------------------|----------------|-------|
| Poly-N-isopropylacrylamide (PNIPAM)/PEGDA                       | n/a                    | 10         | 3D-extrusion          | Multilayer, layer thickness 260 µm                                 | Water            | n/a | RT        | Yes, reversible between 20-50 °C | 1              | No                         | 0.1-3          | [124] |
| PEGDA/poly-(ε-caprolactone) (PCL)                               | 1                      | 15         | 3D-SLA-DLP            | n/a                                                                | Water            | n/a | RT        | No                               | 3              | No                         | 5              | [67]  |
| GelMA/PEGDA                                                     | 0.7                    | 0-15       | Bulk and 3D-extrusion | Multilayer, layer thickness 200 µm                                 | PBS              | 7.4 | 36.1      | No                               | 1              | 7 days                     | 4-7            | [139] |
| PEGDA/poly(ethylene glycol) methyl ethyl methacrylate (PEGMEMA) | 0.25 ,0.575            | 45-100     | 3D-SLA                | Multilayer, layer thickness 50 µm, dia 9 mm, structure height 1 mm | PBS              | n/a | RT        | No                               | 1              | No                         | 0.03-0.74      | [140] |
| PEGDA/poly(3,4-ethylenedioxythiophene) (PEDOT)                  | 0.575                  | 50, 70, 90 | 3D-SLA                | Multilayer                                                         | PBS              | n/a | 37        | No                               | 1              | No                         | 0.13-0.35      | [141] |
| Poly(ε-caprolactone) maleic acid (PGCL-Ma) & PEGDA              | 2, 3.6 & 8             | 0-100      | Bulk                  | no specific shape, a section of 25 mg weight                       | PBS              | 7.4 | 37        | N/a                              | 40             | No degradation for 20 days | 4.1- 73        | [142] |

| PEGDA containing copolymer                                                                    | Molecular weight (kDa) | Conc (%)   | Bulk/3 D | Sample type                                                          | Storage solution    | pH       | Temp (°C) | Volumetric change Yes/No               | Duration (day) | Degradation                | Swelling ratio | Ref        |
|-----------------------------------------------------------------------------------------------|------------------------|------------|----------|----------------------------------------------------------------------|---------------------|----------|-----------|----------------------------------------|----------------|----------------------------|----------------|------------|
| PEGDA/Gelatin methacrylate (GelMA)                                                            | 0.7                    | 5-20       | Bulk     | Nanospheres                                                          | PBS                 | n/a      | 37        | No                                     | n/a            | n/a                        | 6-10           | [143]      |
| PEGDA/SiO2                                                                                    | 0.575                  | n/a        | 3D-SLA   | Multilayer, layer thickness 25,50,100 µm                             | Water               | n/a      | 25        | Yes, 22% in thickness and 15% in width | 4              | No                         | 0.35           | [144]      |
| PEGDA                                                                                         | n/a                    | 10         | Bulk     | Cylinder, dia 15 mm, structure height 3 mm                           | Deionised water     | n/a      | 37        | No                                     | 3              | n/a                        | 5-13.5         | [145]      |
| PEGDA/GelMA                                                                                   | 1, 4, 8                | 15, 20, 30 | Bulk     | n/a                                                                  | PBS                 | 4,7, 7.4 | 37        | No                                     | 3              | No degradation for 21 days | 10-12          | [146]      |
| PEGDA                                                                                         | 0.7                    | 20         | 3D-SLA   | Multilayer, layer thickness 20 µm, dia 6 mm, structure height 5 mm   | Deionised water     | n/a      | RT        | No                                     | 1              | No                         | 5.1-5.6        | [147, 152] |
| PEGDA/tendon tECM                                                                             | n/a                    | 10         | 3D-SLA   | Multilayer                                                           | Water               | n/a      | 25        | No                                     | 1/2            | No                         | 0.06-0.12      | [148]      |
| water-miscible acrylate (HBA)/poly(ethylene glycol) methyl ethyl methacrylate (PEGMEMA)/PEGDA | 0.575, 0.7             | 2.5,7.5    | 3D-SLA   | Multilayer, layer thickness 50 µm, dia 9 mm, structure height 0.8 mm | Mili-Q waer and PBS | n/a      | RT        | Yes                                    | 3-24 hrs       | No                         | 0.1-0.8        | [150]      |

| PEGDA containing copolymer     | Molecular weight (kDa) | Conc (%) | Bulk/3D | Sample type                                   | Storage solution        | pH  | Temp (°C) | Volumetric change Yes/No                             | Duration (day) | Degradation | Swelling ratio | Ref   |
|--------------------------------|------------------------|----------|---------|-----------------------------------------------|-------------------------|-----|-----------|------------------------------------------------------|----------------|-------------|----------------|-------|
| PEGDA                          | 0.575                  | 2-80     | 3D-DLP  | Multilayer, layer thickness 50,100, 500 µm    | Deionised water         | n/a | RT        | Yes                                                  | 1              | No          | 0.01-0.2       | [151] |
| PEGDA                          | 0.7, 3.4               | 10,20,40 | Bulk    | Cylinder, dia 10 mm, structure thickness 1 mm | Deionised water         | n/a | RT        | No                                                   | n/a            | No          | 2-9            | [153] |
| GeIMA-PEGDA                    | 20                     | 10       | Bulk    | Cylinder, dia 10 mm, structure thickness 3 mm | Collagenase type II     | n/a | RT        | No                                                   | 2              | Yes         | 0.01-0.8       | [156] |
| PEGDA                          | 0.575                  | n/a      | 3D-SLA  | Multilayer, layer thickness 100 µm            | Water                   | n/a | 4-15      | Yes, reversible depending on degree of cross-linking | n/a            | n/a         | 0.2-2.3        | [161] |
| PEGDA                          | 0.575                  | N/a      | 3D-SLA  | Multilayer, cantilevers                       | Ethanol, Water, acetone | n/a | RT        | n/a                                                  | 60 mins        | no          | 1.2-1.7        | [169] |
| Calcium phosphates (Cap)/PEGDA | 0.250, 0.575, 0.7      | n/a      | 3D-SLA  | Multilayer, layer thickness 20 µm, dia 6 mm   | Water                   | n/a | RT        | No                                                   | 7              | No          | 0.20-0.60      | [163] |
| PEGDA                          | 0.7, 3.4, 5, 10        | 40       | 3D-SLA  | Cylinder, layer thickness 100 µm, dia 5 mm,   | PBS                     | 7.4 | 37        | No                                                   | 1              | No          | 5-35           | [95]  |

| PEGDA containing copolymer | Molecular weight (kDa) | Conc (%) | Bulk/3 D | Sample type                                            | Storage solution | pH  | Temp (°C) | Volumetric change Yes/No | Duration (day) | Degradation   | Swelling ratio | Ref   |
|----------------------------|------------------------|----------|----------|--------------------------------------------------------|------------------|-----|-----------|--------------------------|----------------|---------------|----------------|-------|
| PEGDA/Acrylic acid (AA)    | 1, 4, 10               | 9-36     | Bulk     | structure height 1 mm<br>Sheet, layer thickness 0.4 mm | PBS              | 7.4 | RT        | Yes                      | 3              | after 4 weeks | 1-3.5          | [170] |
| PEGDA                      | 10                     | 7.5      | Bulk     | Tubes, dia 3.85-7.2 mm                                 | Mili-Q           | 7.4 | 37        | Yes                      | 2              | No            | 30             | [162] |

### **2.3.2 Mechanical Properties of PEGDA Hydrogels**

An ideal 3D matrix should mimic the mechanical properties of the tissue or pathological environment being studied. Methods have been created for evaluating the elastic modulus of in vitro scaffolds, specifically hydrogels that are extensively employed in 3D culture owing to their adjustable properties and compatibility with biological systems [171,172].

#### **2.3.2.1 Bulk Characteristics of PEGDA Hydrogels**

The tensile test is a commonly used method for evaluating the Young's modulus of materials, which is measured as the ratio of applied stress to resultant strain (Figure2-7a). Soft biological tissues tend to exhibit greater resistance to deformation as the applied stress increases (Table2-2). When conducting tensile tests on biological samples, it is crucial to measure the strain at a specific stress while assuming the material behaves as a perfectly elastic one (Figure2-7b) [173]. 3D printed PEGDA hydrogel using SLA tested on tensile machine reported an elastic modulus of 1.4 MPa [174]. A major drawback of hydrogels is their low mechanical stability at physiological conditions, but it can be fused with other polymeric materials to overcome this [175]. In the same study, blending the PEGDA with tetraethoxysilane nanoparticles increased modulus by at least 50% [174]. Another study showed that a bilayer of PNIPAM/PEGDA produced by extrusion based 3D printing had a Young's modulus of 36 kPa [124]. Even though the mechanical properties of PNIPAM/PEGDA was considerably low but the addition of PNIPAM has resulted in having a thermoresponsive composite hydrogel that reacts differently at temperatures ranging from 20 to 50 °C. A study that used a combination of PEGDA/GelMA hydrogels produced through bulk

cross-linking for researching neurodegeneration found that the modulus measured during a tensile test was in the range of 10 kPa. This value is consistent with the range required for neural stem cell therapies, which is typically between 1-7 kPa [156,176].

Another method commonly used to measure the elastic modulus of PEGDA hydrogels is the compression test. Similar to tensile tests, samples must be prepared with a definite shape and dimension for accurate results which are normally in a cylindrical shape (Figure 2-7c) [177]. Multiple studies found that PEGDA hydrogels created by both bulk cross-linking and 3D printing with similar molecular weight of 700 Da had compression moduli in the range of 0.02 to 1.29 MPa. The compressive modulus has an inverse relationship with polymer molecular weight and direct relationship with polymer concentration (Figure 2-7d,e) [95]. A bulk characterisation of GelMA/PEGDA (700 Da) hydrogel using compression test showed a compressive modulus of 5-20 MPa. A combination of GelMA/PEGDA (20 kDa) also produced by bulk cross-linking reported a considerably lower compressive modulus compare to GelMA/PEGDA (700 Da) as low as 0.8 kPa [156]. Such a change in compression modulus could also be due to the different strain rate used in the studies [178]. A composite of 3D printed PEGDA hydrogels with SiO<sub>2</sub> nanoparticles using SLA had a compressive modulus of 50-85 MPa which is considerably higher than what was reported in Literature review [144]. Such nanocomposite with enhanced mechanical properties can be explained by the fact the high surface area-to-volume ratio surface interaction between nanofillers and polymers [179].



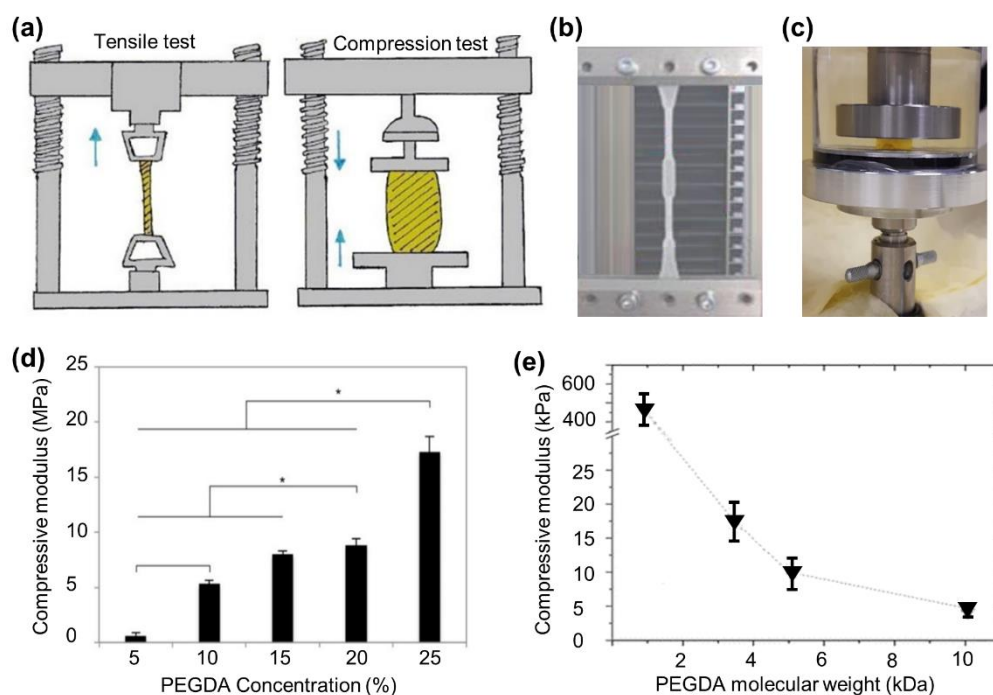


**Figure 2-7 (a) Schematic of tensile and compression. [180]. Reproduced with permission from ref [180]. Copyright 2017 Elsevier. (b) Image of PEGDA hydrogel under tensile tension [111]. Reproduced with permission from ref [111]. Copyright 2022 De Gruyter. (c) Image of 3D PEGDA hydrogel under compression. (d) Compressive moduli of hydrogels with varying PEGDA concentrations [143]. Reproduced with permission from ref [143]. Copyright 2018 IOP Publishing. (e) Mechanical properties of laser-polymerised PEGDA hydrogels were measured for 20% PEGDA hydrogels as a function of molecular weight (0.7, 3.4, 5, and 10 kDa) [95]. Reproduced with permission from ref [95]. Copyright 2010 Royal Society of Chemistry.**

The mechanical properties of PEGDA hydrogels produced using photo-cross-linking can be altered by changing the UV light dosage. Increasing the UV light dosage may result in a decrease in the compressive modulus due to over-cross-linking. This was observed in studies where PEGDA hydrogels were exposed to 20, 40, and 60 seconds of UV light. The compression modulus increased from

0.49 to 0.70 MPa when exposed to 20 and 40 seconds, respectively, but dropped to 0.64 MPa when exposed to 60 seconds of UV light [145]. The mechanical properties of 3D printed cell-laden cartilage constructs were tested using bulk samples, which may not accurately represent the mechanical properties at a small scale, even though the intended use of the material is for 3D bioprinting [143,163]. To obtain accurate surface mechanical properties at the micro and nanoscale, advanced techniques such as AFM and nanoindentation are commonly employed in various studies [181]. Although indentation testing may produce comparable results to compression testing in terms of elastic modulus, it differs significantly due to differences in loading geometry and boundary conditions [182–184].

**Table 2-2 Bulk characterisation of PEGDA hydrogels**

| PEGDA containing copolymer                | Molecular weight (kDa) | Conc (%) | Bulk/3D      | Sample shape                                                   | UV variations | Storage solution | Temperature (°C) | Mechanical test                                               | Modulus      | Ref       |
|-------------------------------------------|------------------------|----------|--------------|----------------------------------------------------------------|---------------|------------------|------------------|---------------------------------------------------------------|--------------|-----------|
| Poly-N-isopropylacrylamide (PNIPAM)/PEGDA | n/a                    | 10       | 3D-extrusion | Multilayer, layer thickness 260 µm                             | No            | Hydrated         | 25               | Tensile strength, uniaxial tension, loading rate 2 mm/s       | 85 kPa       | [124]     |
| PEGDA/poly-(ε-caprolactone) (PCL)         | 1                      | 15       | 3D-SLA-DLP   | Disks, layer thickness 100 µm, dia 8 mm, structure height 3 mm | Yes           | n/a              | n/a              | Compressive modulus, 1 N load cell, 0.1-30% strain            | 50-250 kPa   | [67]      |
| PEGDA/Gelatine methacrylate (GELMA)       | 0.7                    | 5-20     | Bulk         | n/a                                                            | No            | Dry              | RT               | Compression/100 N load, 2 mm/min                              | 5-20 MPa     | [143]     |
| PEGDA/SiO <sub>2</sub>                    | 0.575                  | n/a      | 3D-SLA       | Multilayer, layer thickness 25,50,100 µm                       | No            | Swollen (water)  | 25               | Compression testing, 2 mm/min                                 | 50-85 MPa    | [144]     |
| PEGDA                                     | n/a                    | 10       | Bulk         | Cylinder, dia 15, structure height 3 mm                        | Yes           | Dry              | RT               | Compression/ 2 kN, 0.5 mm/min                                 | 0.49-0.7 MPa | [145]     |
| PEGDA/GELMA                               | 1, 4, 8                | 15,20,30 | Bulk         | n/a                                                            | No            | Hydrated (water) | 37               | Storage modulus, Rheometry, 1 to 400 rad/s, amplitude of 0.3% | 1-8 kPa      | [146]     |
| PEGDA                                     | 0.7                    | 20       | 3D-SLA       | Multilayer, layer thickness 20 µm, dia 6 mm,                   | No            | Swollen (water)  | RT               | Compression testing shear modulus, 0.5 mm/min                 | 0.43 MPa     | [147,152] |

| PEGDA containing copolymer     | Molecular weight (kDa) | Conc (%) | Bulk/3D | Sample shape                                                                                                    | UV variations | Storage solution        | Temperature (°C) | Mechanical test                                                                | Modulus                | Ref   |
|--------------------------------|------------------------|----------|---------|-----------------------------------------------------------------------------------------------------------------|---------------|-------------------------|------------------|--------------------------------------------------------------------------------|------------------------|-------|
| PEGDA/tendon tECM              | n/a                    | 10       | 3D-SLA  | structure height 5 mm<br>Multilayer                                                                             | No            | Dry                     | RT               | Compression, compressive modulus                                               | 0.2-0.3 MPa            | [148] |
| PEGDA                          | 0.7, 3.4               | 10,20,40 | Bulk    | Cylinder, dia 10 mm, structure height 3 mm                                                                      | No            | Swollen (water)         | RT               | Confined compression, 19 µm/min, 10% deformation                               | 22-118 kPa             | [153] |
| GelMA-PEGDA                    | 20                     | 10       | Bulk    | Cube for tensile, 30 x 10 mm, structure height 3 mm. Cylinder for compression, dia 10 mm, structure height 5 mm | No            | PBS                     | 37               | Tensile modulus, max strain at 50%, 1mm/min<br>Compressive modulus, 0.5 mm/min | 10-60 kPa<br>0.8-6 kPa | [156] |
| Calcium phosphates (Cap)/PEGDA | 0.250, 0.575, 0.7      | n/a      | 3D-SLA  | Multilayer, layer thickness 200 µm, dia 6 mm, structure height 12 mm                                            | No            | Dry and swollen (water) | 25 and 37        | Compression testing, 1 mm/min                                                  | 0.21-19.87 MPa         | [163] |
| PEGDA                          | 0.7, 3.4, 5, 10        | 40       | 3D-SLA  | Disk, layer thickness 100 µm, dia 5 mm, structure height 1 mm                                                   | No            | PBS                     | 25               | Compressive modulus, compression test, 1 mm/s, strain at 10%                   | 5-503 kPa              | [95]  |

| PEGDA containing copolymer | Molecular weight (kDa) | Conc (%) | Bulk/3D | Sample shape                                | UV variations | Storage solution | Temperature (°C) | Mechanical test                                                        | Modulus                   | Ref   |
|----------------------------|------------------------|----------|---------|---------------------------------------------|---------------|------------------|------------------|------------------------------------------------------------------------|---------------------------|-------|
| PEGDA/Acrylic acid (AA)    | 1, 4, 10               | 9-36     | Bulk    | Sheet, 2 x 5 cm, structure thickness 0.4 mm | No            | PBS              | RT               | Tensile test, elastic modulus, 1.5 mm/min                              | 68-214 kPa                | [170] |
| PEGDA                      | 0.6                    | n/a      | 3D-DLP  | Cube, 10 x 80 mm, structure height 0.7 mm   | No            | n/a              | n/a              | Tensile test, Young's modulus, load cell 500 N                         | 1.4 MPa                   | [174] |
| PEGDA                      | 0.258                  | 7-40     | Bulk    | Cylinder, dia 11 mm, structure height 8 mm  | No            | Dry<br>wet       | RT               | Tensile test, elastic modulus, 20 N, Compression test, elastic modulus | 4-20 MPa<br>0.05-3.19 MPa | [181] |

### **2.3.2.2 Localised Characteristics of PEGDA Hydrogels**

Nanoindentation and AFM are widely used to test mechanical properties of soft biological tissue materials and optical thin layers. They are particularly beneficial for testing hydrogels, as they require only small samples, can be performed on a single sample, and reduce differences between samples. Both techniques involve pressing a probe onto the surface of the hydrogel and creating a force-indentation distance curve, from which the hydrogel's Young's modulus can be obtained [185,186]. The probe characteristics, such as material, spring constant, radius, and cone angle, have a significant impact on the measurement's resolution, accuracy, and sensitivity [187–189]. AFM has been used to assess the elastic modulus of PEG based hydrogels (Figure 2-8a) [190–192]. For instance, AFM results for PEGDA hydrogels with varying molecular weight showed an increase in elastic modulus [193], contradicting bulk compression test results of an inverse relationship between molecular weight and compression modulus [153]. Therefore, it could possibly be a difference in results obtained in macroscale such as compression testing and nanoscale such as AFM. The elastic modulus of bulk-cross-linked PEGDA hydrogels increased from 30 to 80 kPa as the concentration of the PEGDA monomer increased from 20 to 50%, as determined by AFM analysis (Figure 2-8b) [194]. The mechanical properties of 3D printed PEGDA hydrogels produced by SLA and direct laser writing (DLW) were analysed using AFM. The results showed that the elastic modulus of these hydrogels was in the range of 1-10 MPa (Figure 2-8c) [161,195]. Longer UV light dosage result in higher elastic modulus as it dictates the degree of cross-linking. AFM measurements on 3D PEGDA hydrogel show that elastic modulus increases

linearly with increased UV light dosage and increased degree of cross-linking (Figure 2-8d) [194].

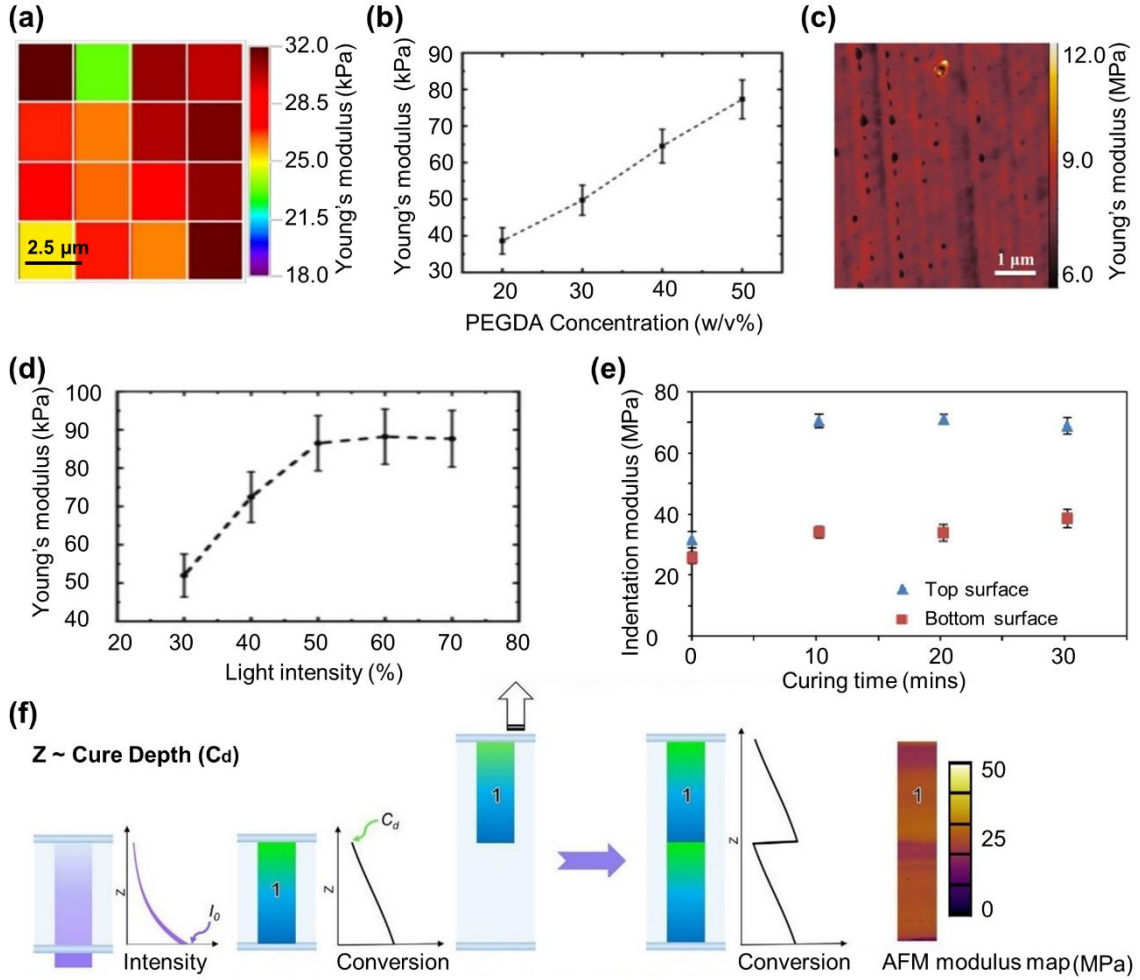


**Figure 2-8 Localised mechanical properties of PEGDA hydrogels measured using AFM and nanoindentation techniques. (a) Elastic modulus map of bulk hydrogels showing variations between 18-32 kPa [192]. Reproduced with permission from ref [192]. Copyright 2011 Elsevier. (b) Young's modulus of a hydrogel with change in PEGDA concentration between 20 to 50 w/v% measured using AFM [194]. Reproduced with permission from ref [194]. Copyright 2022 Elsevier. (c) AFM topographic image of 3D hydrogels produced using DLW using PeakForce QNM mode showing elastic modulus ranging between 6 to 12 MPa in dry state [195]. Reproduced with permission from ref [195]. Copyright 2021 Elsevier. (d) The**

influence of light intensity on the mechanical properties of hydrogel is examined by AFM showing an overall positive trend [194]. Reproduced with permission from ref [194]. Copyright 2022 Elsevier. (e) Plots of nanoindentation for 3D printed hydrogel using inkjet printing showing the difference in nanomechanical properties at top and bottom of the hydrogel after post-curing process. The results show that unidirectional post-curing results in heterogeneity in elastic modulus across the sample [81]. Reproduced with permission from ref [81]. Copyright 2016 Wiley. (f) Multilayer hydrogels produced by DLP printer with irradiance intensity ( $I_0$ ), and layer thickness ( $Z$ ) parameters used to control cure depth ( $C_d$ ). AFM used for mapping elastic modulus cross-sectioned of hydrogels showing how incorporating a light absorber, can result in exponential decay in light intensity with penetration depth, resulting in a limited thickness of resin that undergoes gelation [196]. Reproduced with permission from ref [196]. Copyright 2021 Wiley.

Nanoindentation on the other hand can map the elastic properties of soft materials at a microscopic scale, using an indenter tip to apply a defined force and measuring the indentation depth with sub-nanometer accuracy [197]. It is useful for analysing the microenvironment's effect on the mechanical behaviour of the matrix. For soft samples, flat punches and spherical indenters with a large radius have been developed [90,198]. The elastic modulus of 3D PEGDA hydrogels used as a scaffold for bone marrow mesenchymal stem cells, produced by two-photon polymerisation, increased from 100-1500 kPa as laser intensity and raster speed were increased [199]. In another study, 3D PEGDA structures were created using a technique called mask projected excimer laser SLA, without adding a photoinitiator to the prepolymer solution. This method relies on high levels of light energy and resulted in a significant increase in elastic modulus, as



measured by nanoindentation. [200]. 3D PEGDA hydrogels produced by this technique without photoinitiator showed a 10-fold increase in elastic modulus when analysed by nanoindentation [201]. Such advancement in 3D printing helps with reducing the risk of cytotoxicity from the photoinitiator molecules during cell proliferation [201]. Nanoindentation measurements provide insight into the time dependent behaviour of hydrogels and how surface characteristics affect cell behaviour when in contact with the hydrogel surface. These measurements have shown that hydrogels used for multicellular spheroid culturing exhibit time-dependent stress curves, indicating viscoelastic behaviour that leads to better cell adhesion, spreading and tissue formation [202]. Nanoindentation was used to compare the mechanical characteristics of the top and bottom surface of a 3D printed PCL/PEGDA hydrogel, revealing that the top surface had a higher elastic modulus than the bottom surface (Figure 2-8e). This difference can be attributed to the reduced intensity of UV light reaching the bottom surface during 3D printing, resulting in a lower degree of cross-linking [81]. This suggests that 3D printing can result in structures with variations in cross-linking and mechanical properties. It was found that 3D printed PEGDA hydrogels produced by DLP printing have a mechanical gradient across the layers, as confirmed by measuring the elastic modulus along the surface of cross-sectioned 3D-printed structures (Figure 2-8f) [196]. Moreover, examining samples in their hydrated and dried state also showed to report different elastic modulus with dry samples always have a higher modulus in comparison with swollen hydrated samples [144,163]. Nanoindentation research on PEGDA hydrogels showed that the elastic modulus of a dried PEGDA hydrogel was 5 MPa, while the modulus of hydrated samples

was slightly above 1 MPa [203]. Table 2-3 summarises the variations in elastic modulus as reported in various studies, based on the utilisation of different printing parameters, fabrication methods, and starting prepolymer solutions.

**Table 2-3 Localised characterisation of PEGDA hydrogels using AFM and Nanoindentation**

| PEGDA containing copolymer | Molecular weight (kDa) | Conc (%) | Bulk/3D                       | Sample shape                               | UV variations | Storage solution | Temperature (°C) | Mechanical test                                  | Modulus       | Ref   |
|----------------------------|------------------------|----------|-------------------------------|--------------------------------------------|---------------|------------------|------------------|--------------------------------------------------|---------------|-------|
| PEGDA                      | 0.575                  | n/a      | 3D-SLA                        | Multilayer, layer thickness 100 $\mu$ m    | Yes           | n/a              | n/a              | AFM, elastic modulus                             | 1.25-4 MPa    | [161] |
| PEGDA                      | 0.6                    | n/a      | 3D-DLP                        | Multilayer, layer thickness 10-100 $\mu$ m | No            | dry              | n/a              | Nanoindentation, radius 1 $\mu$ m diameter,      | 7 MPa         | [174] |
| PEGDA                      | 0.258                  | 7-40     | Bulk                          | Cylinder, dia 11 mm, structure height 8 mm | No            | Wet              | RT               | Nanoindentation, flat punch. 54 $\mu$ m diameter | 1.84-3.29 MPa | [181] |
| PEGDA/Hyaluronic acid (HA) | 0.575                  | n/a      | Bulk                          | n/a                                        | No            | No               | 25               | AFM,                                             | 2.5-25 kPa    | [192] |
| PEGDA                      | 0.258, 0.575, 0.7      | n/a      | Bulk                          | Sheet, structure height 1.5 mm             | No            | water            | n/a              | AFM                                              | 2.48-4.33 MPa | [193] |
| PEGDA                      | 0.575                  | 5-20     | Bulk                          | Sheet, structure height 70 $\mu$ m         | No            | water            | 20               | AFM, 2.5 $\mu$ m radius spherical tip            | 2.8-228.9 kPa | [204] |
| PEGDA                      | 0.7                    | n/a      | 3D-Direct Laser writing (DLW) | n/a                                        | Yes           | No               | n/a              | AFM, elastic modulus                             | 6.9-9.5 MPa   | [195] |

| PEGDA containing copolymer                    |       | Molecular weight (kDa) | Conc (%) | Bulk/3D            | Sample shape                                                                                     | UV variations | Storage solution | Temperature (°C) | Mechanical test                                             | Modulus                         | Ref   |
|-----------------------------------------------|-------|------------------------|----------|--------------------|--------------------------------------------------------------------------------------------------|---------------|------------------|------------------|-------------------------------------------------------------|---------------------------------|-------|
| PEGDA                                         |       | 0.7                    | 50       | 3D-DLW             | Multilayer cube, 100 x 100 $\mu\text{m}$ , structure height 50 $\mu\text{m}$                     | Yes           | water            | RT               | Nanoindentation, spherical 28 $\mu\text{m}$ radius          | 100-1500 kPa                    | [199] |
| PEGDA photoinitiator)                         | (no   | 0.7                    | n/a      | 3D-laser SLA       | Multilayer, layer thickness 100 $\mu\text{m}$                                                    | Yes           | dry              | n/a              | Nanoindentation, Berkovich tip                              | 10-100 MPa                      | [201] |
| Polycaprolactone methacrylated (PCLDMA)/PEGDA | di-   | 0.250                  | 0-50     | 3D-Inkjet printing | Multilayer cube, layer thickness 5 $\mu\text{m}$ , 100 layers, 5 x 5 mm, structure height 0.5 mm | Yes           | n/a              | RT               | Nanoindentation, spherical indenter 50 $\mu\text{m}$ radius | 25-75 MPa                       | [81]  |
| PEGDA                                         |       | 0.7                    | n/a      | 3D-DLP             | Multilayer, layer thickness 10, 30, 100 $\mu\text{m}$                                            | Yes           | dry              | RT               | AFM                                                         | 9.5-34.5 MPa                    | [196] |
| PEGDA                                         |       | 6, 20                  | 15       | 3D-SLA             | Multilayer, layer thickness 250 $\mu\text{m}$                                                    | No            | Water, dry       | RT               | Nanoindentation                                             | 5 MPa (dry)<br>1 MPa (hydrated) | [203] |
| PEGDA                                         |       | 0.7                    | n/a      | 3D-SLA             | Multilayer, dia 300 $\mu\text{m}$ , structure height 600 $\mu\text{m}$                           | No            | n/a              | n/a              | AFM                                                         | 200 MPa                         | [205] |
| PEGDA fibrinogen)                             | (PEG- | 0.4, 4                 | 5-25     | Microfluidic       | n/a                                                                                              | No            | n/a              | n/a              | AFM                                                         | 0.7-50 kPa                      | [206] |

| PEGDA containing copolymer | Molecular weight (kDa) | Conc (%) | Bulk/3D      | Sample shape                              | UV variations | Storage solution | Temperature (°C) | Mechanical test | Modulus                                | Ref   |
|----------------------------|------------------------|----------|--------------|-------------------------------------------|---------------|------------------|------------------|-----------------|----------------------------------------|-------|
| PEGDA                      | 0.7                    | 20-50    | Bulk         | Cuboid, 6 x 6 mm, structure height 0.4 mm | Yes           | n/a              | n/a              | AFM             | 35-95 kPa                              | [194] |
| Polyacrylamide (PAM)/PEGDA | 0.7                    | 5-16     | Microfluidic | Particles                                 | No            | Water, dry       | RT               | AFM             | 2-7 kPa (hydrated)<br>25-180 kPa (dry) | [207] |

## **2.4 3D PEGDA Hydrogel for Cardiac Tissue Engineering**

As discussed previously, PEGDA hydrogel has been used for various applications within the field of tissue engineering [37,208,209]. In the area of organ-on-chip devices it has been tested for 3D cell culture platforms such as skeletal muscle microtissues (SMT) [67,210–212]. Designing functional in vitro SMT involves two crucial elements: scaffold architecture and functionality. The scaffold should facilitate muscle differentiation and myogenesis leading to aligned, dense, and well-oriented myofibers. The scaffold's functionality can be improved through artificial electrical stimulation promoting myogenic cell transformation into functional myofibers [213–216].

The popular scaffold design for contractile cardiac tissue resembles Polydimethylsiloxane (PDMS) moulded cantilever beams (posts), with tissue hanging from both ends. It evolved from 2D to representative 3D tissue studies and multiple commercial systems exist (Figure2-9a-h) [217–224]. The technique measures the deflection of vertical posts caused by the contraction of the SMT. The tissue construct attached to the top of the posts pulls them together, creating a horizontal displacement, allowing calculation of the contraction force [225–227].

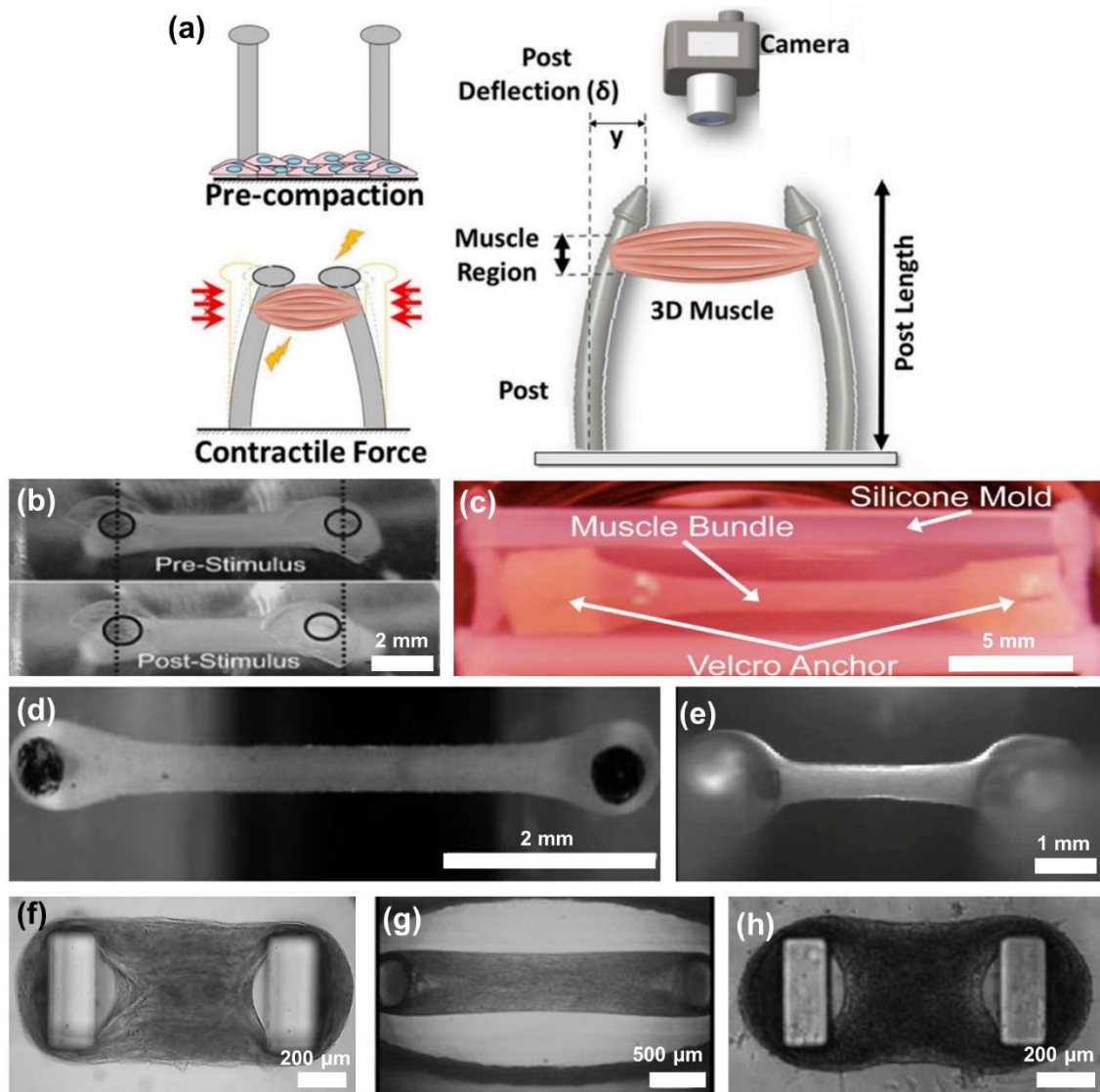


**Figure 2-9 State-of-the-art in muscle tissue strip (MTS) formation based on the quantification of the deflection of a pair of vertical cantilever PDMS beams (posts) for functional read-out assays. (a) Schematic of contractile force measurement for MTS that is grown in vitro between flexible cantilevers (posts) serving as tendons. Force is quantified by tracking post displacements in response to stimulation and knowing platform mechanics [212]. Reproduced with permission from ref [212]. Copyright 2022 eLife. (b) Moulding process was employed to make caps for the posts, using primary mouse myoblasts tissue strips were created encased in either a collagen/matrigel or fibrin scaffold [217]. Reproduced with permission**

from ref [217]. Copyright 2008 Wiley. (c) Skeletal muscle bundle of rats myblasts embedded with matrigel/fibrinogen and collagen I [218]. Reproduced with permission from ref [218]. Copyright 2011 Elsevier. (d) Human engineered cardiac tissues (hECT) created using differentiated human pluripotent stem cells in multi-tissue bioreactor [219]. Reproduced with permission from ref [219]. Copyright 2016 JoVE. (e) Atrial-like engineered heart tissue generated from human induced pluripotent stem cells (hiPSC) [228]. Reproduced with permission from ref [228]. Copyright 2016 ICCSR. (f) Muscle tissue was created by embedding either C2C12 cells or iPSC-derived cardiomyocytes in a fibrin-based scaffold [229]. Reproduced with permission from ref [229]. Copyright 2018 Elsevier. (g) The transformation of the ECM by human myoblasts forming a human muscle micro-tissue held by two posts for a 96-well culture platform [222]. Reproduced with permission from ref [222]. Copyright 2020 Nature. (h) Miniaturised hiPSC-based cardiac tissue formed around micropillars [223]. Reproduced with permission from ref [223]. Copyright 2020 IEEE.

As a better alternative to PDMS, which absorbs lipophilic compounds, PEGDA hydrogels limit nonspecific adsorption of proteins, DNA, and lipophilic drugs, making them suitable for drug testing devices [147]. Additionally, techniques such as masked photolithography and mould based fabrication are both inefficient and unsuitable for large-scale production. These methods lack flexibility when it comes to creating complex shapes with reliable and repeatable mechanical properties that match living cells. On the other hand, 3D printing using micro-SLA and DLP offer a faster and more dependable manufacturing approach. These techniques involves the LbL fabrication of 3D PEGDA hydrogel posts. The concept originated from miniaturised walking robots that were constructed from



a 3D-printed PEGDA hydrogel base and cantilever with two different molecular weights. Subsequently, the cantilever was seeded with contractile cardiomyocyte cells [230]. The cantilever was initially polymerised, then its base was 3D printed, and its surface was functionalised with collagen. The thickness of the cantilever was altered to assess its performance. Further development led to the creation of cantilevers with varying molecular weights, as a correlation between substrate stiffness and its impact on cardiomyocyte contractile behaviour was established (Figure 2-10a,b) [231–233]. It was discovered that increasing the molecular weight of the PEGDA monomer in the prepolymer solution creates more compliant structures with elasticity similar to native myocardium, measured in kiloPascals [231]. Such knowledge led to creation of 3D printed flexible bio-bot that can hold a muscle strip together and respond to optical and electrical pulses [234]. The same printing technique was further developed to help investigate fabrication and cryopreservation of muscle tissue strip (MTS) and multicellular spinal cord-muscle bioactuator using 3D PEGDA hydrogels (Figure 2-10c) [126,235]. In another study the focus was to optimise 3D PEGDA hydrogel as an artificial muscle, focus was on adjusting its stiffness by changing molecular weight and measuring its contractile strength and durability [170]. It was discovered that the hydrogels had a decline in performance after 4 weeks due to the gradual degradation of the material. In a most recent work 3D PEGDA microenvironment was printed to facilitate and support the formation of MTS [147]. The study aimed to investigate the behaviour of MTS when the diameter of 3D PEGDA micro-cantilevers and the concentration of PEGDA in the prepolymer solution are altered. It was established that increasing the diameter of the 3D PEGDA micro-

cantilevers resulted in a reduction in the ability of the formed tissue to exert sufficient contractile forces to bend the cantilever, while increasing the concentration of PEGDA in the prepolymer solution from 20% to 50% had a similar effect. The exerted tissue force on the cantilever was acquired through the use of Euler-Bernoulli theory based on cantilever stiffness and deflection (Figure 2-10d) [147].

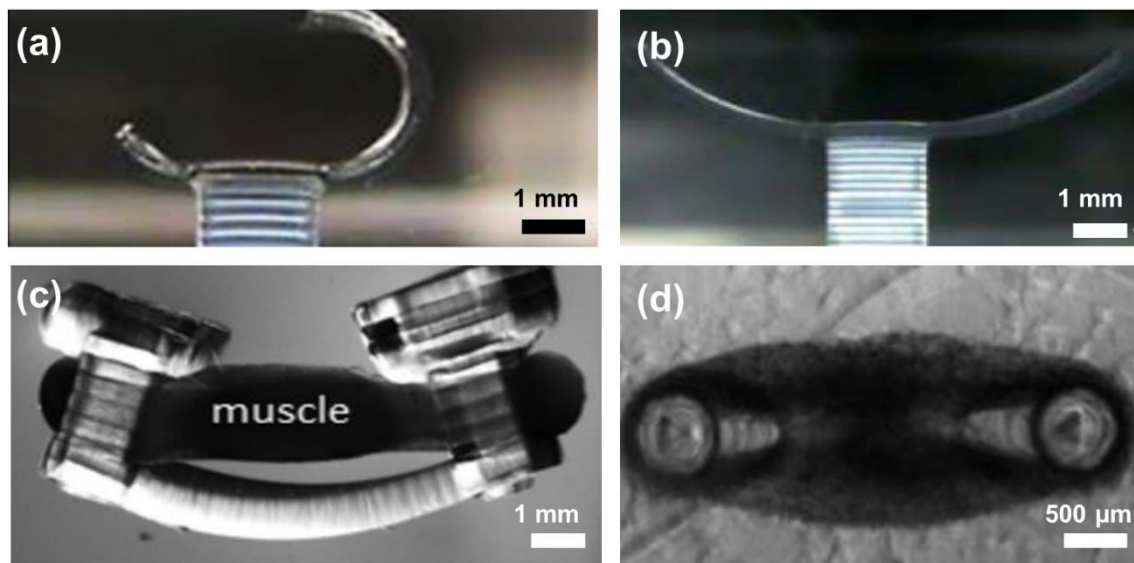


**Figure 2-10 3D printed PEGDA cantilevers using SLA for contractile tissue studies.**

**(a)** Bio-bot 3D printed PEGDA cantilever with cardiac cell sheet seeded on bottom of the cantilever [230]. Reproduced with permission from ref [230]. Copyright 2012 Nature. **(b)** Multi-material 3D PEGDA cantilever and base with cardiomyocytes seeded on the cantilever. Deflection occurs due to intrinsic stress in the formed tissue [231]. Reproduced with permission from ref [231]. Copyright 2012 Nature. **(c)** Spinobot from the spinal cord and C2C12 myoblasts with tissue attached to the hydrogel skeleton which causes bending by generating passive tension [126]. Reproduced with permission from ref [126]. Copyright 2020 AIP Publishing. **(d)** Muscle tissue created by embedding contractile cells of C2C12 hanging on 3D

**printed PEGDA hydrogels for multi assay platform [147]. Reproduced with permission from ref [147]. Copyright 2019 American Chemical Society.**

To achieve higher maturity in the MTS for enhanced cardiomyocyte research a study proposed the integration of a flexible support structure made of the same material as the 3D printed PEGDA hydrogel beneath the cantilevers to enhance mechanical stimulation of the tissue for its maturity [236]. This was achieved by stretching the flexible structure, which in turn horizontally stretches the cantilever and mechanically contracts the MTS, thus promoting tissue maturation. In addition to mechanical stimulation, electrical and optical stimulation are widely recognised as key techniques for promoting maturity in mechanically-tensioned tissues [237–239].

## **2.5 Knowledge Gap and Future Perspective**

Although advanced SLA, DLP, microfluidic, and inkjet printing techniques have allowed for the creation of complex 3D printed PEGDA microstructure hydrogels, the understanding of the impact of LbL fabrication on their behaviour and properties is still limited. Several critical gaps in knowledge need to be addressed, including thermal response, isotropic behaviour, homogeneity, stability, and surface nanomechanical properties (Figure 2-11a,b). One of these gaps pertains to the dimensional stability of these hydrogels, as well as their anisotropy and isotropy. Previous studies have not fully explored these aspects, and further research is essential to comprehend their behaviour and properties in different physiological environments. Another area that requires further investigation is the impact of swelling on 3D printed PEGDA hydrogels. Studies on swelling have primarily focused on water and PBS, with little exploration of the effects of cell

culture media on these hydrogels. Therefore, there is a need to investigate their behaviour and properties in a realistic cell culture setting. The long-term stability of these hydrogels is also crucial for future research. Although earlier studies have examined their stability over shorter time periods, longer-term studies spanning months or even years are required to establish their shelf life. Moreover, the relationship between different environments, such as temperature and cell culture medium, and the behaviour of these hydrogels needs to be thoroughly studied. That includes the reversibility of 3D printed PEGDA hydrogels as a result of temperature change. There is some evidence that these hydrogels may show hysteresis when stored at different temperatures, which could impact their physical stability. Understanding the effect of temperature on the behaviour of 3D PEGDA hydrogel for both storage, transport and final application is vital. It is crucial to improve understanding of the fundamental nanomechanical properties of 3D PEGDA hydrogels, and how altering fabrication parameters like layer thickness and UV dosage can impact their surface nanomechanical behaviour uniformity. This knowledge is essential because it can significantly impact cell adhesion, differentiation, and integration with the host microstructure. Therefore, it is imperative to conduct further research to enhance the effectiveness of these materials in biomedical applications, particularly in the study of contractile tissue studies, where ensuring dimensional and mechanical stability, as well as structural integrity, is critical for achieving positive outcomes.

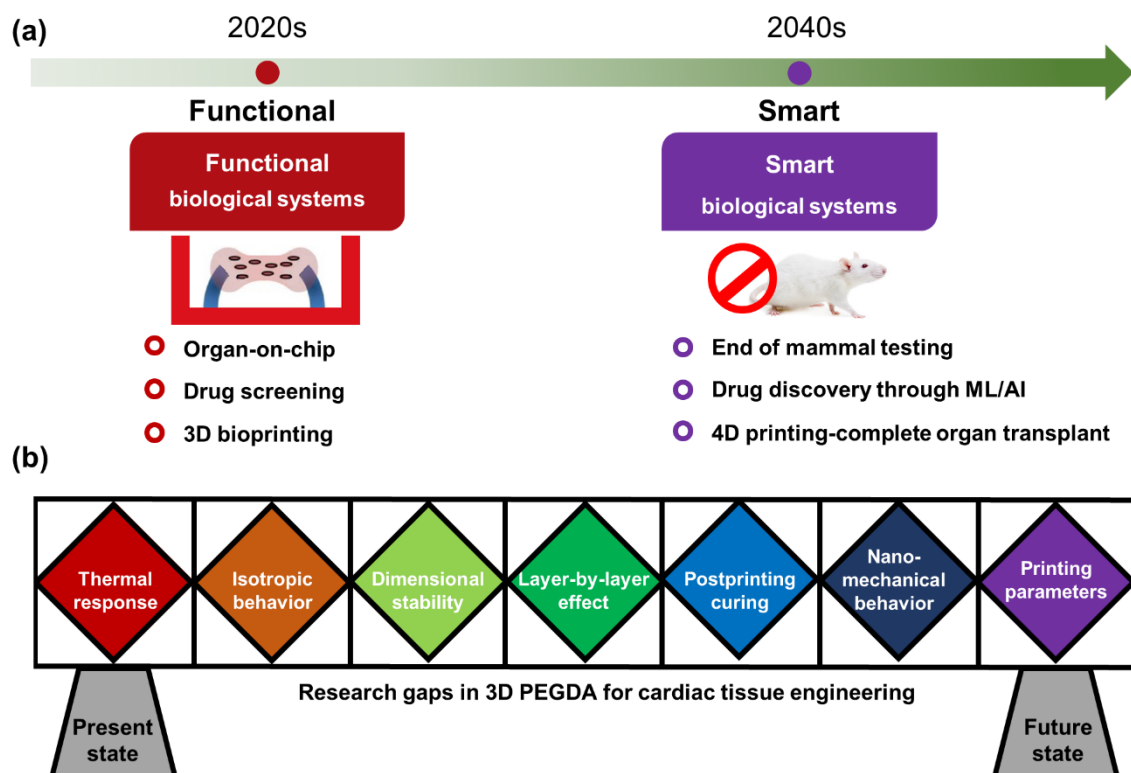


Figure 2-11 (a) Roadmap of 3D printing technologies and their role end of mammal testing and complete 4D organs. (b) Identified research gaps in 3D PEGDA hydrogels and ways to bridge the gap towards animal-free contractile tissue studies.

## 2.6 Conclusions

In conclusion, this review has highlighted the recent advances in additive manufacturing of PEGDA hydrogels and their potential for tissue engineering applications. The various printing techniques, such as extrusion-based, inkjet-based, and SLA-based printing, have been discussed, along with their advantages and limitations. The physicochemical properties of PEGDA hydrogels and biocompatibility have been shown to be highly tunable and can be tailored to match specific tissue engineering requirements. The reviewed literature indicates that PEGDA hydrogels possess many favorable properties

that make them suitable for use in tissue engineering, such as excellent biocompatibility, high water content, and the ability to support cell growth and proliferation. However, there are still many research gaps that need to be addressed in the future, such as investigating the optimal conditions for PEGDA hydrogel fabrication and functionalisation, as well as the effects of different printing techniques on the resulting structures and properties. Additionally, the potential of LbL printing to improve the mechanical properties and functionality of PEGDA hydrogels should be further explored. Further in-depth in vitro and in vivo studies are needed to elucidate the mechanisms underlying the interactions between cells and PEGDA hydrogels and optimise the design of scaffolds for specific tissue engineering applications. Despite these challenges, continued exploration and advancement in the area of additive manufacturing of PEGDA hydrogels will lead to significant breakthroughs in tissue engineering, and ultimately improve the quality of life for patients in need of tissue replacement therapies. As the technology continues to improve and become more widely available, PEGDA hydrogels have the potential to become a versatile platform for various biofunctional living systems such as artificial organs, organ-on-chip, microphysiological systems and drug testing models.

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# Chapter 3

## Thermal Response of Multilayer UV Cross-Linked PEGDA Hydrogels

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## **Abstract**

Poly(ethylene glycol) diacrylate (PEGDA) hydrogels are ubiquitously used in a wide variety of applications in tissue engineering. In this study, the thermal response of multilayer PEGDA hydrogels was investigated under various conditions of the temperature-controlled environments (8, 20, 37, and 45 °C) through gravimetric and volumetric methods. These multilayer hydrogels were produced using a computer-controlled projection lithography and compared to the monolithic hydrogels fabricated through bulk photo-cross-linking. The Normalised Weight Fraction (NWF) of the hydrogels increased to +14% at lower 8 °C temperature, whereas the NWF decreased to −11.1% at 45 °C. It was found that the PEGDA hydrogels exhibited significant changes in weight and volume with temperature variations, due to alterations in the polymer network's hydrophobicity and hydrogen bonding. These findings highlight the importance of considering polymerisation conditions and temperature variations in design and fabrication processes. It was observed that the volume of multilayer PEGDA hydrogels increased to about 10% at a temperature of 8 °C, while their volume decreased by 8% and 12% when stored at 37 °C and 45 °C, respectively. The study also noted anisotropic behaviour where the axial dimensional change was approximately 43% higher than the lateral dimension. While the multilayer PEGDA hydrogels showed reversibility in their behaviour at certain temperatures, further investigation is needed to understand their degradation over repeated cycles. This finding is critical to inform the design and fabrication of PEGDA hydrogels to compensate for the axial and lateral volume changes during the application at different temperatures.



### **3.1 Introduction**

Rapid advances in biomedical engineering are underpinned by the development of smart functional materials that exhibit excellent biocompatibility and tunable mechanochemical properties [1–5]. The mechanical properties of hydrogel can be precisely controlled to mimic a desired tissue microenvironment for use as tissue scaffolds with behaviour similar to that of an extracellular matrix [6–9]. Their physicochemical properties, such as compactness and low interfacial tension with aqueous media, resemble living tissues due to their high affinity to water [10–12]. These properties have allowed hydrogels to be widely used in many biomedical applications including drug delivery and tissue engineering [13,14].

The emergence of poly (ethylene glycol) diacrylate (PEGDA) as candidates for biomaterials can be attributed to their approved low-immunogenic biocompatibility and ability to be photo-cross-linked using appropriate photoinitiator [15,16]. With the recent development in lithographic additive manufacturing [17,18], the multi-length scale structure and morphology of PEGDA can be tailored to exhibit unique biofunctionality of complex 3D scaffolds [19]. In addition, the biomechanical properties of PEGDA can be tuned by controlling the molecular weight, light irradiation dosage, and concentration of photoinitiator and photoabsorber during the cross-linking process [20,21].

To date, the fundamental understanding of the long-term thermomechanical behaviour of PEGDA has not been established [22–24]. The aim of this study was to elucidate the effect of external temperature on the long-term dimensional

stability of photo-cross-linked PEGDA to address their thermal response in various environmental conditions that differ to the fabrication conditions. We report herein that the equilibrium volume of PEGDA hydrogels increases by 10% when they are in the cold storage temperature of 8 °C and decreases by 8% when they are in the body temperature of 37 °C. We also demonstrate that their thermal response is fully reversible, which expand the possibility of mass production and scale-up use of photo-cross-linked PEGDA hydrogels. Our approach and analysis offer insights into long term stability of hydrogels that can be extrapolated further to other biomaterials for the benefit of many biomedical engineering applications.

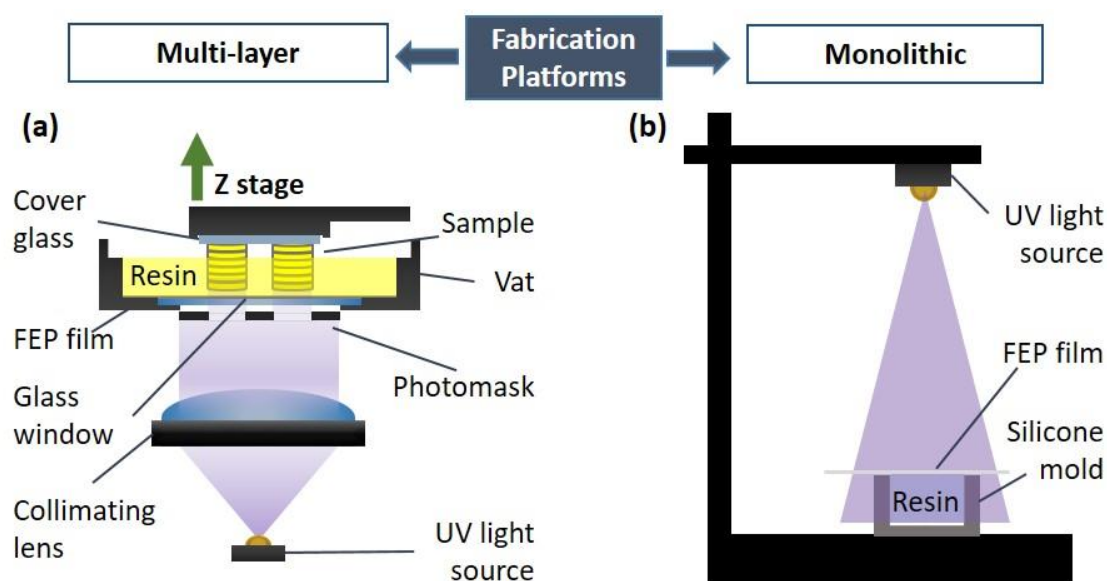


**Figure 3-1 Schematic of fabrication platforms of (a) computer-controlled projection lithography for multilayer hydrogel structures and (b) fixed-photo-cross-linking setup for monolithic hydrogel structures.**

## 3.2 Materials and Methods

### 3.2.1 Synthesis of PEGDA 3D Structures

The starting material for the aqueous prepolymer solution contained 5 mg/mL Lithium phenyl-2,4,6-trimethylbenzoylphosphinate photoinitiator (LAP; Merck 900889,  $\geq 95\%$ ) and 200 mg/mL Poly (ethylene glycol) diacrylate (PEGDA; Merck 455008,  $M_n$  700 g/mol). Multilayer PEGDA hydrogel samples were created by projecting an image of a photomask (10k resolution from Photo Data) using a custom-made computer-controlled projection lithography (Figure 3-1a). The system uses a projection of a static image, attached at the bottom of the vat, to construct a layer-by-layer structure until the required height is reached (Figure 3-1a). Layer thickness was set at nominal thickness of 20  $\mu\text{m}$  and 150  $\mu\text{m}$  and the manufactured hydrogel structures are denoted herein as multi-20  $\mu\text{m}$  and multi-150  $\mu\text{m}$ , respectively. For fabrication of multi-20  $\mu\text{m}$  and multi-150  $\mu\text{m}$ , 9 and 1.8 mg/mL Quinoline Yellow photoabsorber (QY; Merck 309052) was also added to the prepolymer solutions, respectively [17]. The presence of the QY photoabsorber in the prepolymer solution made the colour of the cross-linked sample yellow (Figure 3-2a,b). Each layer was exposed for 3 s to the UV light with wavelength of 365 nm at an intensity of 20 mW/cm<sup>2</sup>, equivalent to 120 mJ/cm<sup>2</sup> dosage, as each layer was exposed twice (Figure 3-2d,e). The structure was fabricated on the surface of a treated coverslip glass (24×24×1 mm. Agar Scientific AGL4367). The coverglasses were washed in three baths inside a sonication device (10 mins in each bath): 1. deionised water and soap, 2. acetone and 3. isopropanol. They were then dried using an air blower. After that, they were transferred to an oxygen plasma chamber (Diener Atto) for 3 cycles of 2.5

mins at the power of 50 W to remove any organic contaminants. Then, they were placed in a bath of solution mix of 2%v/v of 3-(trimethoxysilyl) propyl methacrylate (TMSPMA; Merck 440159) in 94–96% ethanol for 10 mins, washed with ethanol 99% for 3 times, and then placed on a hot plate set at 110 °C for another 10 mins to dry them off [21]. The treatment created a methacrylate layer on the surface of the coverglass to allow covalent binding with the fabricated hydrogels [25].

As control samples, monolithic structures were created in a 10×10×5 mm silicone mould using single exposure bulk cross-linking with 0.5 mL of PEGDA and LAP mix solution without QY. A thin layer of Fluorinated Ethylene Propylene (FEP) was placed on top of the mould to remove excess solution and to flatten the shape of the intended cuboid (Figure 3-1b). The solution was exposed to 3 s of UV light at 40 mW/cm<sup>2</sup> to equate to the same dosage as for every layer of the multilayer structure that was 120 mJ/cm<sup>2</sup> (Figure 3-2c). The fabricated monolithic hydrogel structures are denoted herein as mono-5 mm. The fracture like interfaces in the monolithic samples were from the surface of silicone mould that the samples were cross-linked in (Figure 3-2f). Two additional types of monolithic samples were fabricated with structure height of 1.5 mm and 3 mm, which are denoted herein as mono-1.5 mm and mono-3 mm. Those samples were used to examine the effect of the overall hydrogel structures' height on the thermal response of the hydrogel.

### **3.2.2 <sup>1</sup>H NMR and DSC Measurements**

All starting materials and the residual fraction from storage of selected multi-20 µm and multi-150 µm and mono-5 mm PEGDA hydrogels were characterised with Proton Nuclear Magnetic Resonance (<sup>1</sup>H NMR) spectroscopy (Bruker

Ascend 400 MHz spectrometer) with a BBFO probe, at an ambient temperature. The solvent peaks were referenced to 4.70 ppm (HDO, D<sub>2</sub>O) using Tetramethylsilane (TMS; Merck 87920, ≥99.5%, MW 88.22 g/mol) as an internal standard. Hydrogel samples were stored in 7 mL of DI water immediately after fabrication, for 24 hrs, allowing time for the unreacted polymer to leach out of the hydrogel structure. The DI water solution was then dried under vacuum to get rid of all moisture. The remaining solid residue was dissolved in 1 mL of Deuterium oxide (D<sub>2</sub>O; Merck 151882, ≥99.9%, MW 20.03 g/mol) and was characterised by <sup>1</sup>H NMR. The glass transition temperature of the PEGDA hydrogels was determined by Differential Scanning Calorimetry (DSC; Mettler Toledo, DSC 3+ STAR System).

### **3.2.3 Gravimetric and Volumetric Measurements**

PEGDA hydrogels thermal response was investigated through gravimetric and volumetric methods. Samples were transferred to individual temperature-controlled water baths at 8, 20, 37 and 45 °C inside a fridge or environmental cabinet. Temperatures 8 and 45 °C were chosen to represent cold-storage and transportation temperatures, 20 °C is the fabrication temperature, and 37 °C was the incubation temperature mimicking the biomedical application environment. For each temperature, 4 samples of multi-20 µm and multi-150 µm cuboid PEGDA hydrogels with dimensions 5 × 5 × 10.05 mm and 4 samples of mono- 5 mm cuboids with dimensions 10×10×5.1 mm were fabricated.

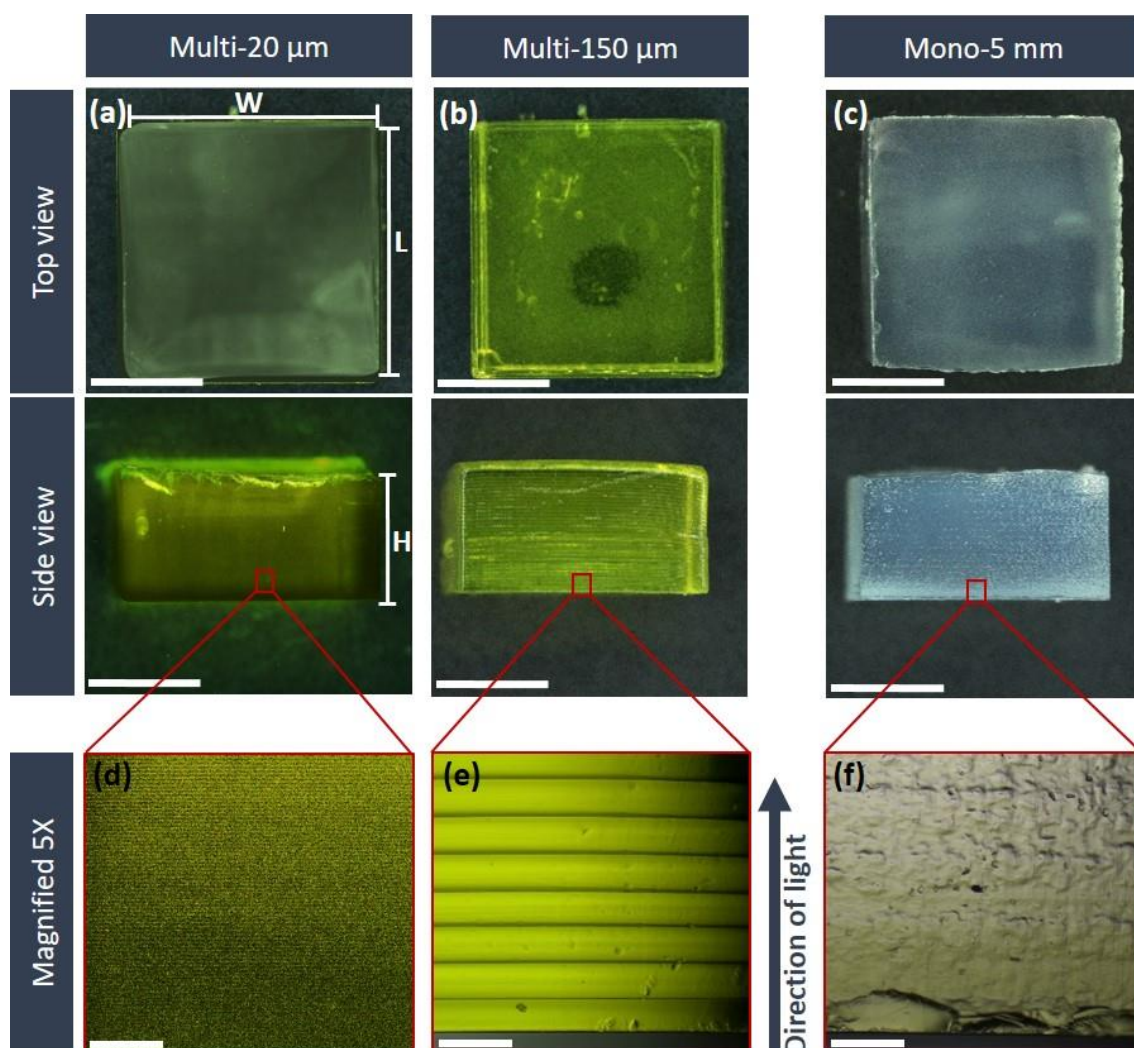


**Figure 3-2** Top and side view photographs of multi- (a) 20  $\mu\text{m}$  and (b) 150  $\mu\text{m}$  and (c) mono-5 mm PEGDA hydrogels. Side view microscopy images of multi- (d) 20  $\mu\text{m}$  and (e) 150  $\mu\text{m}$  and (f) mono- 5 mm PEGDA hydrogels. Scale bars in (a), (b) and (c) are 5 mm and in (d), (e) and (f) are 300  $\mu\text{m}$ .

All hydrogel samples were weighed immediately after fabrication and placed in their designated water bath. At predetermined time points, every 1.5–2 hrs for 6 hrs and every 24 hrs for 168 hrs, the hydrogel samples were removed from their water baths, gently blotted using medical wipes (Kimberly Clark) and weighed. The samples were then dried at the end of the 168 hrs of the study using freeze-

drying. The hydrogels were removed from their water baths and put straight inside a liquid nitrogen bath for 60 s. They were then transferred to a freezer set at  $-20^{\circ}\text{C}$  until all samples were frozen using liquid nitrogen. All samples were transferred into freeze dryer (Christ Alpha 1–2 LD plus) with condenser temperature and sublimation pressure set at  $-60^{\circ}\text{C}$  and  $1.1 \times 10^{-2}$  mbar and kept overnight. Their dried weight ( $M_d$ , unit: mg) was measured the following day using an analytical balance (Ohaus AS60C) with uncertainty of  $\pm 0.1$  mg.

The Normalised Weight Fraction (NWF, dimensionless) was calculated using the equation

$$\text{NWF} = M_s/M_0 \quad (3-1)$$

Where ( $M_s$ , unit: mg) is the weight of the hydrogel at time  $t$ , and ( $M_0$ , unit: mg) is weight of the hydrogel immediately after fabrication, i.e., time 0. The ( $M_s$ , unit: mg) was calculated by adding the solid residue weight ( $M_r$ , unit: mg) for each hydrogel sample to the recorded weight ( $M_t$ , unit: mg) at each time point.

$$M_s = M_t + M_r \quad (3-2)$$

To calculate the solid residue weight ( $M_r$ ), which measures the weight loss due to unreacted prepolymer PEGDA, LAP photoinitiator, and QY photoabsorber leaching out of the cross-linked hydrogels, 4 samples from both multilayer (e.g., multi-20  $\mu\text{m}$  and multi-150  $\mu\text{m}$ ) and monolithic (e.g., mono-5 mm, mono-3 mm and mono-1.5 mm) hydrogels were fabricated and dried immediately. The average dried weights of the samples ( $\overline{M_{d0}}$ , unit: mg) was calculated and compared to the dried weight ( $M_d$ , unit: mg) of each individual hydrogel, which were stored for 168 hrs at each nominal temperature.

$$M_r = \overline{M_{d0}} - M_d \quad (3-3)$$

The change in actual volume of the hydrogel samples was measured using the Normalised Volume Fraction (NVF, dimensionless). It was done by measuring the volume of the hydrogel samples immediately after fabrication ( $V_i$ , unit:  $\text{mm}^3$ ) and every 1.5–2 hrs for 6 hrs and overnight ( $V_t$ , unit:  $\text{mm}^3$ ). The measured volumes were normalised by the initial volume of the hydrogel. The measurements were done by recording the dimensions of the hydrated samples through capturing images, top: Width and length, side: height, using stereo microscope (Zeiss Stemi 305) while they were fully immersed in their water baths [26]. For normalised volume fraction the following formula was used

$$\text{NVF} = V_t/V_i \quad (3-4)$$

The normalised volume fraction percentage change ( $\Delta\text{NVF}$ , dimensionless) represents the volume change after 24 hrs storage of PEGDA hydrogels at temperatures of 8, 20, 37 and 45 °C. The  $\Delta\text{NVF}$  was calculated using the formula below

$$\Delta\text{NVF} = (\text{NVF}_t - 1) * 100\% \quad (3-5)$$

where ( $\text{NVF}_t$ , dimensionless) is the normalised volume fraction after 24 hrs.

The reversible thermal response of the multilayer PEGDA hydrogel to temperature variation was investigated by monitoring the NWF of 4 freshly fabricated multilayer samples by moving them around 3 water baths with temperatures set at (8, 20, and 45 °C), every 24 hrs for 168 hrs.



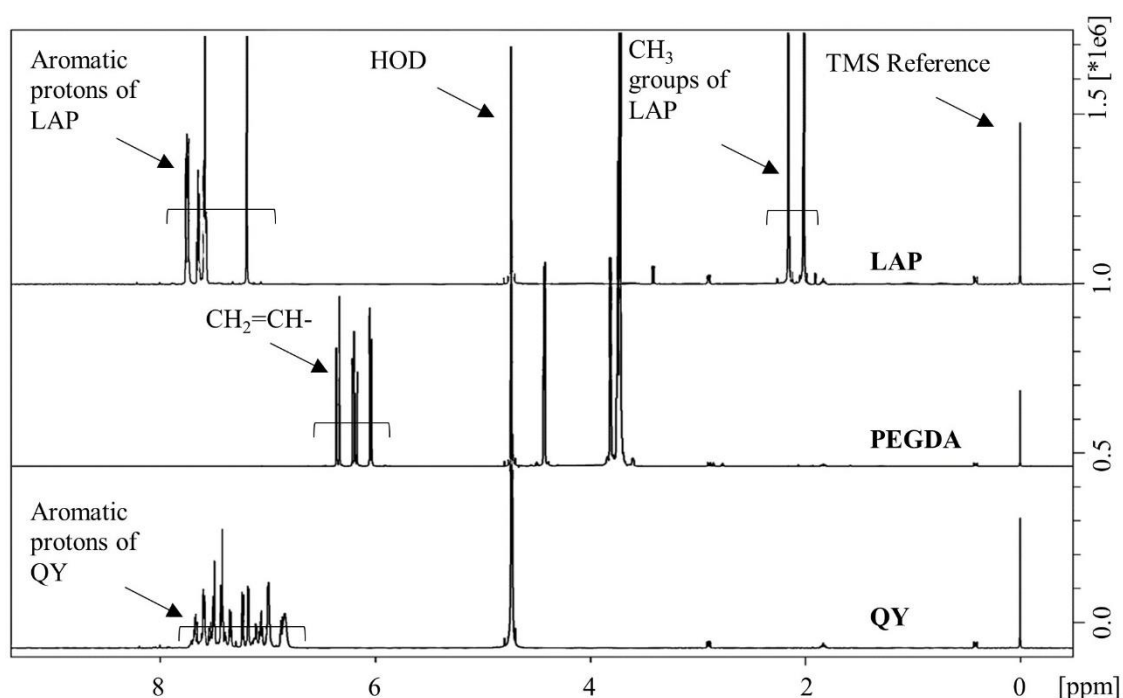


**Figure 3-3**  $^1\text{H}$  NMR spectra of the PEGDA hydrogels starting materials in  $\text{D}_2\text{O}$ . LAP photoinitiator (top) exhibits peaks of  $\text{CH}_3$  groups at 2.00 and 2.25 ppm and aromatic protons between 6.90 and 8.20 ppm. PEGDA monomer (middle) exhibits peaks of the protons of the  $\text{CH}_2=\text{CH}-$  double bonds centred at 6.25 ppm. QY photoabsorber (bottom) exhibits peaks of aromatic protons between 6.90 and 8.20 ppm. The peak at 0.00 ppm is attributed to TMS reference.

### 3.2.4 Scanning Electron Microscopy (SEM)

The interlayers of the multilayer PEGDA hydrogel were imaged using SEM (VEGA3, TESCAN) at an acceleration voltage of 10 kV and 3 nA beam current. The hydrogel samples were prepared using freeze-drying method to remove water. The samples were coated with 20 nm gold using sputter coater.

## 3.3 Results

The  $^1\text{H}$  NMR spectrum of PEGDA prepolymer solution showed two peaks at 3.80 ppm, which are attributed to the protons of  $\text{CH}_2=\text{CH}_2\text{O}$  groups of PEGDA, and 3

set of peaks centred at 6.25 ppm, which are attributed to the protons of  $\text{CH}_2=\text{CH}$ -groups (Figure 3-3). The peaks associated to aromatic protons from the LAP photoinitiator and QY photoabsorber are observed between 6.90 and 8.20 ppm (Figure 3-3). Two peaks at 2.00 and 2.25 ppm are attributed to one  $\text{CH}_3$  and two  $\text{CH}_3$ , respectively, of the LAP photoinitiator. The peak at 4.80 ppm is attributed to the solvent Deuterium hydrogen monoxide (HOD).

The  $^1\text{H}$  NMR spectrum of the solution in  $\text{D}_2\text{O}$ , in which mono-5 mm hydrogel had been stored for 24 hrs, exhibits negligible peaks at 6.25 ppm that can be attributed to the protons of  $\text{CH}_2=\text{CH}$ -groups of PEGDA. As only traces of a soluble PEGDA oligomeric fraction can be observed as two peaks centred at 3.80 ppm, this suggests that the fabrication of mono-5 mm hydrogel yields an almost 100% cross-linking of the PEGDA prepolymer into PEGDA polymer (Figure 3-4). Conversely, the  $^1\text{H}$  NMR spectra of the  $\text{D}_2\text{O}$  solutions after 24 hrs of storage from the multi-20  $\mu\text{m}$  and multi-150  $\mu\text{m}$  hydrogels were observed to exhibit peaks attributed to  $\text{CH}_2=\text{CH}$ - double bonds at 6.25 ppm. This suggests that a small fraction of PEGDA prepolymer was trapped in multilayer hydrogels during the photo-cross-linking process. Repeat measurements on multi-20  $\mu\text{m}$  hydrogels confirmed a similar degree of cross-linking of PEGDA polymer and reproducibility of the cross-linking data. The thermal analysis of the dry multi-20  $\mu\text{m}$ , multi-150  $\mu\text{m}$ , and mono-5 mm PEGDA hydrogel revealed the glass transition temperatures of the all the PEGDA polymers were between  $-41\text{ }^\circ\text{C}$  and  $-43\text{ }^\circ\text{C}$  using DSC (SI, Chapter 3, Figure S3-1a). On the other hand, the glass transition temperature of the prepolymer PEGDA solution was  $-15.4\text{ }^\circ\text{C}$  (SI, Chapter 3, Figure S3-1b).

These findings confirm that cross-linking process has taken place during the photo-cross-linking process.

Note that the amount of solid residue leaching out of the hydrogels into the storing media was observed to be dependent on the storage temperature. The mass of the solid residue within 168 hrs of storage in DI water was observed to be up to 7 mg on average, which is up to 4.4% of the original weight after fabrication. This was calculated through use of normalised dried weight (NDW) (SI, Chapter 3, Eq. (S3-5)) for multi-20  $\mu\text{m}$  and multi-150  $\mu\text{m}$  and mono-5 mm hydrogels which were stored at the nominal temperatures of 8, 20, 37 and 45 °C (SI, Chapter 3, Figure S3-3). Higher storage temperatures resulted in the release of higher mass, where the temperatures of 8 °C and 45 °C yield mass release of about 3.5% and 8%, respectively (SI, Chapter 3, Figure S3-3).

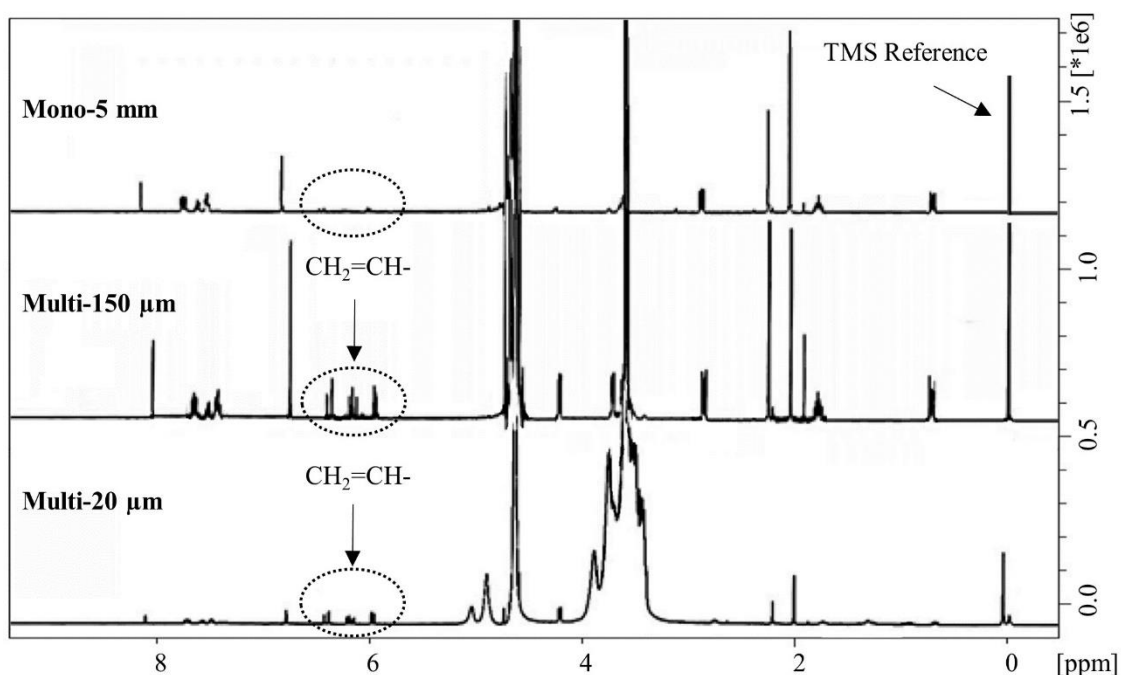


**Figure 3-4  $^1\text{H}$  NMR spectra of the solid residue after 24 hrs storage in  $\text{D}_2\text{O}$  from the PEGDA hydrogels: mono-5 mm (top), multi-150  $\mu\text{m}$  (middle), and multi-20  $\mu\text{m}$  (bottom) in  $\text{D}_2\text{O}$ . The peak at 0.00 ppm is attributed to TMS reference. Major peaks of protons of  $\text{CH}_2=\text{CH}-$  groups of PEGDA are at 6.25 ppm,  $\text{CH}_3$  groups from LAP at 2.00 and 2.25 ppm, and aromatics protons of QY and LAP between 6.90 and 8.20 ppm.**

The weight of multi- 150  $\mu\text{m}$  hydrogels reduced rapidly within the first 4.5 hrs, as indicated by a decrease in NWF (Eq. (3-1)) to 0.96 and 0.90 when the temperature was raised to 37  $^\circ\text{C}$  and 45  $^\circ\text{C}$ , respectively, from the fabrication temperature of about 20  $^\circ\text{C}$ . In contrast, NWF of multi-20  $\mu\text{m}$  hydrogels was surprisingly increased at 45  $^\circ\text{C}$  and 37  $^\circ\text{C}$  to 1.01 and 1.04 in the first 4–6 hrs, but then it dropped slightly to 0.95 and 1 after 24 hrs, respectively (Figure 3-5a). For both multi-20  $\mu\text{m}$  and multi-150  $\mu\text{m}$ , the NWF at a lower temperature of 8  $^\circ\text{C}$  increased

to 1.14 and 1.24 in the first 4.5 hrs and remained relatively constant there after (Figure 3-5a). Samples stored at the fabrication temperature of 20 °C exhibited a similar behaviour to those stored at 8 °C, displaying NWF values of approximately 1.2 and 1.1 for the multi-20 µm and multi-150 µm samples, respectively. All multi-20 µm, multi-150 µm and mono-5 mm PEGDA hydrogels reached constant mass at around 6 hrs of being stored in the temperature-controlled water baths at 8, 20, 37 and 45 °C. For mono-5 mm hydrogels, similar pattern with lower NWF values were recorded (Figure 3-5a). However, monolithic hydrogels appeared to be more thermal sensitive towards elevated temperatures of 37 °C and 45 °C. Note that NWF is a measure of weight fraction in the sample due to water uptake and release at different time points, which is at unity at 0 hrs for all multilayer and monolithic hydrogel samples.

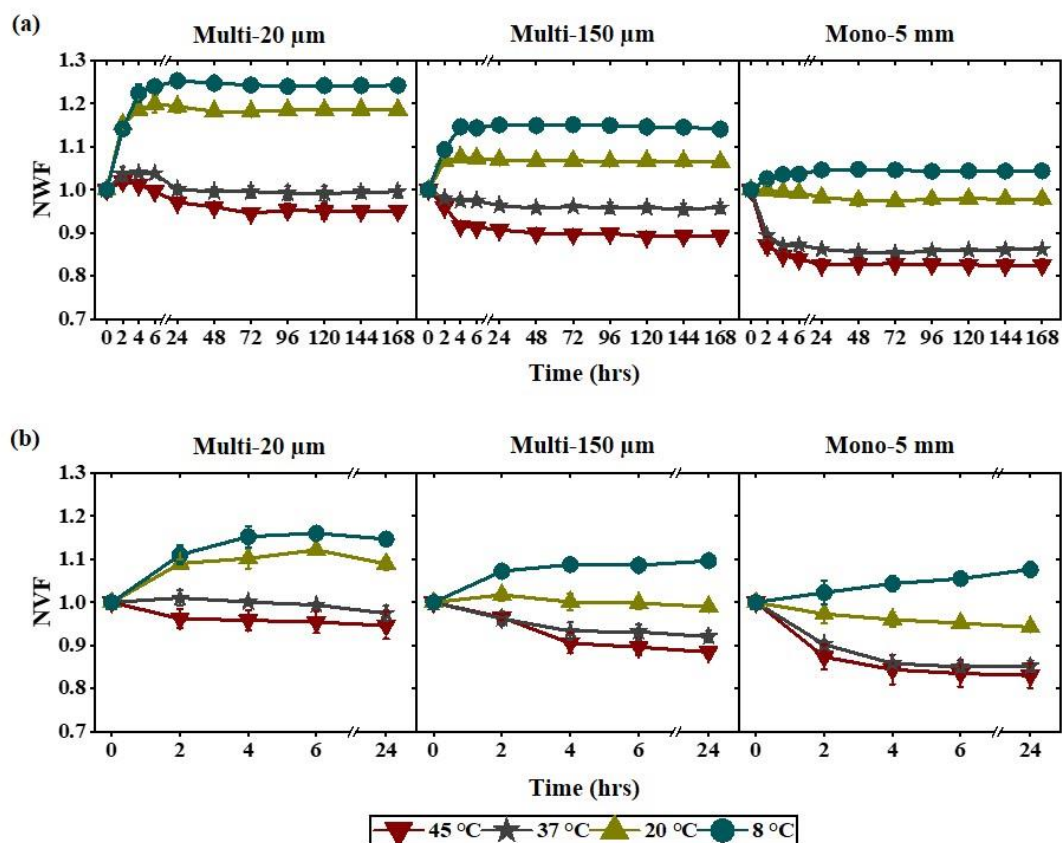


Figure 3-5 (a) NWF of multi-20  $\mu\text{m}$  and multi-150  $\mu\text{m}$  and mono-5 mm PEGDA hydrogels for up to 168 hrs after fabrication, which is denoted herein as the 0 hrs. (b) NVF of multi-20  $\mu\text{m}$  and multi-150  $\mu\text{m}$  and mono-5 mm PEGDA hydrogels samples within 24 hrs of the initial fabricated state. Error bars represent standard deviation from the mean, with  $n = 4$  and  $n = 3$  for independent multilayer and monolithic samples, respectively. Some error bars are not visible as they are smaller than the data point symbols. All lines are guide to the eye only.

A similar behaviour was observed when the thermal response was quantified volumetrically (Figure 3-5b). The NVF in multi-20  $\mu\text{m}$  hydrogels stored at 8  $^{\circ}\text{C}$  was slightly higher than that of the multi-150  $\mu\text{m}$  hydrogels. In the first 2 hrs, the NVF in multi-20  $\mu\text{m}$  increased to 1.11, while that of multi-150  $\mu\text{m}$  increased to 1.07. Although mono-5 mm PEGDA hydrogels had a similar overall behaviour to

the multi-20  $\mu\text{m}$  and multi-150  $\mu\text{m}$  ones, the magnitude of volume fraction for samples stored at 37 and 45  $^{\circ}\text{C}$  exceeded that of multi-20  $\mu\text{m}$  and multi-150  $\mu\text{m}$  hydrogels. In the first 4 hrs, the volume of mono-5 mm samples increased at 37 and 45  $^{\circ}\text{C}$  to reach NVF of 0.86 and 0.84, respectively, while that of multi-20  $\mu\text{m}$  and multi-150  $\mu\text{m}$  samples was found at 0.93 and 0.91, respectively (Figure 3-5b). The multi-20  $\mu\text{m}$  hydrogels, when stored at 20  $^{\circ}\text{C}$  after fabrication, exhibited a pattern akin to those stored at 8  $^{\circ}\text{C}$ , displaying a NWF of approximately 1.10. In contrast, the NWF for the multi-150  $\mu\text{m}$  samples was nearly 1.00. The dimensional change due to the temperature variation at nominal storage temperatures of 45, 37, 20 and 8  $^{\circ}\text{C}$  for multi-150  $\mu\text{m}$  hydrogels can be visually observed (Figure 3-6a). Note that these hydrogels were fabricated at a temperature of 20  $^{\circ}\text{C}$  and this visual observation was carried out 168 hrs after fabrication.

$\Delta\text{NVF}$  (Eq. (3-5)) is observed to be monotonically decreasing as temperature increases with a similar rate for all samples within the first 24 hrs after fabrication (Figure 3-6b). The thinner hydrogel samples exhibit a more positive  $\Delta\text{NVF}$  than the thicker ones. For instance, for multilayer hydrogels, the multi-20  $\mu\text{m}$  hydrogels had a higher  $\Delta\text{NVF}$  than that of multi-150  $\mu\text{m}$  at all temperature. The  $\Delta\text{NVF}$  for multi-20  $\mu\text{m}$  at temperatures of 8  $^{\circ}\text{C}$  and 45  $^{\circ}\text{C}$  was observed to be +15% and -5%, respectively, compared to +10% and -12%, respectively, for the multi-150  $\mu\text{m}$  hydrogels (Figure 3-6b). A similar behaviour was observed with monolithic samples, where the  $\Delta\text{NVF}$  of the mono-1.5 mm was higher than that of the mono-5 mm at temperatures of 8  $^{\circ}\text{C}$  and 45  $^{\circ}\text{C}$  (Figure 3-6b).

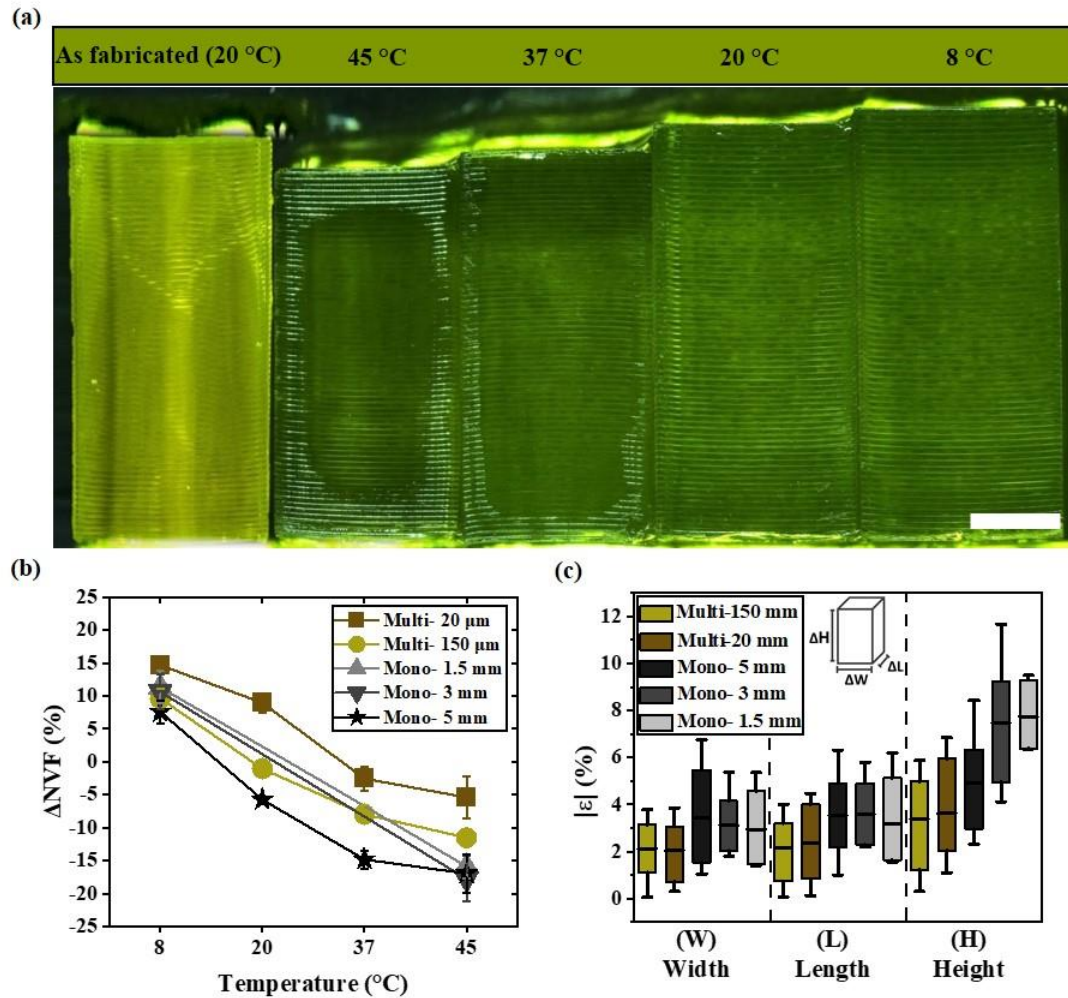


Figure 3-6 (a) Microscopy image of the multi-150  $\mu\text{m}$  hydrogels (composed of total of 67 layers) as fabricated and 168 hrs after fabrication where samples were stored at nominal temperatures of 45, 37, 20 and 8  $^{\circ}\text{C}$ . Image scale bar is 5 mm. (b) Comparison of the change in NVF ( $\Delta\text{NVF}$ ) for both multi- 20  $\mu\text{m}$  and multi-150  $\mu\text{m}$  and mono-5 mm, mono-3 mm and mono-1.5 mm hydrogel samples 24 hrs after fabrication. Error bars represent standard deviation from the mean  $n = 4$  and  $n = 3$  for multilayer and monolithic independent samples, respectively. Some error bars are not visible as they are smaller than the data point symbols. All lines are guide to the eye only. (c) Lateral and axial strain ( $|\epsilon|$ ) (SI, Chapter 3, Eqs. (S3-2–4)) in both multi- 20  $\mu\text{m}$  and multi-150  $\mu\text{m}$  and mono-5 mm, mono-3 mm and mono-1.5 mm hydrogel samples 24 hrs after fabrication. The whiskers represent the 1.5 times



**interquartile range. The median for each data set is identified by a horizontal line and the outliers are shown as diamond symbols.**

The change in volume itself was found to be asymmetric for all samples, where the strain in the axial direction, i.e. height, was considerably higher at approximately 58% on average compared to the lateral change, i.e. width and length (Figure 3-6c). The magnitude of lateral ( $|\epsilon_W|$  and  $|\epsilon_L|$ ) and axial strains ( $|\epsilon_H|$ ) was calculated by equations (SI, Chapter 3, Eqs. (S3-2–4)). This was done by calculating the dimensions change for width, length, and height for each sample within the first 24 hrs. Note that the axial direction of the samples is defined as the direction at which the UV light was applied onto the samples and the lateral direction is the plane of the sample normal to the direction of the UV light. It was observed that the thinner hydrogel samples, both multilayer and monolithic, exhibited slightly lower  $|\epsilon_W|$  and  $|\epsilon_L|$  but higher  $|\epsilon_H|$  than the thicker ones (Figure 3-6c). For multilayer samples,  $|\epsilon_H|$  of multi-20  $\mu\text{m}$  was observed to be up to 5% higher than that of multi-150  $\mu\text{m}$  hydrogels. Similarly for monolithic samples,  $|\epsilon_H|$  of mono-1.5 mm is up to 53.8% higher than that of mono-5 mm hydrogels.

The thermal response of PEGDA hydrogels was observed to be fully reversible within the temperature range of 8–45 °C (Figure 3-7a). For a multi-150  $\mu\text{m}$  hydrogel, the NWF reached 1.02 while being stored at 20 °C for 24 hrs after fabrication. The NWF of the same sample increased to 1.10 when the temperature was decreased to 8 °C. The NWF dropped to 0.85 when the temperature was increased to 45 °C within 24 hrs of storage, which then increased back to 1.02 when subsequently stored at 20 °C for the following 24 hrs of storage. This strongly suggests that the inversed correlation behaviour

between NWF of PEGDA hydrogels and storage temperature, in which NWF monotonically decreases with the increase of temperature, can be fully reversed (Figure 3-7b).

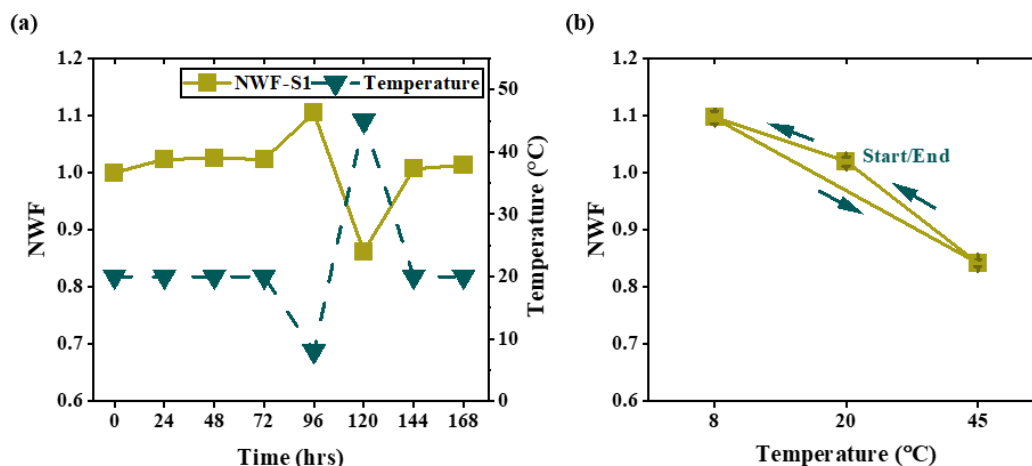


**Figure 3-7 Thermal response of individual multi-150  $\mu\text{m}$  hydrogels to temperature variation for 168 hrs. (a) NWF of a multi-150  $\mu\text{m}$  hydrogel moved between different water baths at 8, 20 and 45  $^{\circ}\text{C}$  as set times. (b) Average NWF of 4 independent multi-150  $\mu\text{m}$  hydrogels stored and moved in between different water baths. Arrows in (b) represent pattern of temperature change in different water baths. Error bars represent standard deviation from the mean  $n = 4$  for (b). Some error bars are not visible as they are smaller than the data point symbols. All lines are guide to the eye only.**

### 3.4 Discussion

This work demonstrated the feasibility of manufacturing multilayer PEGDA hydrogels using projection lithography technique and QY as photoabsorber with a nominal thickness of 20 and 150  $\mu\text{m}$ . QY was utilised to limit the UV light penetration into the prepolymer solution [18], thus a control over the thickness of the cross-linked layer could be enabled by controlling its concentration. At QY

concentrations of 1.8 mg/mL and 9 mg/mL, and with a UV light dosage of 120 mJ/cm<sup>2</sup>, we estimated the cross-linking depth using the extinction coefficient of the QY photoabsorber and the Beer-Lambert law. The cross-linking depth was determined to be  $46 \pm 2 \mu\text{m}$  for multi-20  $\mu\text{m}$  and  $299 \pm 5 \mu\text{m}$  for multi-150  $\mu\text{m}$ , respectively (SI, Chapter3, Figures S3-5, S3-6, and SI, Chapter 5, section 8). The nominal layer thickness was set to be approximately half that of the cross-linking depth at 20  $\mu\text{m}$  and 150  $\mu\text{m}$  for multi-20  $\mu\text{m}$  and multi-150  $\mu\text{m}$ . As the cross-linking depth is about twice of the nominal layer thickness, each layer should, in principle, be exposed twice to UV light as the structure was built top down. It was suggested that the double exposure may enhance bonding between neighbouring layers [18]. NMR spectra showed that the average degree of cross-linking across the structure in multi-20  $\mu\text{m}$  and multi-150  $\mu\text{m}$  and mono-5 mm PEGDA hydrogels were different. The mono-5 mm, which did not contain QY photoabsorber in its prepolymer solution, had the highest degree of cross-linking, while multi-20  $\mu\text{m}$  with the highest QY concentration had the lowest cross-linking. This suggests that a higher QY photoabsorber concentration in the prepolymer solution leads to a lower degree of cross-linking in the resulting polymer. Caution must be taken to avoid overexposure, which may be detrimental to the mechanical properties required for cardiomyocyte studies due to increased cross-linking density which may lead to stiffer hydrogels [18,27–29]. As the mechanical properties of PEGDA are dependent on the degree of cross-linking, this creates an opportunity to tailor the properties of the photo-cross-linked structures by tuning the fabrication parameters, e.g. UV light dosage, QY photoabsorber concentration and number of layers. These parameters, and thus the properties,

can then be optimised to meet the requirement of specific applications [30]. For instance, soft hydrogels for use in cardiac tissue development can be fabricated by high QY photoabsorber concentration and number of layers and low UV dosage [31].

The significance of the slope in the regression line was explored across varying nominal temperatures (8, 20, 37, and 45 °C). Hypothesis testing was conducted for each curve at every time point to ascertain whether the slope equalled zero, employing the Method of Least Squares. This involved calculating the p-value at each time point and comparing it with a predetermined significance level of 0.05, corresponding to a 95% confidence level. If the p-value is equal to or exceeds the significance level, we do not reject the null hypothesis, indicating insufficient evidence to conclude the slope is not zero. This point approximates the onset of equilibrium for each sample. Based on the calculations (SI, Chapter 3, Table S3-1) at 8 °C, the multi-20 µm and multi-150 µm PEGDA hydrogels reached equilibrium after around 4 hrs, respectively (SI, Chapter 3, Table S3-1). At 20 °C, both types of PEGDA hydrogels achieved equilibrium after 6 hrs (SI, Chapter 3, Table S3-1). However, PEGDA hydrogels stored at 45 °C, specifically the multi-20 µm and multi-150 µm varieties, reached equilibrium after 24 and 72 hrs, respectively (SI, Chapter 3, Table S3-1). In contrast, monolithic PEGDA hydrogels reached equilibrium between 4 and 6 hrs at all temperatures (SI, Chapter 3, Table S3-1). This suggests that mass equilibrium for both multilayer and monolithic PEGDA hydrogels can be attained after 24 hrs at temperatures of 8, 20, and 37 °C, consistent with a previous study on 3D printed PEGDA composite hydrogels [27].

PEGDA hydrogels are at equilibrium when the rate of water expulsion slows down, as the osmotic pressure inside and outside the polymeric network reaches similar levels [26]. The variation in the equilibrium water content (EWC) of multi-20  $\mu\text{m}$  and multi-150  $\mu\text{m}$  and mono-5 mm hydrogels can be attributed to the variations in concentration of the PEGDA monomer in the prepolymer solution prior to the photo-cross-linking process (SI, Chapter 3, Figure S3-2). Changes in the concentration of PEGDA monomer in the prepolymer solution has been observed to directly influence the swelling kinetics of the cross-linked hydrogel [32].

While the NVF measurements were showing similar thermal response behaviour to NWF, it was suggested that this is not fully proportional to the gravimetric behaviour [26]. We observed that after 24 hrs, both multi-20  $\mu\text{m}$  and multi-150  $\mu\text{m}$  and mono-5 mm PEGDA hydrogels did not reach volumetric equilibrium. The network polymer chain itself may take much longer period of time to rearrange itself and dimensionally stabilise. It has been reported that some hydrogels, e.g. poly(N-isopropylacrylamide) PNIPAM, require more than a month to reach volumetric equilibrium [33]. Since the volumetric observations were only taken every 2 hrs for 6 hrs and at 24 hrs after fabrication, there were not enough data for a thorough and statistically reliable equilibrium hypothesis testing based on slope of the regression line. Therefore, it is inconclusive to suggest if PEGDA hydrogels have reached volumetric equilibrium within 24 hrs. For applications where dimensional stability of the cross-linked hydrogel is essential, e.g. for in vivo and in vitro tissue studies, the fabricated hydrogels may need to be prestored at constant 37 °C for more than 24 hrs before proceeding with experimentation.

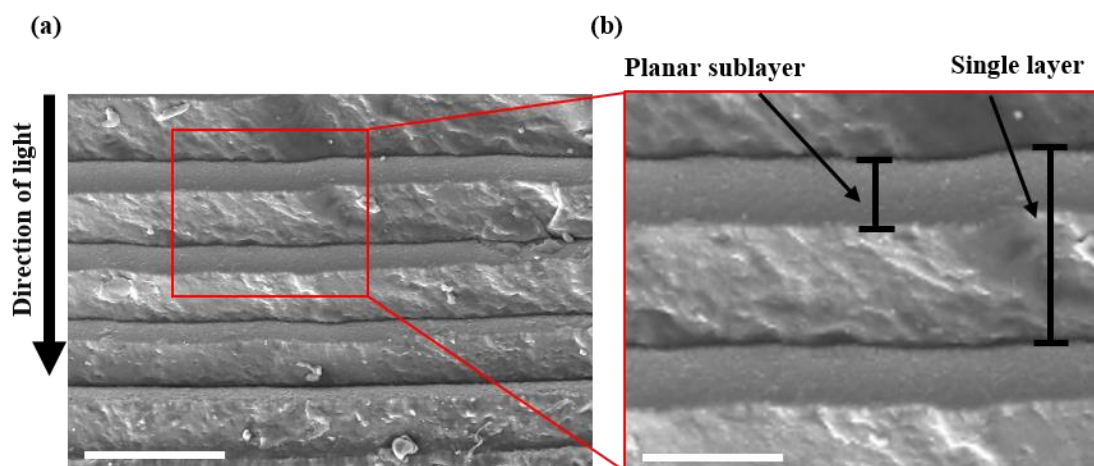


**Figure 3-8 SEM image of a freeze dried multi-150  $\mu\text{m}$  PEGDA hydrogel showing the highly cross-linked planar sublayers at the first surface of each layer. Scale bar for (a) and (b) are 200  $\mu\text{m}$  and 100  $\mu\text{m}$ , respectively.**

The difference in NVF between the multilayer and monolithic hydrogels may be attributed to variations in the degree of cross-linking in the fabricated PEGDA hydrogels. The higher degree of cross-linking leads to a higher volumetric percentage change in the monolithic hydrogels compared with multilayer hydrogels. The fact that the volume changes but mass remains the same suggests that the density of the hydrogel is not constant before reaching volumetric equilibrium. This is vital for the calculation of theoretical mesh size or porosity, as it is typically assumed that the polymer density is constant.

It is important to note that the strain in the axial direction ( $|\epsilon_H|$ ) was considerably higher than that of the lateral ( $|\epsilon_W|$ ) and ( $|\epsilon_L|$ ) for both multilayer and monolithic PEGDA hydrogels (Figure 3-6c). The anisotropic characteristic can be attributed to the formation of highly cross-linked planar sublayers at the interface of each layer. We hypothesise that these planar sublayers are thermally and structurally more stable than the remaining part of each layer. This leads to the formation of

an isostrain condition in lateral direction and an isostress condition in the axial direction. When the fabricated hydrogels undergo thermally induced volumetric strain, the highly cross-linked planar sublayers are able to limit the strain in the isostrain condition better than in the isostress condition. Therefore, the strain in the lateral direction is expected to be much less than that in the axial direction. This is in agreement with our observation where the swelling and shrinkage are higher in the axial direction rather than in the lateral direction (Figure 3-6c). Further observation on the Figure 3-6c, suggest that the multi-20  $\mu\text{m}$  hydrogels have higher axial dimensional change compared with the multi-150  $\mu\text{m}$ . This further supports our hypothesis that the increased number of highly cross-linked planar sublayers within the hydrogel structure, dependent on the number of printed layers in the structure, results in a more stable structure along the direction of the sublayers, i.e. lateral direction, while maintaining compliance in the direction normal to the sublayers, i.e. axial direction. SEM images of the multilayer hydrogel confirm the presence of highly cross-linked planar sublayer at the first surface of each layer (Figure 3-8).

The UV light diminishes in intensity as it passes through a solution containing a photoabsorber and controlling the concentration of the photoabsorber limits UV penetration, thus regulating the cross-linked layer's thickness. This leads to an exposure gradient in each layer, causing higher cross-linking at the layer's top, closest to the UV source, while the bottom might not fully cross-link. This may suggest either the described cross-linking depth is overestimated or there is a critical dosage threshold at which cross-linking start to occur. Additionally, we set the nominal layer thickness to be 20  $\mu\text{m}$  and 150  $\mu\text{m}$  for multi-20  $\mu\text{m}$  and multi-

150  $\mu\text{m}$ , respectively, assuming two exposures for each layer to improve adhesion. However, it was found that only a small upper section of the previously formed layer underwent double exposure during printing, which creating a highly cross-linked planar sublayer. The thickness of the highly cross-linked sublayer for both multi-20  $\mu\text{m}$  and multi-150  $\mu\text{m}$  was estimated to be  $3 \pm 2 \mu\text{m}$  and  $80 \pm 16 \mu\text{m}$  respectively (SI, Chapter 5, Section 9, Figure S5-9 and Figure S5-10), This gradient in cross-linking density across each layer adversely impacts mechanical properties throughout the layer's thickness.

A similar behaviour is also observed on the monolithic samples. The strain of three monolithic hydrogels, i.e. mono-5 mm, mono-3 mm and mono-1.5 mm, show that shorter structures correspond to higher  $|\epsilon_H|$  (Figure 3-6c). This suggest that the degree of cross-linking varies across the heights of monolithic samples in a similar fashion to the multilayer samples. Such a variation can be attributed to the cross-linking depth of the UV irradiation at a wavelength of 365 nm and a dosage of 120 mJ/cm<sup>2</sup> into the PEGDA hydrogels.

Note that the thermal response of all three monolithic hydrogels exhibits similar expansion and shrinkage behaviours to each other, as indicated by the similarity of their ECW, NWF, and NVF (SI, Chapter 3, Figure S3-4). This finding contradicts earlier findings where the UV photo-cross-linked PEGDA hydrogels with no photoabsorber were classed as isotropic gels [34]. For a collimated or slightly diverging UV irradiation source, the highly cross-linked sublayer is planar and normal to the direction of the impinging UV light. Therefore, PEGDA hydrogels manufactured by projection lithography are expected to be anisotropic due to the presence of these sublayers. This anisotropy may be reduced by



maximising the portion of the highly cross-linked sublayers within the hydrogels by post fabrication multiaxial UV exposure. Further investigation is needed to fully elucidate the underlying mechanism governing this anisotropy behaviour.

A previously reported study suggests a correlation in these polymers, particularly regarding the relationship between cross-linking density, elastic modulus, and CTE. This correlation implies that increased cross-linking density leads to higher stiffness and lower CTE [35]. Therefore, an alternative explanation for the observed anisotropic behaviour could be linked to the potential impact of differences in cross-linking density due to the attenuation of light intensity as it passes through the layer. These fluctuations in cross-linking density might suggest the presence of differing coefficients of thermal expansion (CTE) within each layer, spanning from the highly cross-linked sublayer to the rest of the layer.

Our gravimetric and volumetric analysis suggests that the thermal response of PEGDA hydrogels can be attributed to changes in hydrogen bonds strength at different temperatures. PEGDA comprises of hydrophilic (O-CH<sub>2</sub>-CH<sub>2</sub>) repeating unit backbone called oxyethylene compound [36] and acrylates side chains containing hydronutral vinyl groups [37]. The oxyethylene chain interacts favourably with the low temperature water structures and less favourably with the high temperature forms of water [38], as its average conformation changes from being more polar at low temperatures to being less polar at high temperatures [39]. This phenomenon has been consistently observed in samples stored at both the fabrication temperature of 20 °C and lower temperatures, such as 8 °C. At the fabrication temperature of around 20 °C, the oxyethylene chain in the backbone continues to exhibit hydrophilic behaviour, actively absorbing water

molecules until it reaches a state of equilibrium. With increased temperatures the oxyethylene chain undergoes drastic change from hydrophilic to hydrophobic because the negative entropy of water molecules around the nonpolar regions of PEGDA dominates [40]. Even though the hydrogel multilayer PEGDA hydrogels are fully immersed in water, the hydrogen bonds are weakened, and water molecules are desorbed and expelled from the polymer chain due to the dominant hydrophobic interactions between backbone groups at an elevated temperature [41,42]. The desorption of the water molecules results in shrinkage in the PEGDA polymer network (Figure 3-9). In addition, the outside region of the membrane would be affected first and thus the hydrophobic interactions amongst the vinyl groups in the outer regions become stronger. This leads to the rapid shrinkage of the outermost surface, forming a dense skin layer that prevents the free water from diffusing out and thus prolonging the response rate [41,43,44]. The opposite behaviour was also observed, where the increased polarity in lower temperatures results in stronger hydrogen bonds between the water molecules and the polymer network, hence more absorption of water molecules into the polymer network and higher swelling [45–47].

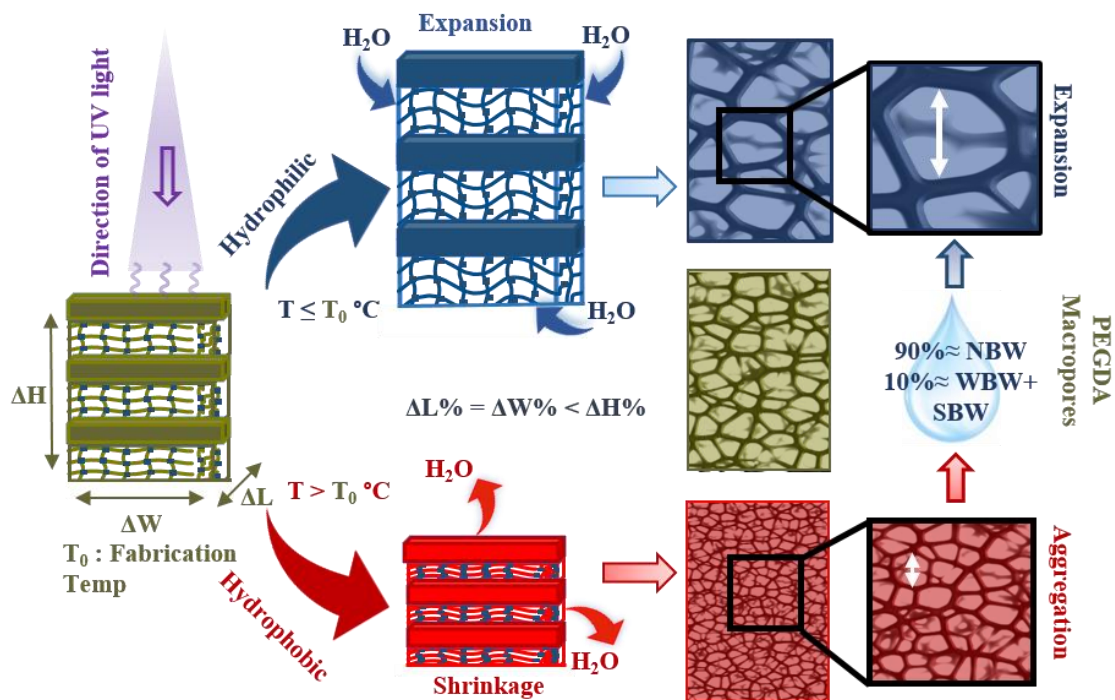


**Figure 3-9 Schematic of anisotropic thermoresponsive behaviour of PEGDA hydrogels, where above and below fabrication temperature, an unequal ( $L\% = W\%$  (lateral)  $< H\%$  (axial)) volume change is observed. Below fabrication temperature, due to higher NBW absorption and stretching of macropores, there is a volume expansion. Above fabrication temperatures, the NBW and WBW are expelled from the network, macropores aggregate and hydrogel shrinks.**

As multilayer PEGDA hydrogels respond reversibly to temperature changes and undergo reversible phase transition [45], they can be potentially used in a range of temperatures without suffering from thermal degradation by repeated temperature changes. A thermal responsive PEGDA hydrogel exhibits a reversible expansion/shrinkage transition where the polymer can revert back to its original state of equilibrium, within a certain temperature range, above or below the critical gel transition temperature (CGTT) [42]. It has been reported that PEGDA with molecular weight of 400 had a CGTT between 34 and 36 °C [46].

Even though it was not investigated herein, we can speculate that the CGTT of our polymer would have similar values to that of PEGDA 400. This suggests that the mass lost/gained through the thermal expansion/shrinkage process may be recuperated within the 24 hrs period if the storage temperature goes above or below 34–36 °C.

All the aforementioned findings suggest that PEGDA is a macroporous hydrogel that absorbs water molecules into its pores. The water absorption behaviour is strongly dependant on temperatures, as it is influenced by the contributions of free water, non-bound water (NBW), weakly bound water (WBW), and strongly bound water (SBW) (Figure 3-9) [48]. The main portion of water is attributable to free water and NBW located within macropores. As the temperature increases above the fabrication temperature ( $T_0$ ), polymeric chains start to aggregate, phase separation takes place and the shrunk micropores expel the free water, NBW and WBW from the PEGDA polymer network [33,49]. The SBW which forms the primary hydration shell around the PEGDA polymer chains that is located within the micro and nanopores in the macropore walls is not effected by range of temperature in the study, as they are strongly bound to the network chain and are normally trapped within the nanopores [26,48,50]. The size, distribution and alignment of the pores in the hydrogel is of relevance for in vitro studies, as they affect attachment of the living tissues, muscles, and their performance [51].

### **3.5 Conclusions**

This study was directed to investigate the thermal response of multilayer and monolithic photo cross-linked PEGDA hydrogels. The equilibrium weight and volume of photo cross-linked PEGDA hydrogels at storage temperatures of 8–45

°C was observed to change between +14% and -19%. At 37 °C, which represents the incubation temperature, a negative 5–15% weight and volume change was observed. The Normalised Weight Fraction (NWF) of the multi-20 µm and multi-150 µm hydrogels increased to +14% at lower 8 °C temperature, whereas the NWF decreased to -11.1% at 45 °C. As a result of the water uptake and release, the volume of the PEGDA hydrogels also changed. The NVF results showed that the multi-20 µm and multi-150 µm layered hydrogels experienced expansion of approximately +10% at 8 °C, while they shrunk at 45 °C to -11.5%. This expansion and shrinkage were observed to be due to change in the oxyethylene chain of the polymer network when temperature changes. The backbone of the PEGDA polymer network undergoes drastic change from hydrophilic to hydrophobic at elevated temperatures. This weakens the hydrogen bonds and water molecules are expelled from the polymer chains due to the dominant hydrophobic interactions between backbone groups. The desorption of the water molecules results in shrinkage in the PEGDA polymer network. At lower temperatures, an opposite behaviour was seen, where stronger hydrogen bonds between the water molecules and the polymer network due to increased polarity, results in more absorption of water molecules into the polymer network and higher polymer expansion.

These findings suggest that differences in the degree of polymerisation and application temperatures have to be considered in the design and fabrication process. In addition, the observed anisotropic behaviour of photo cross-linked PEGDA hydrogel between the axial and lateral directions must be taken into account into the design of the UV irradiation direction during the polymerisation

process. A projection lithography of PEGDA hydrogels using a single UV irradiation source leads to a large anisotropic characteristic, where the thermal responses in axial dimension was about 58% higher than those in lateral dimension. The multilayer PEGDA hydrogels were observed to have reversibility behaviour at temperatures of 8–45 °C. However, further investigation with high number of cycles or frequency needs to be undertaken to fully understand the effect of the temperature cycles on the degradation of the hydrogels. The findings presented herein should be considered as a basis for further studies into the effect of fabrication protocol and environmental conditions on the long term physicochemical and thermomechanical behaviour of the PEGDA hydrogels, which are crucial to many future biomedical and bioengineering applications.

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## Supplementary Information (Paper 2)

### 1. DSC measurements for 3D printed PEGDA hydrogels

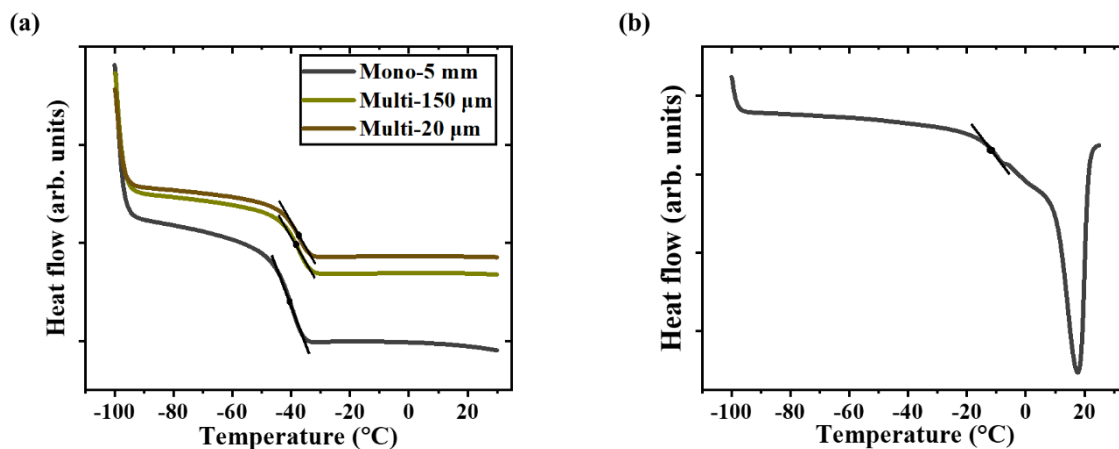


Figure S3- 1 (a) Thermogram of multi-20  $\mu\text{m}$  (blue line, top spectrum), multi-150  $\mu\text{m}$  (red line, middle spectrum) and mono-5 mm (black line, bottom spectrum) PEGDA hydrogel second heating from -100 °C to 25 °C, at 10 °C/min (before swelling in  $\text{D}_2\text{O}$ ) with glass temperatures recorded between -41 °C and -43 °C. (b) Thermogram of PEGDA monomer, second heating from -100 °C to 25 °C, at 10 °C/min. Glass transition for PEGDA monomer recorded at -15.38 °C.

## 2. Calculating equilibrium water content (EWC)

The equilibrium water content (EWC, dimensionless) was calculated using the equation

$$\text{EWC} = (M_s - M_d)/M_s \quad (\text{S3-1})$$

$M_s$ , unit: mg, is the weight of the hydrogel at time  $t$ , and  $M_d$ , unit: mg, is the dry weight of the hydrogel [18].

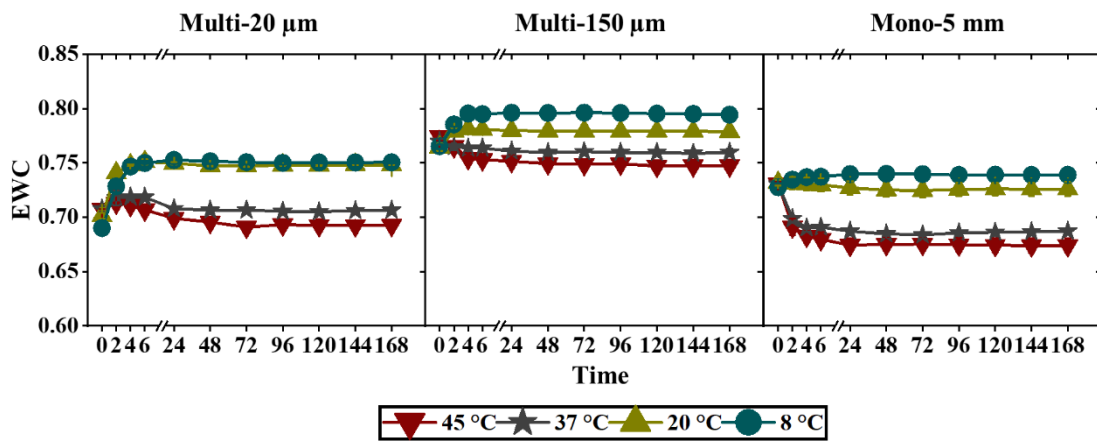


Figure S3- 2 EWC of multi-20 and 150  $\mu\text{m}$  and mono-5 mm PEGDA hydrogel samples, both stored at 8, 20, 37 and 45  $^{\circ}\text{C}$  in DI water for 168 hrs (7 days) measured at predetermined time points immediately after printing (0 hrs). Error bars represent standard deviation from the mean  $n=4$  and  $n=3$  for multilayer and monolithic independent samples, respectively. Some error bars are not visible as they are smaller than the data point symbols. All lines are guide to the eye only.

### 3. Calculating lateral and axial strain

To calculate the lateral and axial strain ( $|\varepsilon_{W,L,H}|$ , dimensionless) the following set of equations were used. First, the percentage width, length and height change was calculated for each sample.

$$|\varepsilon_{Wn}| = \left| \frac{W_t - W_0}{W_0} \right| \times 100 \quad (\text{S3-2})$$

$$|\varepsilon_{Ln}| = \left| \frac{L_t - L_0}{L_0} \right| \times 100 \quad (\text{S3-3})$$

$$|\varepsilon_{Hn}| = \left| \frac{H_t - H_0}{H_0} \right| \times 100 \quad (\text{S3-4})$$

$W_t$ ,  $L_t$  and  $H_t$  and,  $W_0$ ,  $L_0$  and  $H_0$  were the width, length and height (which have the same dimension of length of mm) at time  $t$  (24 hrs) and immediately after printing (0 hrs).

#### 4. Calculating normalised dried weight (NDW)

$$NDW = (\bar{M}_{d0})/M_d \quad (S3-5)$$

To calculate NDW (dimensionless), the average dried weights of the samples ( $\bar{M}_{d0}$ , unit: mg) was calculated and compared to the dried weight ( $M_d$ , unit: mg) of each individual hydrogel which were stored for 168 hrs, at each temperature.

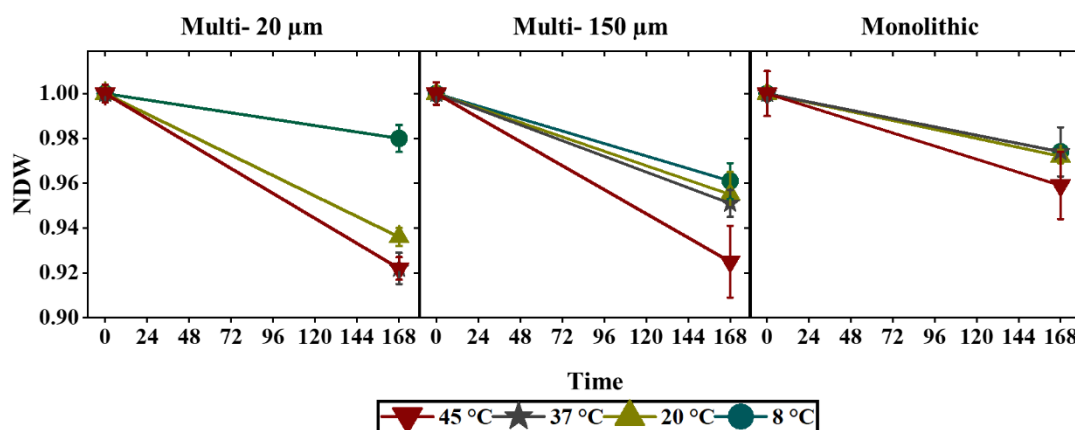


Figure S3- 3 NDW of multi-20 and 150 μm and mono-5 mm PEGDA hydrogel samples measured for all samples stored at 8, 20, 37 and 45 °C. Error bars represent standard deviation from the mean n= 3, 4 and 3 for monolithic, multilayer 150 and 20 μm layer thickness, respectively. Some error bars are not visible as they are smaller than the data point symbols. All lines are guide to the eye only.



## 5. Additional gravimetric measurements

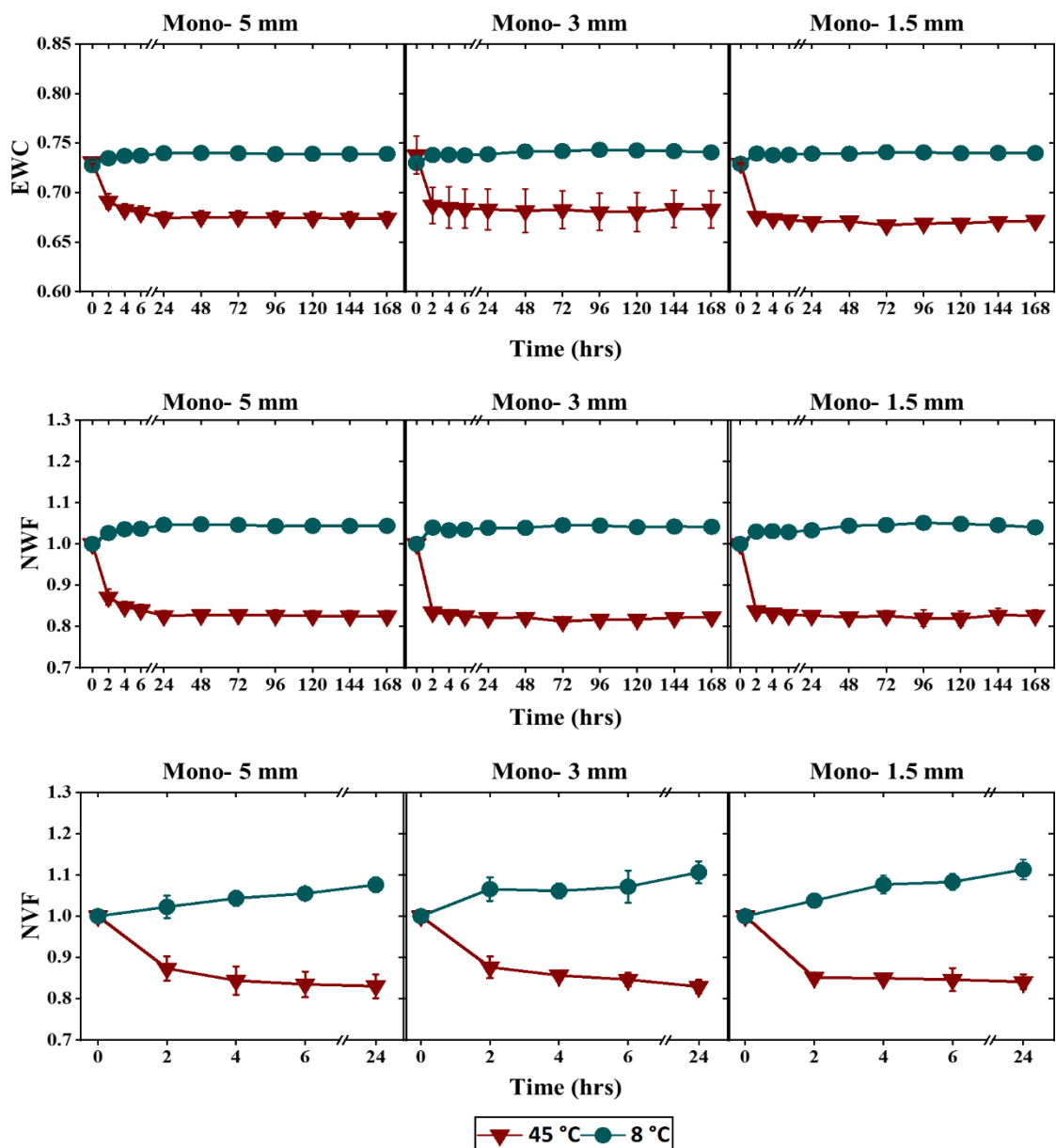


Figure S3- 4 EWC, NWF and NVF of mono-5, 3 and 1.5 mm thicknesses PEGDA hydrogel samples, stored at 8, 20, 37 and 45 °C in DI water for 168 hrs (7 days) measured at predetermined time points immediately after printing (0 hrs). Error bars represent standard deviation from the mean n= 3. Some error bars are not visible as they are smaller than the data point symbols. All lines are guide to the eye only.

## 6. Calculating the depth of cross-linking

First, prepolymer solutions with 1.8 and 9 mg/mL concentration of QY photoabsorber were separately prepared. Then, a treated coverslip glass was placed at the bottom of the vat. The prepolymer solution of one concentrations was poured into the vat and filled almost half of the vat volume. Then, the prepolymer solution was exposed to the same UV light with an equivalent dose of  $120 \text{ mJ/cm}^2$  (light intensity  $20 \text{ mW/cm}^2$  for a period of 6s). The cross-linked hydrogel layer was attached to the coverslip glass, due to being covalently bonded to it. The coverslip glass was attached on a right angle glass prism and the thickness of the cross-linked hydrogel layer was measured using an optical microscope. The thickness of the layer would indicate the cross-linking depth for each concentration.

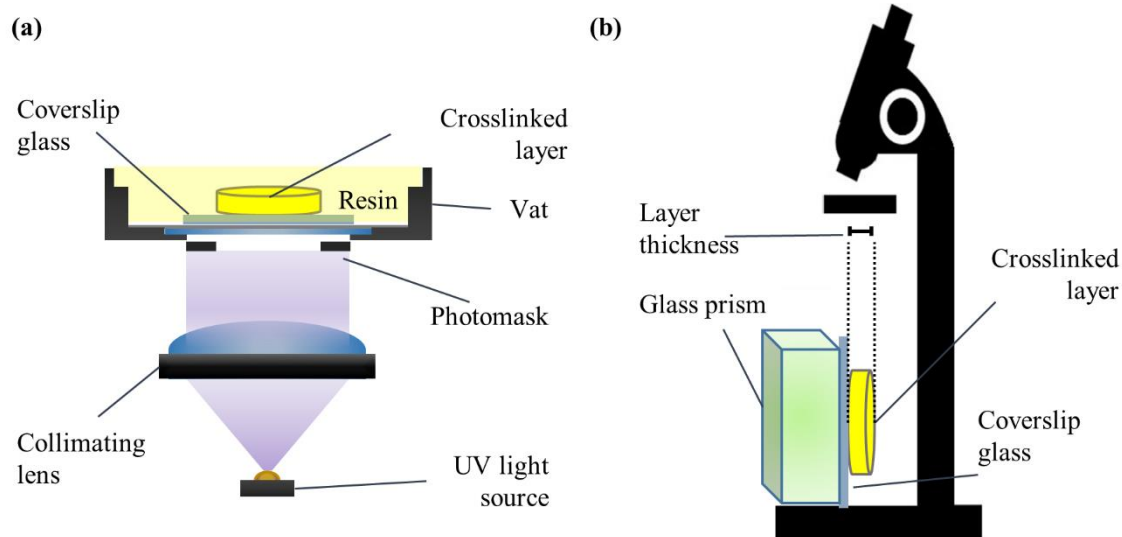


**Figure S3- 5 Schematic of (a) setup for fabricating single layer hydrogels on coverslip glass, (b) Use of optical microscope for determining depth of UV light penetration for pre-polymer solutions with various QY photoabsorber concentrations.**

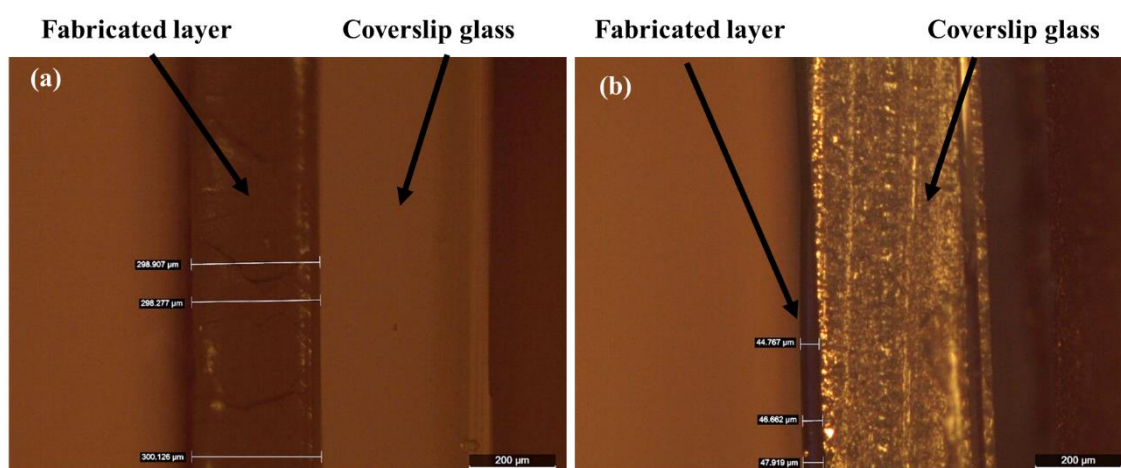


**Figure S3- 6 Microscopy images of the fabricated single layers for pre-polymer solution with (a) 1.8 mg/mL and (b) 9 mg/mL, of QY photoabsorber concentrations.**

## 7. The significance of the regression line slope for equilibrium identification using NWF results: step-by-step guide

### Step 1: Input the data

Enter your dataset into an Excel worksheet. Place the independent variable (timeline) (X) in column A and the dependent variable (NWF results) (Y) in column B.

**Step 2:** Perform Linear Regression Analysis for every single time point from 0 to 11 or 12, by calculate the following:

1. n, number of data points in the data set
2. r, correlation coefficient between the X and Y
3.  $S_x$ , standard deviation of X
4.  $S_y$ , standard deviation of Y
5. b, slope of the regression line

$$b = r \frac{S_y}{S_x} \quad (\text{S3-6})$$

6.  $S_{y.x}$ , standard error of the estimate of the dependent variable Y

$$S_{y.x} = S_y \sqrt{(1 - r^2) \frac{n-1}{n-2}} \quad (\text{S3-7})$$

7.  $S_b$ , standard error of the slope

$$S_b = \frac{S_{y.x}}{S_x \sqrt{n-1}} \quad (\text{S3-8})$$

8. t, t-statistic

$$t = \frac{b}{S_b} \quad (\text{S3-9})$$

9. df, degrees of freedom

$$df = n - 2 \quad (\text{S3-10})$$

10. p-value, probability associated with the observed t-statistic

$$p - \text{value} = \text{TDIST}(\text{ABS}(t), df, 2) \quad (\text{S3-11})$$

**Step 3:** Conduct the Hypothesis Test

Write down the null hypothesis ( $H_0$ ) and alternative hypothesis ( $H_1$ ).

$H_0$ : The slope is equal to zero ( $\beta = 0$ ).

$H_1$ : The slope is not equal to zero ( $\beta \neq 0$ ).

Look for the "P-value" associated with the coefficient of the independent variable in the regression output.

Compare the p-value to the chosen significance level ( $\alpha = 0.05$ ).

If  $p\text{-value} < \alpha$ : Reject the null hypothesis.

If  $p\text{-value} \geq \alpha$ : Fail to reject the null hypothesis.

**Step 4:** If the null hypothesis is rejected, there is evidence that the slope is not zero. And, if it is failed to reject the null hypothesis, there is no sufficient evidence to conclude that the slope is not zero.

Therefore, identify the point at which the p-value exceeds the value of  $\alpha$  and select that time as the identified equilibrium point for the sample. Repeat for each sample to approximate the onset of equilibrium.

**Table S3- 1 Calculations of the p-value for approximation of onset equilibrium point using NWF findings for (a) mono-5 mm, (b) multi-20  $\mu$ m, (c) multi-150  $\mu$ m PEGDA hydrogels. Green boxes are the identified equilibrium time point where p-value is higher than significance level ( $\alpha = 0.05$ ) for every nominal temperatures of 8, 20, 37 and 45  $^{\circ}$ C and sample type meeting the 95% confidence interval requirement.**

| (a) Mono-5 mm |                 |                 |                 |                | (b) Multi-20 $\mu$ m |                 |                 |                 |                | (c) Multi-150 $\mu$ m |                 |                 |                 |                |
|---------------|-----------------|-----------------|-----------------|----------------|----------------------|-----------------|-----------------|-----------------|----------------|-----------------------|-----------------|-----------------|-----------------|----------------|
| Hours         | 45 $^{\circ}$ C | 37 $^{\circ}$ C | 20 $^{\circ}$ C | 8 $^{\circ}$ C | Hours                | 45 $^{\circ}$ C | 37 $^{\circ}$ C | 20 $^{\circ}$ C | 8 $^{\circ}$ C | Hours                 | 45 $^{\circ}$ C | 37 $^{\circ}$ C | 20 $^{\circ}$ C | 8 $^{\circ}$ C |
|               | p-value         |                 |                 |                |                      | p-value         |                 |                 |                |                       | p-value         |                 |                 |                |
| 0             | 0.028           | 0.04            | 0.00            | 0.01           | 0                    | 0.00            | 0.03            | 0.01            | 0.03           | 0                     | 0.00            | 0.00            | 0.29            | 0.03           |
| 2             | 0.007           | 0.04            | 0.01            | 0.04           | 2                    | 0.00            | 0.00            | 0.00            | 0.05           | 1.5                   | 0.00            | 0.00            | 0.02            | 0.04           |
| 4             | 0.013           | 0.17            | 0.04            | 0.19           | 4                    | 0.01            | 0.02            | 0.01            | 0.43           | 3                     | 0.01            | 0.00            | 0.00            | 0.54           |
| 6             | 0.059           | 0.47            | 0.19            | 0.64           | 6                    | 0.03            | 0.08            | 0.14            | 0.40           | 4.5                   | 0.00            | 0.01            | 0.00            | 0.33           |
| 24            | 0.141           | 0.38            | 1.00            | 0.03           | 24                   | 0.09            | 0.18            | 0.64            | 0.05           | 6                     | 0.00            | 0.04            | 0.01            | 0.24           |
| 48            | 0.007           | 0.01            | 0.06            | 0.07           | 48                   | 0.87            | 0.72            | 0.02            | 0.24           | 24                    | 0.01            | 0.12            | 0.02            | 0.01           |
| 72            | 0.022           | 0.02            | 0.13            | 0.33           | 72                   | 0.42            | 0.89            | 0.06            | 0.89           | 48                    | 0.04            | 0.50            | 0.12            | 0.01           |
| 96            | 0.103           | 0.00            | 0.52            | 0.31           | 96                   | 0.38            | 0.15            | 0.23            | 0.12           | 72                    | 0.16            | 0.26            | 0.20            | 0.00           |
| 120           | 0.387           | 0.06            | 0.61            | 1.00           | 120                  | 0.31            | 0.13            | 0.54            | 0.34           | 96                    | 0.34            | 0.81            | 0.53            | 0.02           |
| 144           |                 |                 |                 |                | 144                  |                 |                 |                 |                | 120                   | 0.33            | 1.00            | 0.15            | 0.22           |
| 168           |                 |                 |                 |                | 168                  |                 |                 |                 |                | 144                   |                 |                 |                 |                |
|               |                 |                 |                 |                |                      |                 |                 |                 |                | 168                   |                 |                 |                 |                |

# Chapter 4

## Nanoindentation Response of 3D Printed PEGDA Hydrogels in a Hydrated Environment

### Published as:

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## Abstract

Hydrogels are commonly used materials in tissue engineering and organ-on-chip devices. This study investigated the nanomechanical properties of monolithic and multilayer poly(ethylene glycol) diacrylate (PEGDA) hydrogels manufactured using bulk polymerisation and layer-by-layer projection lithography processes, respectively. An increase in the number of layers (or reduction in layer thickness) from 1 to 8 and further to 60 results in a reduction in the elastic modulus from 5.53 to 1.69 and further to 0.67 MPa, respectively. It was found that a decrease in the number of layers induces a lower creep index ( $C_{IT}$ ) in three-dimensional (3D) printed PEGDA hydrogels. This reduction is attributed to mesoscale imperfections that appear as pockets of voids at the interfaces of the multilayer hydrogels attributed to localised regions of unreacted prepolymers, resulting in variations in defect density in the samples examined. An increase in the degree of cross-linking introduced by a higher dosage of ultraviolet (UV) exposure leads to a higher elastic modulus. This implies that the elastic modulus and creep behaviour of hydrogels are governed and influenced by the degree of cross-linking and defect density of the layers and interfaces. These findings can guide an optimal manufacturing pathway to obtain the desirable nanomechanical properties in 3D printed PEGDA hydrogels, critical for the performance of living cells and tissues, which can be engineered through control of the fabrication parameters.



## 4.1 Introduction

Engineered hydrogel bioscaffolds have been extensively used in the area of biomedical engineering including drug delivery and tissue engineering due to their biocompatibility and the ability to tune their mechanical properties [1–4]. Additive manufacturing, such as three-dimensional (3D) printing, of bioscaffolds offers the ability to customise morphological and mechanical properties for specific tissue engineering applications such as cell encapsulations and 3D tissue formation [5–8]. A large body of literature reports that cell behaviours, including growth, migration, proliferation, differentiation, and tissue formation are all strongly influenced by mechanical cues at the substrate interface on which the cells are cultured [9–11]. For instance, the fate of stem cells can be directed by engineering the surface elastic modulus, topography, and adhesion of the hydrogel substrates [12–14]. The cell elongation direction and sarcomere alignment of muscle tissues can also be influenced by the surface elastic modulus of hydrogel substrates [15–18]. A projection lithography 3D printing technique enables rapid fabrication of photo-cross-linkable hydrogels with complex geometrical features, e.g., high aspect ratio pillars, lattices, or overhangs, and an alteration in associated mechanical characteristics [19,20]. This makes it critical to fully understand the surface mechanical properties of hydrogels for designing and engineering smarter bioscaffold structures expanding functionality [21–23]. A previous study has demonstrated the contractile force measurement of muscle tissue strips using 3D printed poly(ethylene glycol) diacrylate (PEGDA) hydrogel cantilevers [24], underpinned by the ability to engineer the elastic modulus and

selective adhesion at the interface between the tissue strip and the PEGDA cantilever.

While bulk mechanical measurements such as uniaxial compression testing and flexural bending tests provide useful information about the mechanical properties of 3D printed PEGDA structures [7,24–26], they are unsuitable for assessing the spatial variation in surface mechanical properties critical for cell adhesion and tissue formation [27–29]. Moreover, bulk measurement on 3D printed structures with complex geometry and anisotropic characteristics, e.g., high aspect ratio and multilayer [20], is nontrivial to ensure precision alignment of the applied force vector to the principal axes of the layers [30]. Such misalignment can lead to imprecise measurements from buckling or inhomogeneous surface loading and maybe more pronounced in multilayer structures with layer thickness in sub-tens of microns [30]. In contrast, methods such as nanoindentation and atomic force microscopy (AFM) are more suitable for assessing the surface properties of multilayer 3D printed structures [27,31,32], particularly when the alignment between the applied force and layer orientation is critical [33–37].

In this study, the effect of layer number and thickness on nanomechanical and creep behaviour of multilayer PEGDA hydrogel surfaces printed by layer-by-layer (LbL) projection lithography was investigated. It is demonstrated herein that the surface elastic modulus increases from 0.67 to 5.53 MPa by increasing the layer thickness from 20 to 1200  $\mu\text{m}$ . This finding is attributed to structural imperfections introduced by LbL fabrication using projection lithography and is in contrast to the extant understanding of the mechanical behaviour of 3D multilayer hydrogels with the common conception of “the thinner the stronger” [38–40]. This new

understanding is critical for predicting the functionality of 3D printed PEGDA hydrogels, which are of great interest in biomedical applications, as their nanomechanical characteristics may significantly affect the behaviour and performance of living cells and tissues.

## **4.2 Materials and Methods**

### **4.2.1 Fabrication of PEGDA 3D Structures**

A detailed description of methods of hydrogel sample printing, glass treatment, and drying has been described in a previous work [20]. The aqueous prepolymer solution contained 200 mg/mL poly(ethylene glycol) diacrylate (PEGDA, Mn 700 g/mol) and 5 mg/mL photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphine (LAP, ≥95%). For LbL printing of multilayer PEGDA hydrogels with variable layer thickness (multi-20  $\mu\text{m}$  and multi-150  $\mu\text{m}$ ), two prepolymer solutions with addition of 9 and 1.8 mg/mL quinoline yellow (QY) photoabsorber were prepared, respectively. An ultraviolet (UV) light source (wavelength of 365 nm) with an intensity of 20 mW/cm<sup>2</sup> was used. Each layer was exposed for 3 or 15 s, depending on the required dosage of either 120 or 600 mJ/cm<sup>2</sup>, respectively, to ensure cross-linking of every layer of the multilayer structure using a custom-made automated projection lithography printer.

### **4.2.2 Multilayer Samples**

All multilayer samples were printed on a 15 mm diameter laser cut and surface-treated glass slide to fit the well of a nanoindentation liquid cell. The cuboid multilayer sample dimensions were 5 × 5 × 1.2 mm (L × W × H). To maintain a sample thickness of 1.2 mm, samples with 20  $\mu\text{m}$  layer thickness consisting of

60 layers and samples with 150  $\mu\text{m}$  layer thickness consisting of 8 layers were prepared. Hence, the number of layers was inversely proportional to the nominal layer thickness. The samples were printed using LbL projection lithography with a 3 s UV exposure time. The samples were labelled herein as multi-20  $\mu\text{m}$ -3 s and multi-150  $\mu\text{m}$ -3 s (Figure 4-1a,b). The printed samples were stored in either deionised (DI) water (DIW, resistivity of 18.2  $\text{M}\Omega\cdot\text{cm}$ ) or cell culture medium (CCM) for 24 and 720 hrs at room temperatures between 20 and 21  $^{\circ}\text{C}$ . The CCM (Gibco RPMI 1640 Medium, Fisher scientific, Loughborough, U.K., with l-glutamine) is a standard cell culture medium, which uses a sodium bicarbonate buffer system of 2 g/L for in vitro diagnostic use [41]. Additionally, for investigating the effect of the number of layers on the mechanical properties, multilayer hydrogel samples with 4 and 32 layers were fabricated, labelled multi-300  $\mu\text{m}$ -3 s and multi-37.5  $\mu\text{m}$ -3 s, respectively. The QY photoabsorber concentration in the hydrogel samples, multi-300  $\mu\text{m}$ -3 s and multi-37.5  $\mu\text{m}$ -3 s, was kept the same as the sample multi-150  $\mu\text{m}$ -3 s at 1.8 mg/mL in the prepolymer solution. This eliminated the effect of variation in the QY photoabsorber concentration in the hydrogels produced. Moreover, a set of multilayer samples with a 15 s UV exposure time with 20 and 150  $\mu\text{m}$  layers was printed and named multi-20  $\mu\text{m}$ -15 s and multi-150  $\mu\text{m}$ -15 s, respectively (Table 4-1). The samples were stored in DIW for 24 hrs before indentation measurements.

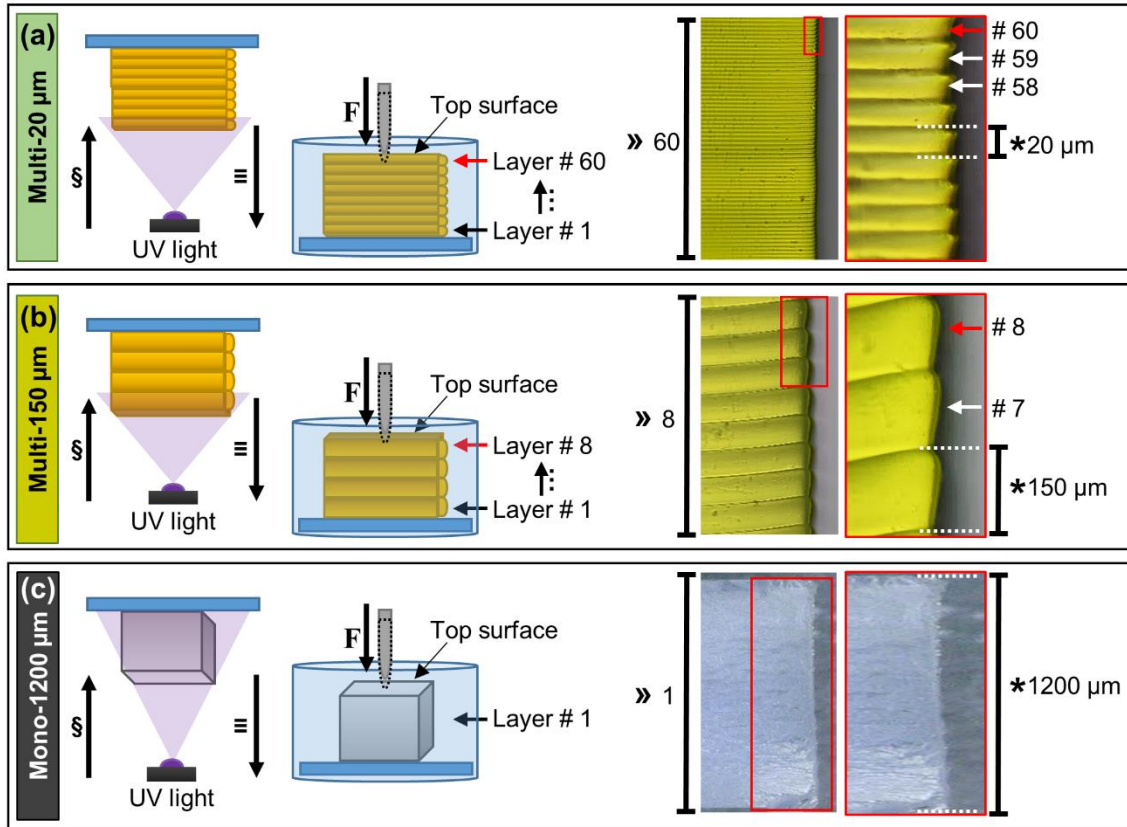


**Figure 4-1** Schematics (left) of the light and print direction and (right) side-view optical microscopy photographs of (a) multi-20  $\mu\text{m}$ , (b) multi-150  $\mu\text{m}$ , and (c) mono-1200  $\mu\text{m}$  PEGDA hydrogel structures. Samples stored in deionised water (DIW) and cell culture medium (CCM) at room temperature (20–21  $^{\circ}\text{C}$ ) at a predefined storage time points (0–720 hrs after fabrication) before indentation. (S) Projection light direction, ( $\equiv$ ) printing direction, (F) indentation direction, ( $\gg$ ) total number of layers, (\*) individual layer thickness. Note that the total structural height for all samples was 1200  $\mu\text{m}$ .

#### 4.2.3 Monolithic Structures

For single-layer monolithic samples, a prepolymer solution of PEGDA and LAP was poured into a vat, and the sample stage was moved to a height of 1200  $\mu\text{m}$ . Cuboid monolithic structures of dimensions  $5 \times 5 \times 1.2 \text{ mm}$  (L  $\times$  W  $\times$  H) were fabricated with UV exposure times of 3 and 15 s, labelled mono-1200  $\mu\text{m}$ -3 s and

mono-1200  $\mu\text{m}$ -15 s, respectively (Table 4-1). The fabricated samples were then stored in DI water for 24 hrs before measurements (Figure 4-1c). The hydrogel structures were fabricated on the surface of a treated microscope glass slide ( $76 \times 26 \times 1\text{--}1.2$  mm, Fisher Scientific, Loughborough, U.K.) cut to  $24 \times 24 \times 1\text{--}1.2$  mm to fit the sample holder.

**Table 4-1 List of multilayer and monolithic samples printed for the nanoindentation measurements in this study<sup>a</sup>**

| Sample name                   | QY<br>(mg/mL) | # of<br>layers | Layer<br>thickness<br>( $\mu\text{m}$ ) | UV<br>exposure<br>time (s) | UV<br>dosage<br>( $\text{mJ}/\text{cm}^2$ ) |
|-------------------------------|---------------|----------------|-----------------------------------------|----------------------------|---------------------------------------------|
| Multi-20 $\mu\text{m}$ -3 s   | 9             | 60             | 20                                      | 3                          | 120                                         |
| Multi-20 $\mu\text{m}$ -15 s  | 9             | 60             | 20                                      | 15                         | 600                                         |
| Multi-150 $\mu\text{m}$ -3 s  | 1.8           | 8              | 150                                     | 3                          | 120                                         |
| Multi-150 $\mu\text{m}$ -15 s | 1.8           | 8              | 150                                     | 15                         | 600                                         |
| Multi-300 $\mu\text{m}$ -3 s  | 1.8           | 4              | 300                                     | 3                          | 120                                         |
| Multi-37.5 $\mu\text{m}$ -3 s | 1.8           | 32             | 37.5                                    | 3                          | 120                                         |
| Mono-1200 $\mu\text{m}$ -3 s  | 0             | 1              | 1200                                    | 3                          | 120                                         |
| Mono-1200 $\mu\text{m}$ -15 s | 0             | 1              | 1200                                    | 15                         | 600                                         |

<sup>a</sup>The overall structure height was maintained at 1.2 mm for all samples.

#### 4.2.4 Nanoindentation Measurements

Nanoindentation experiments were carried out using a Hysitron Bioindenter (Bruker Hysitron, Minneapolis) fitted with a 50  $\mu\text{m}$  diameter, spherical diamond tip. Samples were mounted on coverslip glasses and fixed to a standard glass slide. Tests were carried out in a hydrated environment using a custom-designed liquid cell that fits over a glass slide to create a well for the solution. PEGDA hydrogels were stored in DIW and CCM for 24 and 720 hrs before testing. After mounting the hydrogel in the liquid cell inside the machine's enclosure, the

sample was left for at least 2 hrs to reach equilibrium to minimise effect of thermal drift. The indentations were carried out in a load-controlled manner with a maximum load of up to 20  $\mu\text{N}$ . The load profile of 5 s load, 2 s hold period, and 5 s unload segments was used. For each sample, 12 indents were performed at random locations on top of the hydrogel samples. The indenter was set with a 2  $\mu\text{N}$  preload to ensure that the tip was in contact with the sample surface [42]. The surface of the sample in contact with the probe is the final layer, top surface, exposed to UV light during printing (Figure 4-1). The samples were analysed by extracting  $P-h$  (Figure S4-1) curves from the Hysitron Triboscan Analysis software, and the elastic modulus ( $E$ ) was estimated using the Oliver and Pharr method by analysing the unloading curve of the nanoindentation curve using (SI, Chapter 4, Eqs. (S4-1–6)). For quantifying the creep behaviour, the normalised simple isothermal creep index ( $C_{IT}$ ) obtained from (SI, Chapter 4, Eq. (S4-7)) and creep strain rate ( $\epsilon$ ) calculated using (SI, Chapter 4, Eq. (S4-8)) [43,44].

#### 4.2.5 Gravimetric Measurements

In a previous study, the thermal response of 3D multilayer PEGDA hydrogels was investigated through a gravimetric method for samples stored in deionised water (DIW) [20]. As 3D PEGDA hydrogels are intended for use in biological applications, their thermal response was expanded to include the hydrogel stored in cell culture medium (CCM) baths at 8, 20, and 37 °C. Two cuboid samples of multi-20  $\mu\text{m}$  (comprising of 255 layers) and two multi-150  $\mu\text{m}$  (comprising of 34 layers) PEGDA hydrogels with dimensions 5 × 5 × 5.10 mm were fabricated, using 3 s UV exposure time, for each temperature in DIW and CCM baths.

Immediately after fabrication, 3D multilayer PEGDA hydrogel samples were washed with DIW to remove unreacted prepolymers from the surface, gently blotted using medical wipes, weighed, and placed in their assigned DIW and CCM baths at their controlled-temperature environment (Figure 4-2). The weight of the hydrogel samples was monitored every 2 hrs over the first 6 hrs and then at 24, 48 and 720 hrs (30 days). At each time point, the hydrogel samples were taken out of their storage solution, gently blotted using tissue paper, and weighed before returning to the same storage solution. The samples were vacuum-dried in a desiccator for 24 hrs at room temperature at the end of 720 hrs study. Their dried weight ( $M_d$ ) was measured the following day using an analytical balance.

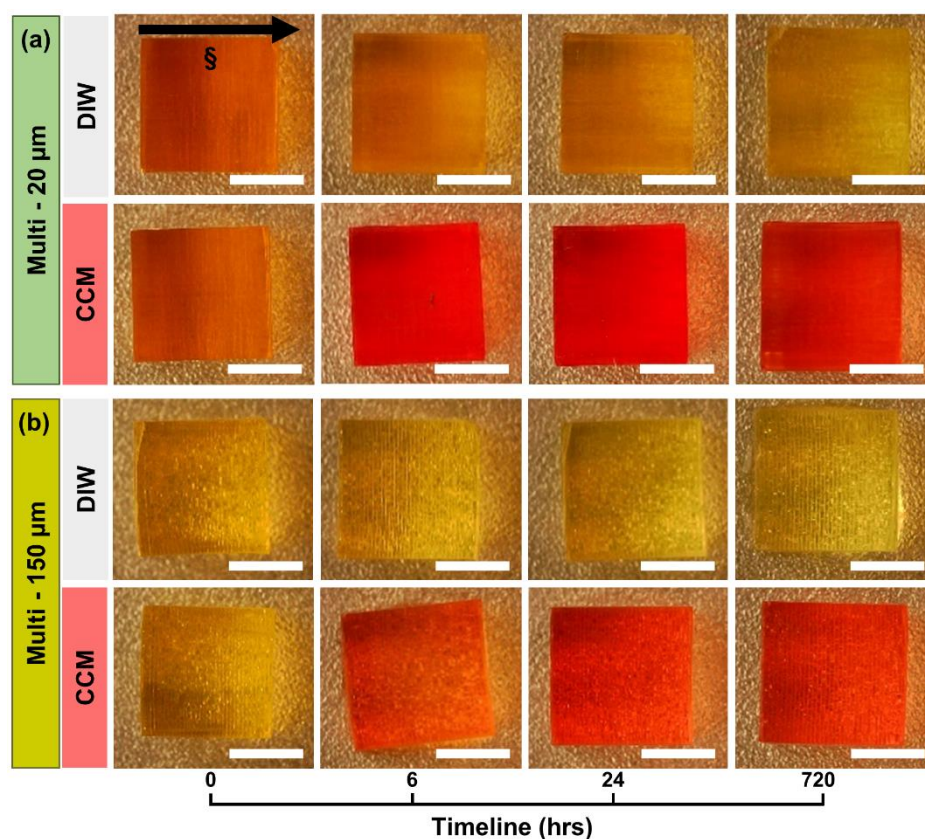


**Figure 4-2** Side-view photographs of 3D multilayer (a) multi-20  $\mu\text{m}$ -3 s and (b) multi-150  $\mu\text{m}$ -3 s PEGDA hydrogel samples stored in DIW (upper) and CCM (lower)



at room temperature at predefined storage time points (0–720 hrs after fabrication), demonstrating the inward diffusion of CCM into the hydrogel system and outward diffusion of the QY photoabsorber from the hydrogel into the DIW storage solution. Zero hr is the time immediately after printing. The scale bars in all images are 5 mm. Note that the dark shades within the hydrogels are due to uneven illumination of the microscope. Direction of projection light (§) is from top to bottom of the structure.

The normalised weight fraction (NWF, dimensionless) was used to calculate the weight change using Eq. (4-1) [20],

$$\text{NWF} = M_s/M_0 \quad (4-1)$$

where ( $M_s$ , unit:mg) and ( $M_0$ , unit: mg) are the weight of the hydrogel at time  $t$  and weight of the hydrogel immediately after fabrication, i.e., time 0, respectively.

#### 4.2.6 $^1\text{H}$ NMR Measurements

All starting materials, PEGDA monomer, LAP photoinitiator, and QY photoabsorber were characterised by proton nuclear magnetic resonance ( $^1\text{H}$  NMR) spectroscopy in deuterium oxide ( $\text{D}_2\text{O}$ ; Merck 151882,  $\geq 99.9\%$ ,  $M_w$  20.03 g/mol), at ambient temperature, using a Bruker Ascend 400 MHz spectrometer with a BBFO probe and tetramethylsilane (TMS; Merck 87920,  $\geq 99.5\%$ ,  $M_w$  88.22 g/mol) as an internal standard (Figure S4-2). The soluble fractions isolated from the swelling experiments of both 3D multilayer hydrogel samples with 5 mm of diameter and 2.5 mm of thickness (samples multi-20  $\mu\text{m}$ -3 s, multi-20  $\mu\text{m}$ -15 s, multi-150  $\mu\text{m}$ -3 s, and multi-150  $\mu\text{m}$ -15 s) and monolithic hydrogel samples (samples mono-1200  $\mu\text{m}$ -3 s and mono-1200  $\mu\text{m}$ -15 s) fabricated using

projection lithography at UV exposure times 3 and 15 s were characterised by  $^1\text{H}$  NMR. The procedure to obtain the soluble fractions involved vacuum-drying hydrogel samples immediately after fabrication. They were stored in 7 mL of water, for 24 hrs, allowing time for the hydrogel to swell and any residual starting materials to leach out of the structure. The dried residue was then characterised by  $^1\text{H}$  NMR in  $\text{D}_2\text{O}$  solution.

#### **4.2.7 Differential Scanning Calorimetry (DSC) Measurements**

The glass transition temperature ( $T_g$ ) of the 3D PEGDA hydrogels was determined by differential scanning calorimetry (DSC; Mettler Toledo, DSC 3+ STAR System) using a heating rate of  $10\text{ }^\circ\text{C}/\text{min}$  under an inert  $\text{N}_2$  atmosphere. The test temperature was cycled twice between  $-100$  and  $25\text{ }^\circ\text{C}$ . The variation of the heat flow in the samples was recorded as a function of temperature and time. Multilayer hydrogel samples, multi- $20\text{ }\mu\text{m}$ -3 s and multi- $150\text{ }\mu\text{m}$ -3 s, respectively, with 5 mm of diameter and 2.5 mm of thickness, previously stored in DIW and CCM for 24 and 720 hrs, were dried and then characterised in triplicate (approximately 9–14 mg). Additionally, multilayer, multi- $20\text{ }\mu\text{m}$ -3 s, multi- $20\text{ }\mu\text{m}$ -15 s, multi- $150\text{ }\mu\text{m}$ -3 s, and multi- $150\text{ }\mu\text{m}$ -15 s, and monolithic, mono- $1200\text{ }\mu\text{m}$ -3 s and mono- $1200\text{ }\mu\text{m}$ -15 s, PEGDA hydrogels exposed to 3 and 15 s UV light, stored in DIW for 24 hrs, were characterised after being dried.

#### **4.2.8 Scanning Electron Microscopy (SEM)**

SEM (VEGA3, TESCAN) at an acceleration voltage of 20 kV was used to image the cross section of vacuum-dried PEGDA hydrogels. The layers of the multilayer hydrogels were exposed by manually cutting the sample vertically along the printing direction using a  $100\text{ }\mu\text{m}$  thick stainless steel blade. A 20 nm gold film

was deposited on each sample using a sputter coater prior to imaging. The backscattered images were captured at  $\times 150$  and  $\times 600$  magnifications.

### 4.3 Results

As shown in Figure 4-3a, the ( $P-h$ ) plot of indents made on the multi-20  $\mu\text{m}$ -3 s hydrogel revealed a gentle slope of 7.2 N/m that was less than half and less than quarter compared to the plot of multi-150  $\mu\text{m}$ -3 s and mono-1200  $\mu\text{m}$ -3 s hydrogels of 16.1 and 32 N/m, respectively. The highest indentation depth, 2.4  $\mu\text{m}$  was seen on multi-20  $\mu\text{m}$ -3 s, compared to the multi-150  $\mu\text{m}$ -3 s and mono-1200  $\mu\text{m}$ -3 s hydrogels where the indentation depth was about 1.2 and 0.6  $\mu\text{m}$ , respectively. Figure 4-3b shows that  $E$  of the multi-20  $\mu\text{m}$ -3 s (60 layers) is approximately 2.5 times lower than that of the multi-150  $\mu\text{m}$ -3 s (8 layers). The decrease in  $E$  was correlated negatively with the increase in the number of layers or interfaces within the PEGDA hydrogel structures. As shown in Figure 4-3b, the monolithic PEGDA hydrogel, mono-1200  $\mu\text{m}$ -3 s, had an  $E$  of  $5.53 \pm 0.37$  MPa that was about 3-fold and 8-fold higher than those of the multi-150  $\mu\text{m}$ -3 s and multi-20  $\mu\text{m}$ -3 s, respectively. Figure 4-3b also shows that  $E$  of both multilayer hydrogels increased by (96 and 20%) and  $E$  in the monolithic hydrogel increased by 84% when the UV exposure time was increased from 3 to 15 s. The increase in UV dosage from 3 to 15 s may lead to an increase in the degree of cross-linking in both multilayer and monolithic PEGDA hydrogels.

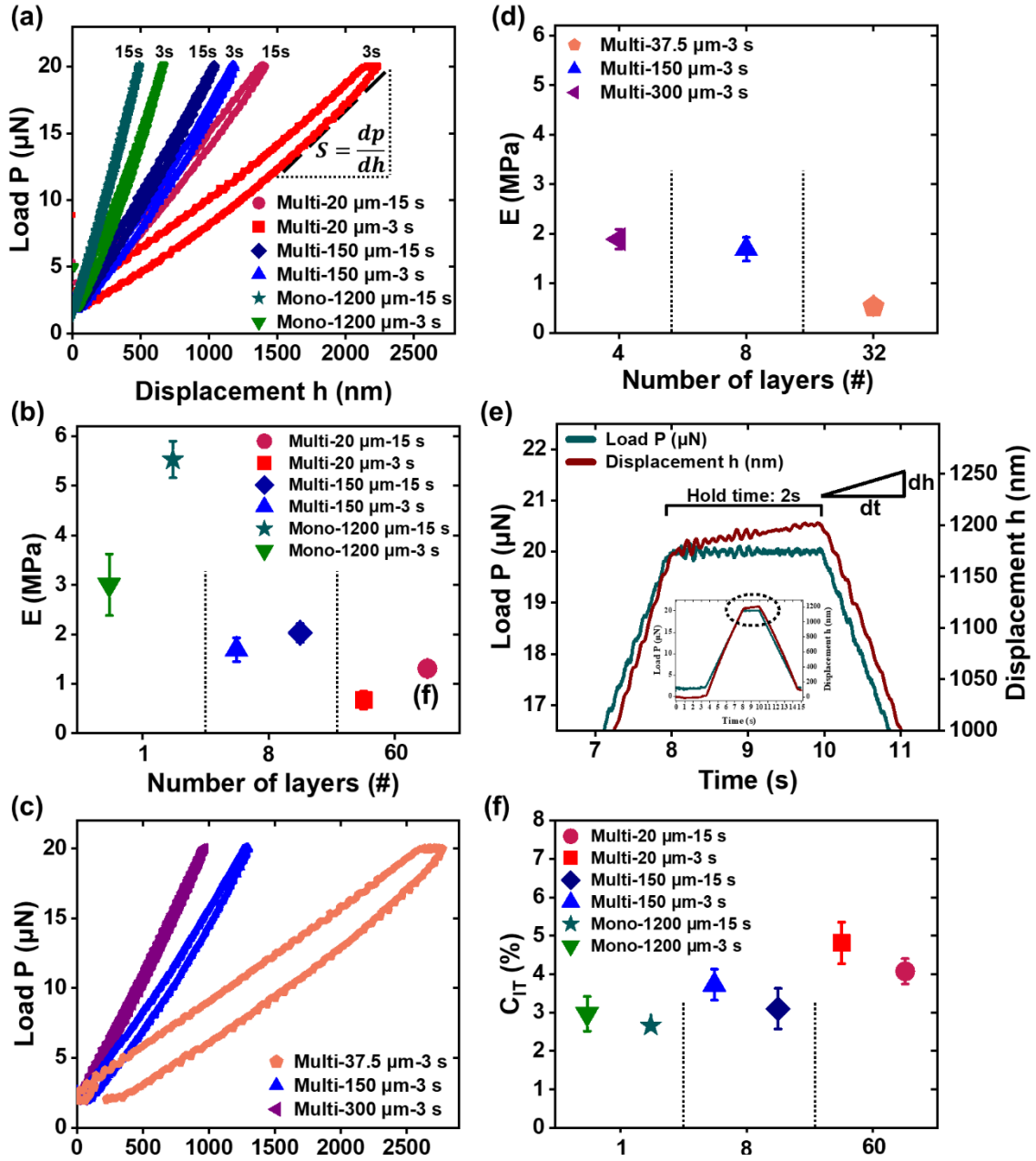


Figure 4-3 (a, c) Representative  $(P-h)$  curves and (b, d) elastic modulus ( $E$ ); (f) creep index ( $C_T$ ) obtained from nanoindentation of 3D multilayer PEGDA hydrogels, multi-20  $\mu\text{m}$ -3 s (■), multi-20  $\mu\text{m}$ -15 s (●), multi-150  $\mu\text{m}$ -3 s (▲), multi-150  $\mu\text{m}$ -15 s (◆), multi-300  $\mu\text{m}$ -3 s (◀), and multi-37.5  $\mu\text{m}$ -3 s (◆) and monolithic hydrogels, mono-1200  $\mu\text{m}$ -3 s (▼) and mono-1200  $\mu\text{m}$ -15 s (★) after 24 hrs of storage in DIW post printing. (e)  $(P-h)$  curve demonstrates the creep depth profile under a constant load for 2 s. The error bars in panels (b) and (d) represent

combined uncertainty of samples  $n = 2$  with 12 indents in each  $n$ , except mono-1200  $\mu\text{m}$ -3 s, mono-1200  $\mu\text{m}$ -15 s, multi-20  $\mu\text{m}$ -15 s, and multi-150  $\mu\text{m}$ -15 s where  $n = 1$  with 12 indents for each sample. The error bars in panel (e) represent the standard deviation from a mean of  $n = 3$ .

To elucidate the effect of the number of layers on the compliance under an indentation area and its immediate surrounding area, additional samples were fabricated and tested with varying number of layers; these include 4 layers (multi-300  $\mu\text{m}$ -3 s), 8 layers (multi-150  $\mu\text{m}$ -3 s), and 32 layers (multi-37.5  $\mu\text{m}$ -3 s). The overall height of all samples was maintained at 1200  $\mu\text{m}$ . The same QY photoabsorber concentration of 1.8 mg/mL was used for all samples. The  $P$ - $h$  curve for these hydrogels (Figure 4-3c) shows that increasing the number of layers from 4 layers in multi-300  $\mu\text{m}$ -3 s to 32 layers in multi-37.5  $\mu\text{m}$ -3 s results in a 2-fold increase in displacement. This is equivalent to a decrease in  $E$  from 1.89 to 0.53 MPa as the number of layers increases from 4 to 32 (Figure 4-3d).

The effect of increasing the number of layers and UV exposure time was further investigated by analysing the creep behaviour of 3D printed PEGDA hydrogels. During a 2 s holding period, where the indentation load was kept constant, the indentation depth continues to increase, which led to a creep deformation (Figure 4-3e). The multi-20  $\mu\text{m}$ -3 s PEGDA hydrogels showed the highest average creep displacement of 106 nm, followed by multi-150  $\mu\text{m}$ -3 s and then mono-1200  $\mu\text{m}$ -3 s at 43 and 19 nm, respectively. At the beginning of the load holding period, the penetration increased at a very high strain rate between 0.35 and 0.03  $\text{s}^{-1}$ , indicating “transient creep” behaviour (SI, Chapter 4, Figure S4-3b) [45]. A steady-state period follows, where the strain increases linearly with time and

equilibrates at the order of about  $0.01 \text{ s}^{-1}$ , depending on the stiffness of 3D PEGDA hydrogels. The creep index ( $C_{IT}$ ) (SI, Chapter 4, Eq. (S4-7)), which quantifies the creep deformation, for multi-20  $\mu\text{m}$ -3 s was 4.8%, approximately 37.5% higher than that for mono-1200  $\mu\text{m}$ -3 s at 3% (Figure 4-3f). An increase in the UV light exposure time from 3 to 15 s leads to a decrease in  $C_{IT}$  of 10–16%.

The observed dependency on the layer thickness or number of layers motivated an investigation of the interfaces between layers and defect density using SEM. Cross-sectional SEM images of vacuum-dried multi-150  $\mu\text{m}$ -15 s and multi-20  $\mu\text{m}$ -15 s PEGDA (Figure 4-4ai,bi) showed delamination of individual layers at multiple locations, indicating weaker interlayer interfaces compared to the hydrogel network within the layer itself. Interface failure could be due to the residual stress at the interface between the top section of the previously printed layer and the bottom section of the newly formed layer. The top section is assumed to be sufficiently cross-linked as it is closer to the UV light, while the bottom section is farther away from the UV light and is assumed to be less cross-linked in comparison [46,47]. Any inhomogeneity or gradient in cross-linking within each layer is attributed to the absorption of the UV light by the photoabsorber in the prepolymer solution resulting in a spatial decay of light intensity during the cross-linking process [48,49]. There was no obvious change in the monolithic mono-1200  $\mu\text{m}$ -3 s and mono-1200  $\mu\text{m}$ -15 s in terms of their visual appearance, as there is no interlayer interface within the structure (Figure 4-4cii, right). High magnification cross-sectional SEM images of vacuum-dried multilayer PEGDA showed imperfections that appear as pockets of voids, both at interfaces and within printed layers [39]. These structural imperfections (Figure

4-4aii,bii) are more evident in the multi-20  $\mu\text{m}$ -3 s than in the multi-150  $\mu\text{m}$ -3 s and not apparent in SEM images of monolithic PEGDA hydrogels (Figure 4-4c).

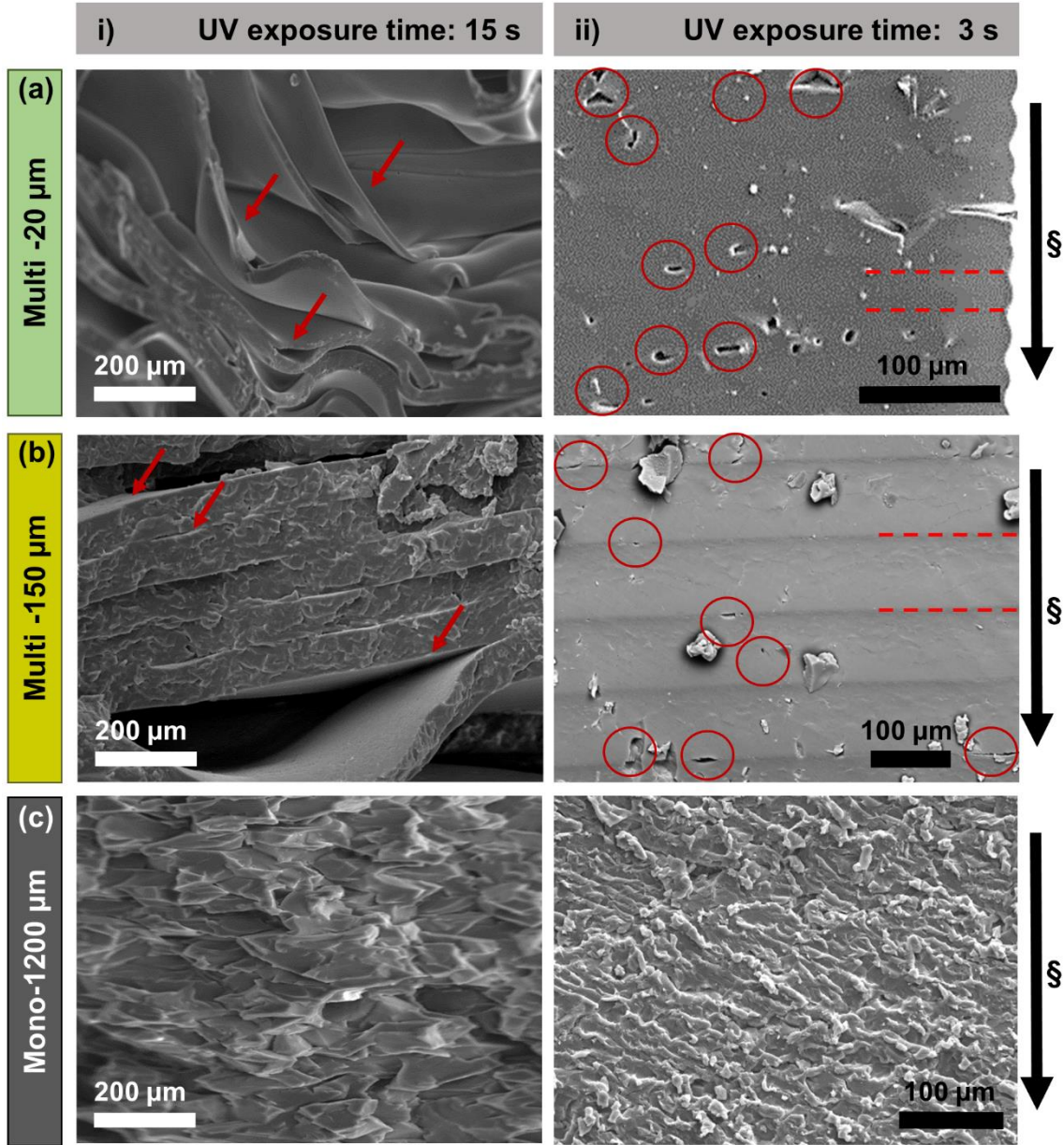


**Figure 4-4 . SEM images of cross section of vacuum-dried 3D PEGDA hydrogels:**  
 (a) (i) multi-20  $\mu\text{m}$ -15 s and (b) (i) multi-150  $\mu\text{m}$ -15 s showing delamination of layers (left-side images with red arrows) with samples exposed to 15 s of UV light. (a) (ii) Multi-20  $\mu\text{m}$ -s and (b) (ii) multi-150  $\mu\text{m}$ -3 s showing structural imperfections and localised pockets of voids due to unreacted prepolymers (right-side images with

red circles) at the interfaces and within the layers with samples exposed to 3 s of UV light. The dark areas are the interfaces of hydrogels (indicated with red dotted lines). (c) (i) Mono-1200  $\mu\text{m}$ -15 s and (ii) mono-1200  $\mu\text{m}$ -3 s PEGDA hydrogels showing a smearing effect due to blade cutting the hydrogel to reveal the cross section. The direction of the projection light (§) is from top to bottom of the structure.

The presence of voids observed in SEM images of the multilayer PEGDA hydrogels was seen to impact the mechanical properties [50]. In this study, the defect density of multilayer PEGDA structures was approximated by apparent void surface area per unit area assuming uniform distribution in a lateral direction (SI, Chapter 4, Figure S4-4 and Table S4-2). The defect density was approximated to be almost 7-fold greater in the multi-20  $\mu\text{m}$ -3 s than that in the multi-150  $\mu\text{m}$ -3 s (Figure 4-5a). This agrees with the negative correlation between  $E$  and the number of layers (Figure 4-3b), where the monolithic mono-1200  $\mu\text{m}$ -3 s showed significantly higher  $E$  compared to the multilayer PEGDA hydrogels with no apparent defects in the dried state.

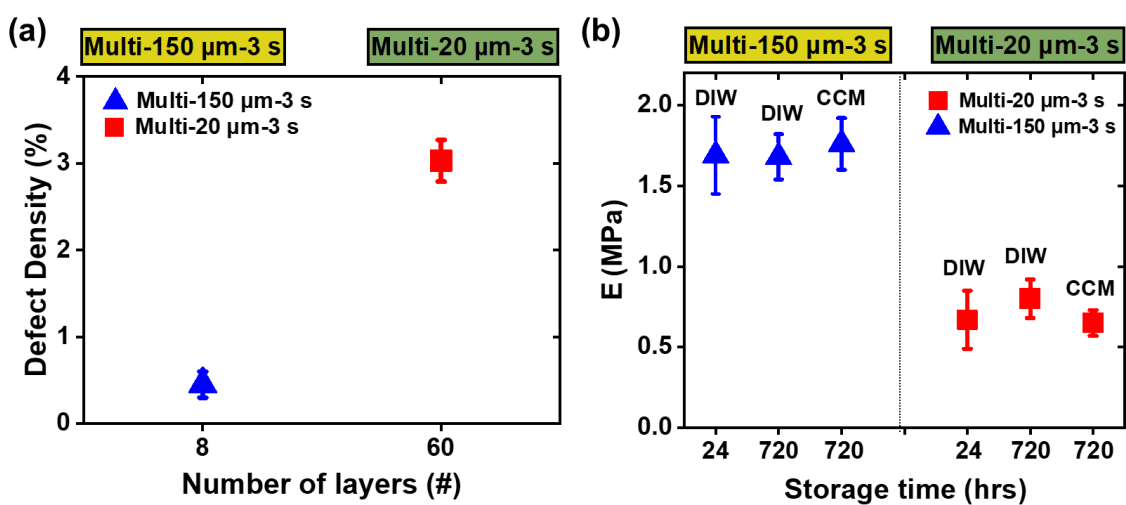




**Figure 4-5 (a) Defect density in  $100\ \mu\text{m}^2$  for both multi-20  $\mu\text{m}$  (■) and multi-150  $\mu\text{m}$  (▲) PEGDA hydrogel samples based on the measurement of defect areas captured in SEM images (Figure S4-4). (b) Nanomechanical stability of multilayer PEGDA hydrogels.  $E$  for both 3D multi-20  $\mu\text{m}$ -3 s (■) and multi-150  $\mu\text{m}$ -3 s (▲) PEGDA hydrogels stored in DIW and CCM for 720 hrs, compared with samples stored only for 24 hrs in DIW. The error bars represent (a) uncertainty of samples  $n = 2$  and (b) combined uncertainty of samples  $n = 2$  with 12 indents in each  $n$ .**

Storage in DIW and CCM solutions over 720 hrs had a negligible effect on the mechanical stability of the hydrogel samples (Figure 4-5b). For 3D multi-20  $\mu\text{m}$ -3 s hydrogels, the average  $E$  increased by + 20%, from  $0.67 \pm 0.18$  to  $0.80 \pm 0.12$  MPa, when the samples stored in DIW for 720 hrs compared to the samples stored in DIW for 24 hrs after fabrication (Figure 4-5b). For the same hydrogels stored in CCM for 720 hrs,  $E$  was constant. For the multi-150  $\mu\text{m}$ -3 s PEGDA,  $E$  was constant for samples stored both in DIW and CCM for 24 and 720 hrs (Figure 4-5b). The absence of correlation between storage time and  $E$  of multilayer PEGDA suggests that the fabricated hydrogels may have reached equilibrium within 24 hrs and no further cross-linking or structural modification takes place for 720 hrs after printing. In agreement with our previous study [20], such stability is also reflected by relatively constant gravimetric measurements (SI, Chapter 4, Figure S4-5) and glass transition temperature ( $T_g$ ) (SI, Chapter 4, Figure S4-6a,b) for 720 hrs. This suggests that 3D printed PEGDA hydrogels are relatively stable under any fabrication and storage conditions and with negligible effect of layer numbers and thicknesses.

To verify the degree of PEGDA cross-linking,  $^1\text{H}$  NMR analysis was used to assess the remaining solid residue recovered after fabrication from the hydrogels in  $\text{D}_2\text{O}$  after swelling in water for 24 hrs. The  $^1\text{H}$  NMR spectra for PEGDA monomers show absorptions of protons of the  $-\text{CH}_2\text{CH}_2\text{O}-$  and  $\text{CH}_2=\text{CH}-$  groups centred at 3.80 and 6.25 ppm, respectively (Figure 4-6a). For all fabricated samples, the  $^1\text{H}$  NMR spectra (Figure 4-6, b-g) show traces of unreacted PEGDA's absorption centred at 3.80 and 6.25 ppm, which corresponds to protons of  $-\text{CH}_2\text{CH}_2\text{O}-$  and  $\text{CH}_2=\text{CH}-$  groups, respectively. The spectra also showed traces of absorption of the LAP photoinitiator at 2.00 and 2.25 ppm ( $\text{CH}_3$  groups), aromatic protons between 6.90 and 8.20 ppm, and QY photoabsorber aromatic protons between 6.90 and 8.20 ppm (SI, Chapter 4, Figure S4-2). These spectra confirm the presence of unreacted monomers that leach out of cross-linked hydrogels over time. Please take note of the variation in TMS reference intensity at 0.00 ppm, especially in Figure 4-6f compared to other spectra. This difference may be influenced by sample preparation, where changes in absorption intensity are associated with the addition of a small crystal of sodium salt of TMS propanesulfonic acid as a reference. Variations in solution concentration or salt quantity can particularly impact the methyl group ( $\text{CH}_3$ ). The second peak around 0.05 ppm in the same Figure 4-6f likely resulted from sample contamination. The prominent signal of HOD at 4.75 ppm confirms the comparability of all spectra. Please refer to (SI, Chapter 4, Section 10, Figure S4-7) for overall  $^1\text{H}$  NMR spectra for all samples.

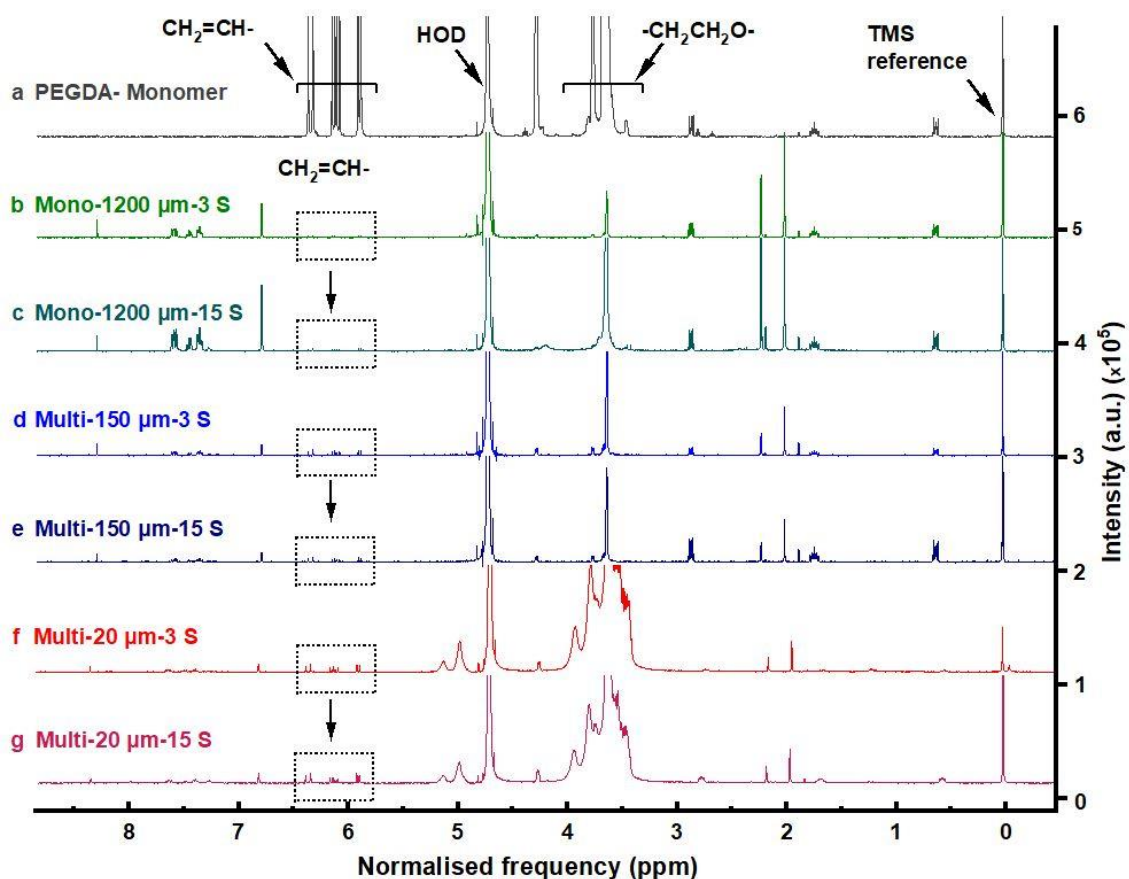


Figure 4-6  $^1\text{H}$  NMR spectra in  $\text{D}_2\text{O}$  with a TMS reference peak at 0.00 ppm of samples. (a) PEGDA monomer showing the proton absorptions of the  $-\text{CH}_2\text{CH}_2\text{O}-$  and  $\text{CH}_2=\text{CH}-$  groups centred at 3.80 and 6.25 ppm, respectively, with an intensity range of  $0-5 \times 10^5$ . (b-g) Soluble fractions recovered from 3D PEGDA hydrogels stored for 24 hrs storage in  $\text{D}_2\text{O}$ : (b, c) samples mono-1200  $\mu\text{m}$ -3 s and mono-1200  $\mu\text{m}$ -15 s, (d, e) samples multi-150  $\mu\text{m}$ -3 s and multi-150  $\mu\text{m}$ -15 s, and (f, g) samples multi-20  $\mu\text{m}$ -3 s and multi-20  $\mu\text{m}$ -15 s. The intensity range for all spectra is  $0-5 \times 10^5$ .

$^1\text{H}$  NMR analysis support the argument that an increased UV light dosage increases cross-linking in the 3D printed PEGDA structures. As previously reported [20], monolithic PEGDA hydrogel samples made in the absence of a QY photoabsorber had the highest degree of cross-linking, whereas multilayer

PEGDA hydrogels, multi-150  $\mu\text{m}$ -3 s and multi-20  $\mu\text{m}$ -3 s, with 9 and 1.8 mg/mL of QY photoabsorber concentrations had lower cross-linking, respectively. In mono-1200  $\mu\text{m}$ -3 s stored in  $\text{D}_2\text{O}$  for 24 hrs and consequently analysed by  $^1\text{H}$  NMR spectroscopy, evidence of traces of protons of  $-\text{CH}_2\text{CH}_2\text{O}-$  and  $\text{CH}_2=\text{CH}-$  groups centred at 3.80 and 6.25 ppm, respectively, was observed, confirming that the amount of unreacted prepolymers was negligible albeit not zero (Figure 4-6b). The peaks of protons of  $\text{CH}_2=\text{CH}-$  groups centred at 6.25 ppm were within the noise level for the mono-1200  $\mu\text{m}$ -15 s (Figure 4-6c), which suggests that the fabrication of 3D monolithic samples with a prolonged UV exposure time of 15 s or 600  $\text{mJ}/\text{cm}^2$  dosage yields the highest degree of cross-linking of the PEGDA prepolymer into PEGDA polymer among all other hydrogels. The increase of absorption centred at 3.80 ppm is due to the  $-\text{CH}_2\text{CH}_2\text{O}$  groups of PEGDA oligomers soluble in  $\text{D}_2\text{O}$ .

In multi-20  $\mu\text{m}$ -3 s and multi-150  $\mu\text{m}$ -3 s made by exposure of 3 s of UV light, the  $^1\text{H}$  NMR shows a slightly higher absorption of the  $\text{CH}_2=\text{CH}-$  protons centred at 6.25 ppm with respect to the 3D monolithic, mono-1200  $\mu\text{m}$ -3 s hydrogels (Figure 4-6d,f). This absorption reduced in 3D multilayer PEGDA hydrogels exposed to 15 s of UV light, confirming that longer exposure to UV light leads to a higher conversion of the unsaturated  $\text{CH}_2=\text{CH}-$  groups compared to saturated  $-\text{CH}_2\text{CH}_2\text{O}-$  groups, which means a higher degree of cross-linking in those hydrogels (Figure 4-6e,g). The UV light promotes the formation of more PEGDA oligomers soluble in  $\text{D}_2\text{O}$  in the sample multi-20  $\mu\text{m}$ -15 s, comprising 60 layers, than that in multi-150  $\mu\text{m}$ -15 s, comprising 8 layers. This suggests that the multi-150  $\mu\text{m}$ -15 s hydrogels have a higher degree of cross-linking than the multi-20

$\mu\text{m}$ -15 s. The  $^1\text{H}$  NMR results indicate that a targeted combination of UV light exposure and number of hydrogel layers has an inverse response to layer thickness, increasing the cross-linking in PEGDA hydrogels.

#### 4.4 Discussion

The  $E$  obtained in this study agrees with those previously reported for PEGDA hydrogels with a similar molecular weight (Table S4-1). As the nanoindentation takes place on the topmost layer in a very shallow area with indentation depths varying between 0.5 and 2.7  $\mu\text{m}$ , the measured  $E$  (Figure 4-3b) represents the material within the indentation and its immediate surroundings rather than the overall mechanical state of the bulk hydrogel (Figure 4-1) [51]. Such a shallow indentation depth was intentionally selected to minimise the influence of the hard substrate on  $E$ . For indentation depths shallower than 10% of the overall structure height, the substrate effect is practically negligible albeit not zero [52,53]. Note that the indentation depth in this study is less than 3  $\mu\text{m}$  on a 1200  $\mu\text{m}$  thick structure (Figure 4-1). As the overall structure heights are the same for all samples, the magnitude of substrate effect is the same across all samples.

In terms of the relationship between  $E$  and the layer thickness, the nanoindentation measurements on 3D printed hydrogels do not agree with the typical “the thinner the stronger” behaviour. The negative correlation between  $E$  and the number of layers may originate from the change in the QY photoabsorber concentration in the prepolymer solution (Figure 4-3b), which was 0 mg/mL for the mono-1200  $\mu\text{m}$ -3 s, 1.8 mg/mL for multi-150  $\mu\text{m}$ -3 s, and 9 mg/mL for the multi-20  $\mu\text{m}$ -3 s. A previous study showed that the variation in the QY photoabsorber concentration affects the degree of cross-linking in the formed

layers [20]. A constant QY photoabsorber concentration at 1.8 mg/mL was therefore used to eliminate the effect of QY and to allow the assumption of the same degree of cross-linking for the multi-300  $\mu\text{m}$ -3 s, multi-150  $\mu\text{m}$ -3 s, and multi-37.5  $\mu\text{m}$ -3 s samples (Figure 4-3d). Based on the initial argument that only the first few microns of the top hydrogel layer are affected by nanoindentation [54],  $E$  is expected to be independent from the number of layers, as they are fabricated from the same chemical formulation. However, the measurements in Figure 4-3d show a 72% reduction in  $E$  when the number of layers increased by 8-fold from 4 to 32 layers. As described above, the degree of cross-linking positively affects the elastic modulus of hydrogel structures (Figure 4-3b and Figure 4-6b–g). This agrees with the literature where 3D printed multilayer hydrogels showed lower compressive  $E$  in comparison with cast monolithic hydrogels [46,55].

To minimise the creep effect on the measured  $E$ , a short holding time of 2 s was selected as suggested by the previous studies [56–58]. However, some degree of creep deformations was still observed in the 3D printed PEGDA samples (SI, Chapter 4, Figure S4-3a). During the holding period at a constant load, the change in the displacement for multi-20  $\mu\text{m}$ -3 s was observed in the range of 4–5% (SI, Chapter 4, Figure S4-3a), while for multi-150  $\mu\text{m}$ -3 s and mono-1200  $\mu\text{m}$ -3 s, it was 2–3%. This suggests that only multi-20  $\mu\text{m}$ -3 s samples fall within the description of a quasi-creep condition, as a change in displacement in the range of 5–10% is required, while the other two fall into a noncreep condition [58]. A longer holding time is required for future studies to fully establish the viscoelastic behaviour of 3D printed PEGDA [59,60].

Note that the curing time of 3 s, equivalent to 120 mJ/cm<sup>2</sup> UV light dosage, was adopted from previous study where high resolution perfusable microchannels were needed [61]. However, the optimisation of the prepolymer solution composition has not covered the effect of different printing parameters on the degree of cross-linking of the 3D multilayer hydrogels. Here, the effect of prolonged UV exposure time on the nanomechanical response of the 3D hydrogels was tested by printing multilayer and monolithic hydrogels where each layer was exposed to 15 s, equivalent to 600 mJ/cm<sup>2</sup> dosage of UV light. The exposure time of 15 s, which is half order of magnitude higher, was chosen to (i) achieve highly cross-linked printed layers, (ii) avoid excessive printing time, and (iii) prevent overexposure, which may lead to nonuniform layers due to over cross-linking [62].

Nanoindentation measurements along with SEM imaging and NMR analysis suggested that the surface nanomechanical properties of 3D printed PEGDA structures are highly dependent on the degree of cross-linking and defect density of the layers and their interfaces. Given that the indentation displacement occurs normal to the printed layers, the applied external force facilitates the collapse of pockets of voids in multiple layers and interfaces immediately beneath the indenter. The observed higher indentation depth ( $h_{\max}$ ) of the multi-20  $\mu\text{m}$ -3 s (Figure 4-7a) suggests that this mechanism is true for 3D printed PEGDA structures with low layer thickness. Thus, the overall nanomechanical response is a combination of response from the topmost layer, the immediate surrounding that includes the preceding printed layers, and the interfaces in between. In contrast, the lower  $h_{\max}$  of multi-150  $\mu\text{m}$ -3 s suggests that the collapse of voids is

limited to only a few layers and interfaces in 3D printed PEGDA structures with low layer thickness, as they are further away from each other (Figure 4-7b). These findings imply that the nanomechanical properties of 3D printed hydrogel structures can be engineered by controlling the layer thickness or the number of layers without necessarily altering the chemical composition of the constituent materials and the UV curing dosage. Future investigation on single-layer PEGDA samples with different layer thicknesses is needed to further discriminate the influence of layer thickness from the number of layers on  $E$ .

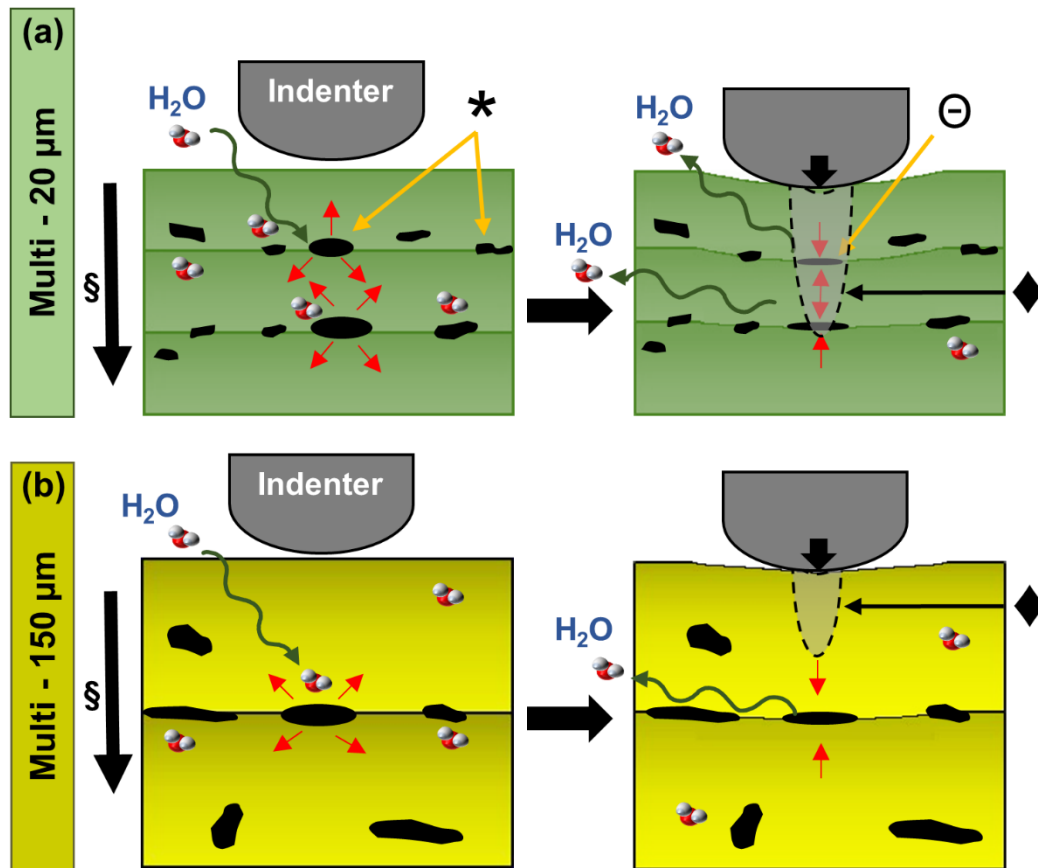


Figure 4-7 Schematic of the effect of the voids in the interface of the multilayer hydrogels (a) multi-20  $\mu\text{m}$  and (b) multi-150  $\mu\text{m}$  PEGDA hydrogels on the indentations and indenter displacement. Direction of projection light ( $S$ ) is from top to bottom of the structure, (\*) empty pockets of voids swollen due to water



uptake, ( $\Theta$ ) collapsed pockets due to localised compression from the indenter, and ( $\diamond$ ) deformation zone of indentation.

## 4.5 Conclusions

Nanoindentation was performed to investigate the influence of the degree of cross-linking and the increased number of layers in multilayer PEGDA structures compared to a monolithic structure. The nanoindentation elastic modulus of multilayer PEGDA structures decreases with an increase in the number of layers. An increase in the layer number from 8 to 32 leads to a decrease in  $E$  by  $(-)$ 218%. The layer-by-layer UV cross-linking process was observed to result in internal defects at the interfaces and within the cross-linked layer, where the defect density as voids increases with the increasing number of layers. Although far from the indentation location, these voids contribute to a reduced  $E$ . The creep index was also observed to increase with the increasing number of layers. An increase in UV light dosage from 3 s (120 mJ/cm<sup>2</sup>) to 15 s (600 mJ/cm<sup>2</sup>) leads to an increase in the degree of cross-linking that ultimately enhances the modulus by 96 and 84%, respectively. This is confirmed by <sup>1</sup>H NMR spectroscopy where samples exposed to 15 s UV light showed a lower absorption of protons of CH<sub>2</sub>=CH– groups, confirming a higher degree of cross-linking. Overall, monolithic samples can be considered as homogeneous structures with higher elastic modulus. In comparison, multilayer samples show heterogeneity and lower elastic modulus due to the interfacial defects, voids, unreacted prepolymers, and residues of the LAP photoinitiator and QY photoabsorber within the polymer network [50]. The findings presented herein should be considered as a basis for further studies into fabrication parameters and structure design to enable

precision engineering of 3D printed multilayer hydrogel structures, which will benefit future tissue engineering and organ-on-chip devices.

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## Supplementary Information (Paper 3)

### 1. Reported elastic modulus of PEGDA in literature

**Table S4- 1 Reported E for PEGDA hydrogels with similar molecular weight using compression, tensile and indentation test methods.**

| <b>MW (KDA)</b> | <b>Sample type</b> | <b>Type of modulus</b> | <b>E (MPa)</b> | <b>Test method</b> | <b>Ref</b> |
|-----------------|--------------------|------------------------|----------------|--------------------|------------|
| 0.4             | Monolithic         | Elastic                | 1.80-3.30      | Nanoindentation    | [1]        |
| 0.7             | Multilayer         | Shear                  | 0.40           | Compression        | [2]        |
| 0.7             | Monolithic         | Elastic                | 3.00           | Compression        | [3]        |
| 0.4             | Monolithic         | Compressive            | 0.10-0.20      | Compression        | [4]        |
| 0.7             | Monolithic         | Elastic                | 0.07           | Tensile            | [5]        |
| 0.7             | Monolithic         | Elastic                | 2.66-2.94      | AFM                | [6]        |
| 0.7             | Multilayer         | Elastic                | 0.67-2.03      | Nanoindentation    | This study |
| 0.7             | Monolithic         | Elastic                | 3.00-5.53      | Nanoindentation    | This study |

## 2. Nanomechanical analysis with Oliver and Pharr method

Oliver and Pharr method was used for estimating the elastic modulus ( $E$ ) of the material from the load-displacement ( $P-h$ ) plot (Figure S4-1) [7,8]. Oliver and Pharr method relies on calculating the projected contact area from a power-law fitted to the unloading curve of the  $P-h$  plot by assuming it as elastic [9]. This method relies on calculating the tip-sample contact size at the top 1/3<sup>rd</sup> part of unloading curve. The slope of the curve which measures the stiffness ( $S$ , unit: N/mm), through  $dP/dh$  where ( $P$ , unit: N) is the contact force or load and ( $h$ , unit: mm) is the relative movement of the centres of the spheres, enables one to obtain the reduced elastic modulus ( $E_r$ , MPa) of the material based on the projected tip-sample contact area ( $A_c$ , unit: mm<sup>2</sup>) through (Eqs. (S4-1 and S4-2)) [10],

$$E_r = \frac{1}{\beta} \frac{\sqrt{\pi}}{2} \frac{S}{\sqrt{A_c}} \quad (\text{S4-1})$$

$$A_c = \pi a^2 \quad (\text{S4-2})$$

where  $\beta = 1$  is a constant for the spherical indenter. The tip-shape function ( $a$ , unit: mm) for a spherical tip with a radius ( $R_i$ , unit: mm) (Eq. (S4-3)) can be obtained by measuring the contact depth ( $h_c$ , unit: mm) at maximum load ( $P_{\max}$ , unit: N) and maximum indenter displacement ( $h_{\max}$ , unit: mm) using (Eq. (S4-4)) [11],

$$a = \sqrt{2R_i h_c - h_c^2} \quad (\text{S4-3})$$

$$h_c = h_{\max} - k \frac{P_{\max}}{S} \quad (\text{S4-4})$$

The ( $k$ , dimensionless) is a geometric constant that was reported to be  $k = 0.75$  for spherical and paraboloid of revolution [7].

Based on the calculated ( $E_r$ ), the elastic modulus ( $E$ , unit: MPa) of the material can be obtained using (Eq. (S4-5)) [12],

$$\frac{1-\nu^2}{E} = \frac{1}{E_r} - \frac{1-\nu_{indenter}^2}{E_{indenter}} \quad (S4-5)$$

where ( $\nu$ ) is Poisson's ratio (dimensionless). Assuming that the indenter is diamond, the  $\nu_{indenter}$  is assumed to be 0.07 and elastic modulus  $E_{indenter}$  is 1141 GPa. Therefore, the second term of the equation can be neglected as the compliance of the indenter is much less than the compliance of the soft material. The equation can be simplified to:

$$E \approx (0.75) \times E_r \quad (S4-6)$$

By assuming the Poisson's ratio of the PEGDA hydrogel to be 0.5, because of the elastomeric character of its network [1,13],  $E_r$  can be multiplied by a constant of 0.75 to calculate  $E$  (Eq. (S4-6)).

Several assumptions were made before the data analysis. Firstly, the indentation behaviour was assumed to be a sink-in type rather than pile-up around the indentation area. Secondly, the variation due to thermal drift was assumed to be negligible because the sample was kept in the same environment for 2 hrs before testing to make sure it has similar temperature as the tip and the enclosure parts. Additionally, each indentation run was set to 12 s in total which reduced risk of thermal drift during the measurement runs.

For calculating creep index ( $C_{IT}$ , dimensionless):

$$C_{IT} = \frac{h_{\max} - h_b}{h_{\max}} \times 100 \quad (S4-7)$$

$$\dot{\varepsilon} = \frac{1}{h_i} \left( \frac{dh}{dt} \right) \quad (S4-8)$$

Where  $h_i$ , is the depth of the indenter (unit: mm) at  $i$ th time step,  $\frac{dh}{dt}$  is the speed of the indenter,  $\dot{\varepsilon}$ , is the creep strain rate (unit:  $s^{-1}$ ),  $h_b$ , is the indentation depths at the beginning of the hold period (unit: mm) and  $h_{\max}$ , is the indentation depths at the end of the hold period (unit: mm).

### 3. Typical force-displacement curve during nanoindentation

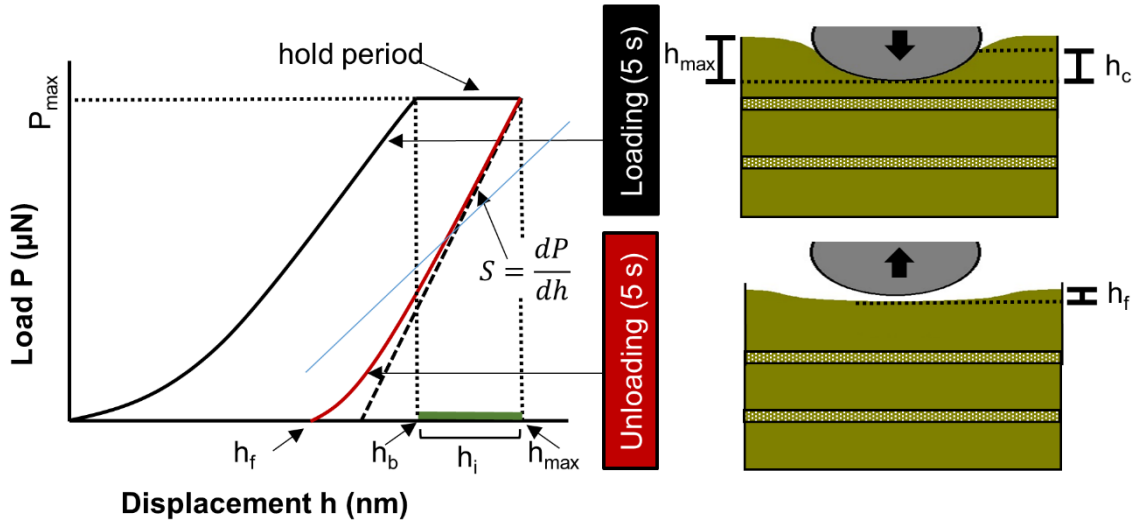


Figure S4-1 Schematic of typical ( $P-h$ ) graph during nanoindentation with a hold period with graphical explanation of loading and unloading of a spherical indenter on a multilayer hydrogel sample.  $P_{\text{max}}$  is the maximum load just before unloading,  $S$  is the top  $1/3^{\text{rd}}$  unloading stiffness,  $h_f$  is the residual depth of nanoindentation,  $h_b$  is the indentation depths at the beginning of the hold period,  $h_{\text{max}}$  is the maximum depth of nanoindentation,  $h_c$  is the contact depth, and  $h_i$  is the depth of the indenter at  $i^{\text{th}}$  time step during the hold period.

#### 4. $^1\text{H}$ NMR spectra for the LAP photoinitiator and the QY photoabsorber

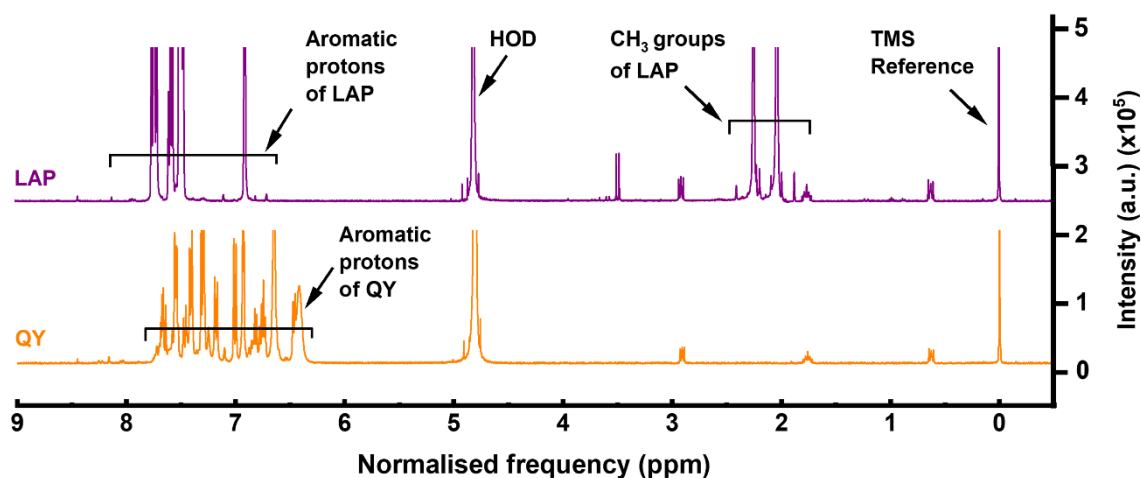


Figure S4-2  $^1\text{H}$  NMR spectra of the starting materials in  $\text{D}_2\text{O}$ . The LAP photoinitiator (top) exhibits peaks of  $\text{CH}_3$  groups at 2.00 and 2.25 ppm and aromatic protons between 6.90 and 8.20 ppm. The QY photoabsorber (bottom) exhibits peaks of aromatic protons between 6.20 and 7.90 ppm. The peak at 0.00 ppm is attributed to TMS reference.

## 5. Detailed creep properties of PEGDA hydrogels

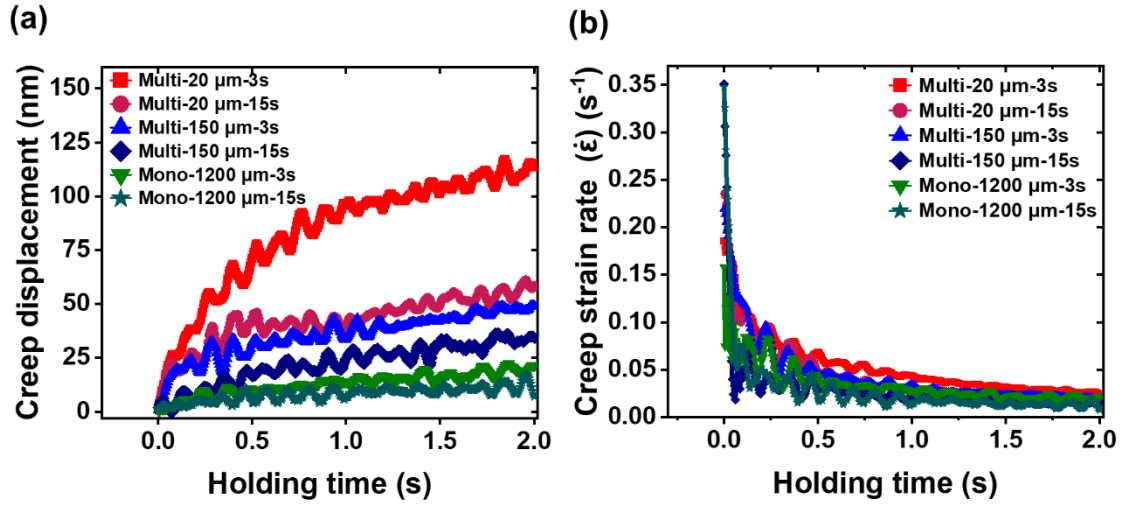


Figure S4-3 Nanoindentation creep properties; Representative (a) creep displacement curves during a constant load holding period, (b) creep strain rate at constant load of 20  $\mu\text{N}$ , for the 3D multilayer, multi-20  $\mu\text{m}$ -3 s ( $\blacksquare$ ), multi-20  $\mu\text{m}$ -15 s ( $\bullet$ ), multi-150  $\mu\text{m}$ -3 s ( $\blacktriangle$ ), and multi-150  $\mu\text{m}$ -15 s ( $\blacklozenge$ ), and monolithic, mono-1200  $\mu\text{m}$ -3 s ( $\blacktriangledown$ ), and mono-1200  $\mu\text{m}$ -15 s ( $\star$ ) PEGDA hydrogels after 24 hrs of storage in DIW post printing. Error bars represent standard deviation from a mean of  $n=3$ .



## 6. Defects identifications and measurements

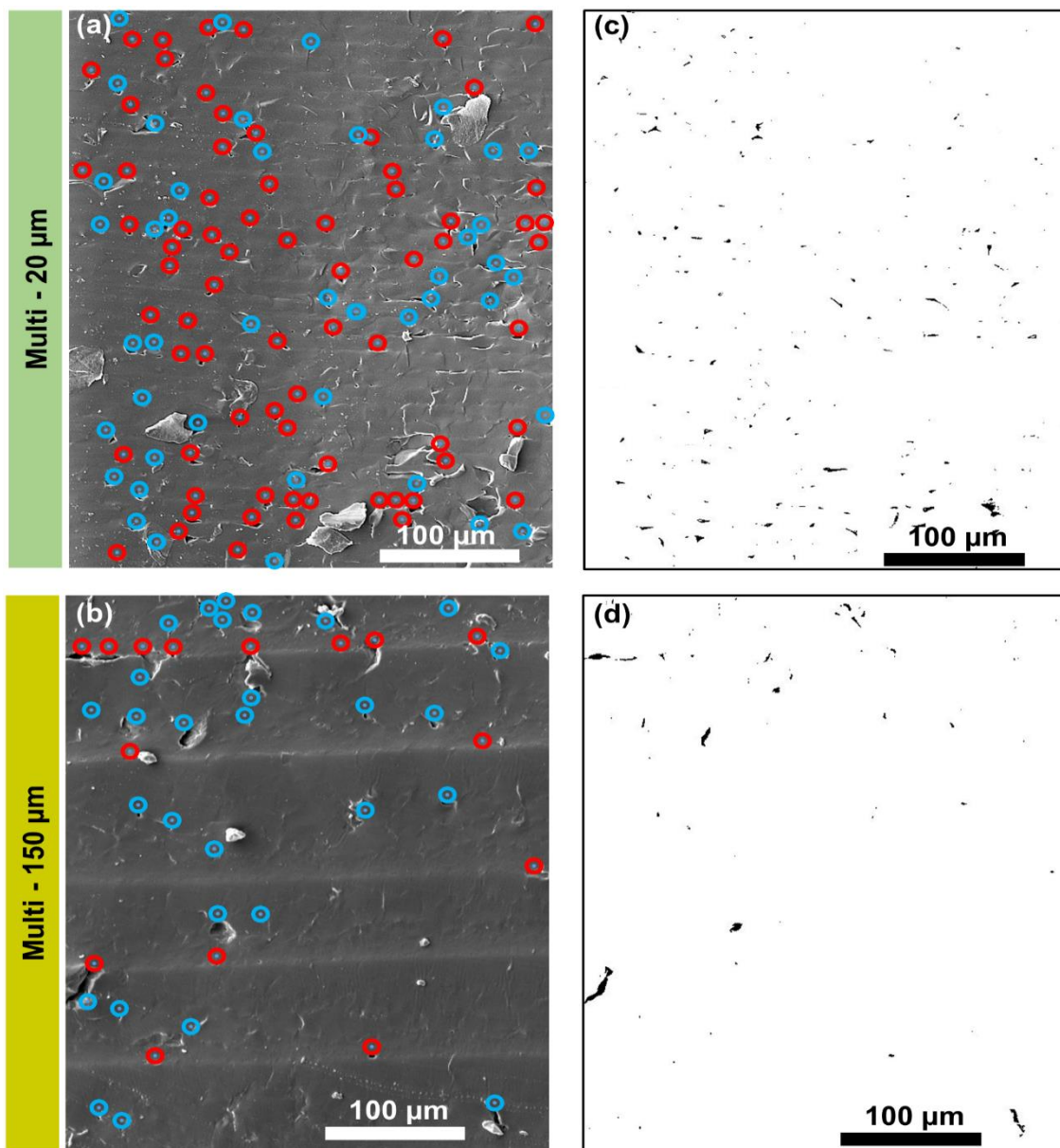


Figure S4-4 (a,b) SEM image of vacuum-dried multi-20  $\mu\text{m}$ -3 s and multi-150  $\mu\text{m}$ -3 s PEGDA hydrogels respectively showing all identified pockets of voids on the interface (red dots) and on the layer (light blue dots). Samples were washed and stored in DI water for 24 hrs prior vacuum-drying to purge any left unreacted prepolymers from the hydrogel. (c,d) Measurement of surface area of the identified pockets of voids using imageJ software.

## 7. Defect density calculations

Table S4-2 Calculation of defect density for (a) multi-20  $\mu\text{m}$ -3 s and (b) multi-150  $\mu\text{m}$ -3 s based on the pockets of voids at the interfaces and on the layers of the SEM images of vacuum-dried 3D PEGDA hydrogels.

| (a)                                                   |         |         |         |
|-------------------------------------------------------|---------|---------|---------|
| Multi-20 $\mu\text{m}$ -3 s                           |         |         |         |
|                                                       | S1      | S2      | Average |
| Total area ( $\mu\text{m}^2$ )                        | 154,000 | 154,000 |         |
| Total apparent voids surface area ( $\mu\text{m}^2$ ) | 4406    | 4930    |         |
| Defect density (%)                                    | 2.86    | 3.20    | 3.03    |

| (b)                                                   |        |        |         |
|-------------------------------------------------------|--------|--------|---------|
| Multi-150 $\mu\text{m}$ -3 s                          |        |        |         |
|                                                       | S1     | S2     | Average |
| Total area ( $\mu\text{m}^2$ )                        | 242377 | 592901 |         |
| Total apparent voids surface area ( $\mu\text{m}^2$ ) | 1342   | 2052   |         |
| Defect density (%)                                    | 0.55   | 0.35   | 0.45    |

## 8. Gravimetric measurements

As the 3D PEGDA hydrogels are intended for use in biological application, their behaviour in a relevant physiological environment were tested. Initially, the weight change NWF (Eq. (4-1)) due to the temperature variations of the 3D multilayer hydrogels over the period 720 hrs (Figure 4-2), 30 days, was measured (SI, Chapter 4, Figure S4-5). For the 3D multilayer, multi-20  $\mu\text{m}$ -3 s, PEGDA hydrogels stored at 8 and 20 °C in both DIW and CCM, there was a weight increase of between +15% to 20%, indicated by increase in NWF between 1.15 and 1.19, within the first 6 hrs of storage, respectively (SI, Chapter 4, Figure S4-5). The change in NWF slowed considerably after the first 24 and at 720 hrs, it has reached 1.16 and 1.20 for samples stored at 8 and 20 °C in DIW and CCM, respectively. The samples stored at 37 °C, both in CCM and DIW, had a subtle weight change over the same period of time with NWF of 1.02 and 1.03 at 24 hrs. However, their final weight at 720 hrs has increased to NWF of 1.05 and 1.07 in CCM and DIW, respectively (SI, Chapter 4, Figure S4-5). The multi-150  $\mu\text{m}$ -3 s PEGDA hydrogels had a similar behaviour to that of the multi-20  $\mu\text{m}$ -3 s at 8 and 20 °C but the magnitude of the change was lower with reported NWF of 1.12 and 1.06 in CCM and DIW, respectively. However, the samples stored at 37 °C had almost 10% water loss in the first 6 to 24 hrs but over time they regained some of the weight loss and reached NWF of 0.96 and 0.94 when stored in CCM and DIW, respectively (SI, Chapter 4, Figure S4-5).

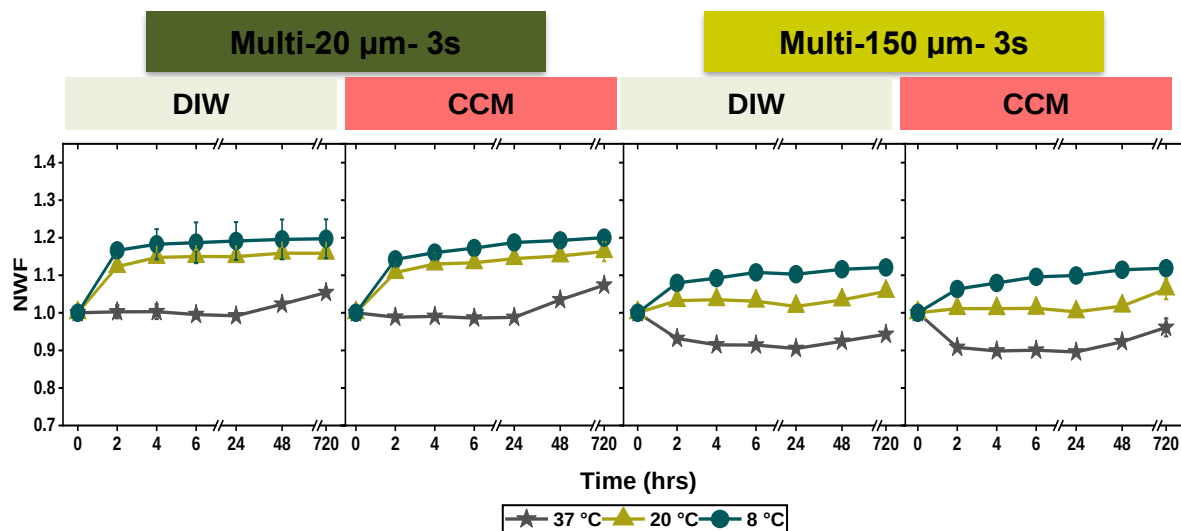


Figure S4-5 NWF of 3D multi-20  $\mu\text{m}$ -3 s and multi-150  $\mu\text{m}$ - 3s PEGDA hydrogel samples, both stored at 8 (●), 20 (▲) and 37 °C (★) in DIW and CCM for 720 hrs (30 days) measured at predetermined time points immediately after printing (0 hrs). Error bars represent standard deviation from the mean, with  $n = 2$  for independent multilayer samples. Some error bars are not visible as they are smaller than the data point symbols. All lines are guide to the eye only.

## 9. Additional DSC measurements

The glass transition temperature ( $T_g$ ) of vacuum-dried multilayer PEGDA hydrogel samples, of multi-20  $\mu\text{m}$ -3 s and multi-150  $\mu\text{m}$ -3 s, at 24 and 720 hrs of storage in DIW and CCM, was recorded. SI, Chapter 4, Figure S4-6b, shows the thermograms of both type of samples and how their  $T_g$  increase of 3  $^{\circ}\text{C}$ , from -40 to -37  $^{\circ}\text{C}$ , when stored in DIW from 24 to 720 hrs. This can be due to the leaching out of more uncross-linked prepolymers from the polymer network and been replaced with DIW which increased the water content of the hydrogel and its purity which resulted in increased  $T_g$  [14]. The  $T_g$  of hydrogel samples which were stored in CCM did not increase of the same amount as the relative samples stored in DIW (Figure S4-6b). This could be due to the availability of various inorganic salts such as sodium bicarbonate in the CCM which either prevented i) the unreacted monomer to leach out from the polymer network and/or ii) the salts infiltrated in the polymer network resulting in higher  $T_g$  in comparison with samples stored in DIW [15].

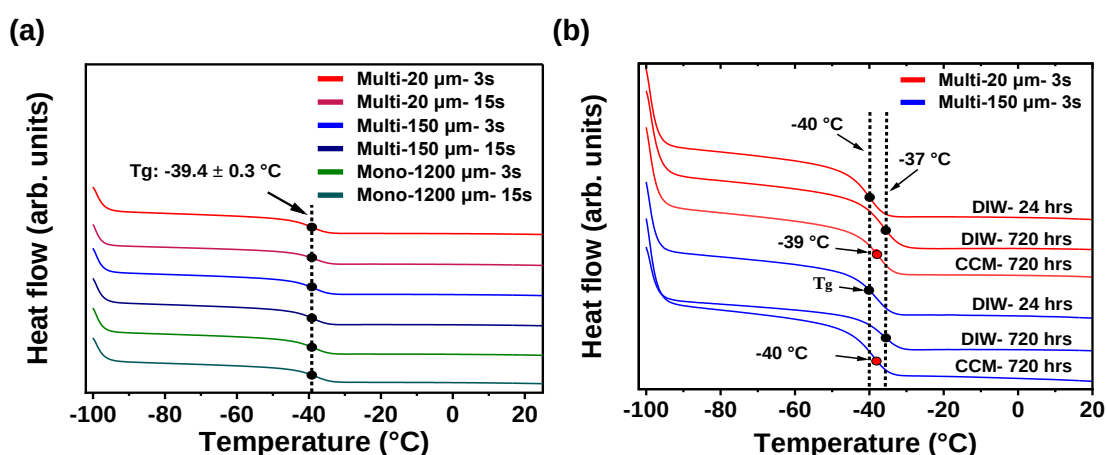


Figure S4-6 (a) Thermograms of 3D multilayer, multi-20  $\mu\text{m}$ -3 s (red), multi-20  $\mu\text{m}$ -15 s (pink), multi-150  $\mu\text{m}$ -3 s (blue), and multi-150  $\mu\text{m}$ -15 s (dark blue) and monolithic, mono-

1200  $\mu\text{m}$ -3 s (■), and mono-1200  $\mu\text{m}$ -15 s (■) PEGDA hydrogels stored in DIW for 24 hrs. (b) Thermograms of multi-20  $\mu\text{m}$ -3 s (red, top three spectrum) and multi-150  $\mu\text{m}$ -3 s (blue lines, bottom three spectrum) at 24 hrs and 720 hrs of storage in DIW of dry PEGDA hydrogels' second heating from -100 °C to 25 °C, at 10 °C/min.

10.  $^1\text{H}$  NMR full spectra for all samples in Figure 4-6

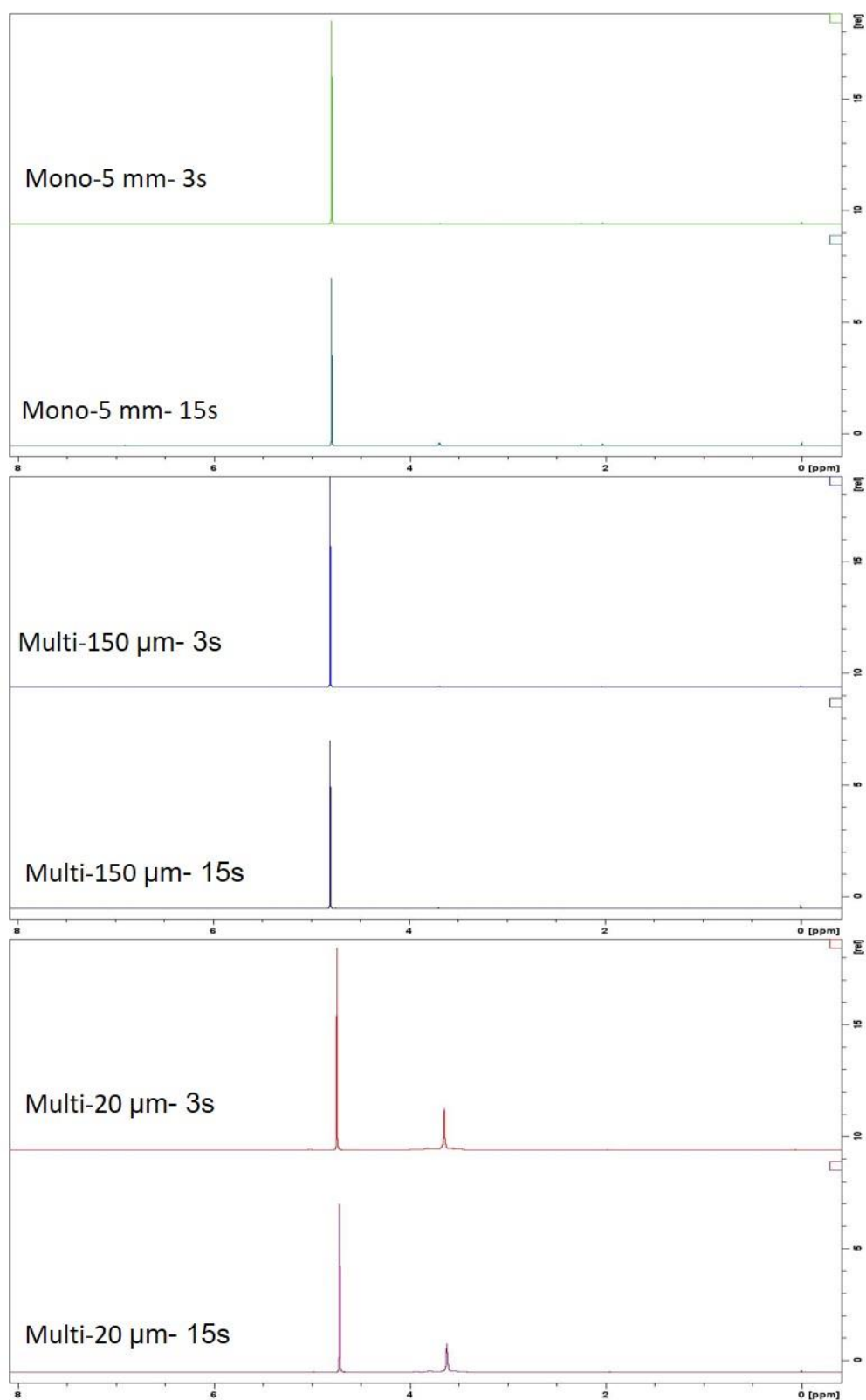


Figure S4-7  $^1\text{H}$  NMR full spectra for all samples corresponding to Figure 4-6.

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# Chapter 5

## Mechanical Behaviour of 3D Printed Poly(ethylene glycol) Diacrylate Hydrogels in Hydrated Conditions Investigated Using Atomic Force Microscopy

### Published as:

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## Abstract

Three-dimensional (3D) printed hydrogels fabricated using light processing techniques are poised to replace conventional processing methods used in tissue engineering and organ-on-chip devices. An intrinsic potential problem remains related to structural heterogeneity translated in the degree of cross-linking of the printed layers. Poly(ethylene glycol) diacrylate (PEGDA) hydrogels were used to fabricate both 3D printed multilayer and control monolithic samples, which were then analysed using atomic force microscopy (AFM) to assess their nanomechanical properties. The fabrication of the hydrogel samples involved layer-by-layer (LbL) projection lithography and bulk cross-linking processes. We evaluated the nanomechanical properties of both hydrogel types in a hydrated environment using the elastic modulus ( $E$ ) as a measure to gain insight into their mechanical properties. We observed that  $E$  increases by 4-fold from 2.8 to 11.9 kPa transitioning from bottom to the top of a single printed layer in a multilayer sample. Such variations could not be seen in control monolithic samples. The variation within the printed layers is ascribed to heterogeneities caused by the photo-cross-linking process. This behaviour was rationalised by spatial variation of the polymer cross-link density related to variations of light absorption within the layers attributed to spatial decay of light intensity during the photo-cross-linking process. More importantly, we observed a significant 44% increase in  $E$ , from 9.1 to 13.1 kPa, as the indentation advanced from the bottom to the top of the multilayer sample. This finding implies that mechanical heterogeneity is present throughout the entire structure, rather than being limited to each layer individually. These findings are critical for design, fabrication and application engineers

intending to use 3D printed multilayer PEGDA hydrogels for in-vitro tissue engineering and organ-on-chip devices.

## 5.1 Introduction

Three-dimensional (3D) printed hydrogel photopolymers fabricated using light processing techniques such as digital light processing (DLP) and stereolithography (SLA) have been extensively used in tissue engineering and organ-on-chip devices [1–3]. These photo-cross-linking techniques are fast, reproducible, and spatially precise for polymerisation of liquid prepolymer solutions into multi-functional hydrogel biostructures with complex shapes [4]. The fabrication involves layer-by-layer (LbL) cross-linking, in which the prepolymer solution is cross-linked by light irradiation, e.g., ultraviolet (UV), one layer at a time to allow a mould-free construction of a freestanding hydrogel structure [5–7]. Photo-cross-linking process also allows property engineering of the biostructures through control of the fabrication parameters such as light dosage, step height, prepolymer molecular weight, and photoabsorber concentration [8–11].

The surface properties of hydrogel biostructures on which the cells are cultured can have a strong influence on cell behaviours, including growth, migration, proliferation, differentiation, and tissue formation [12–14]. For example, the development, organisation, and differentiation of stem cells can be directed by engineering the surface elastic modulus ( $E$ ), topography, and adhesion of hydrogel structures [15–17]. In addition, the ability to measure contractile force of muscle tissue strips using 3D printed poly(ethylene glycol) diacrylate (PEGDA) hydrogel cantilevers requires precise engineering of  $E$  across the cantilever structure and selective tissue adhesion on the cantilever surface [18]. Photo-cross-linked 3D PEGDA hydrogels produced using projection lithography allows

for the spatial and temporal control of cross-linking and fabrication of various complex shapes [19–21].

Despite of the aforementioned advantages, previous studies have reported the existence of cross-linking gradients in photo-cross-linked 3D printed multilayer PEGDA hydrogels that result in inhomogeneous properties, e.g.  $E$ , across the printed layers [22–25]. To date, atomic force microscopy (AFM) measurements were carried out in dried and cryosectioned biostructures [26], hence there is a clear need for improving fundamental understanding of outer surface properties of 3D printed multi hydrogels in realistic conditions. The research reported herein addresses this knowledge gap by systematically and thoroughly mapping the elastic behaviour on the outer surface of 3D printed multilayer PEGDA hydrogels using AFM measurements in hydrated state [27–30]. While the findings presented herein are limited to elastic behaviour, they provide a much-needed baseline understanding of the outer surface variation in  $E$  within the layer and across the entire structure that remained elusive prior to this study. This understanding is critical to allow tailoring of 3D printed hydrogels properties for future design of tissue engineering scaffolds.

We performed AFM measurements on the outer surface of these PEGDA hydrogel samples when fully submerged in deionised (DI) water with a pH similar to a cell culture medium and in their swollen state [31–36]. In this study, we found that hydrated multilayer sample with Mn 700 had an  $E$  ranging from 2.8 to 11.9 kPa within a single layer. This variability was not observed in the monolithic control samples, which  $E$  remained at  $32.3 \pm 2.5$  kPa within the same scale. A crucial finding from our study was that the  $E$  exhibited a substantial 44%

increment, from 9.1 to 13.1 kPa, as the indentation progressed from the bottom to the top of the multilayer sample. This observation suggests that the presence of mechanical heterogeneity extends throughout the entire surface structure and is not confined to individual layers. It highlights the potential for tailoring the mechanical properties of 3D printed PEGDA by controlling the LbL printing process and the importance of considering cross-linking gradients in the design of tissue engineering scaffolds. This understanding provides insight into the surface modulus engineering of hydrogel biostructures critical for selective promotion or repulsion of biological tissues and can be extrapolated to other biopolymeric systems for the benefit of many tissue engineering and organ-on-chip applications.

## **5.2 Materials and Methods**

### **5.2.1 Fabrication of PEGDA 3D Structures**

3D printed multilayer PEGDA samples were synthesised from PEGDA monomer ( $M_n$  700 g/mol) dissolved in DI water (DIW, resistivity of  $18.2 \text{ M}\Omega\cdot\text{cm}$ ) at a weight concentration of 20 wt%. The prepolymer PEGDA solution was prepared by mixing with 5 mg/mL photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP,  $\geq 95\%$ ) and 9 mg/mL photoabsorber Quinoline Yellow (QY). Due to their excellent water solubility and cytocompatibility, LAP and QY were selected for this study [37]. The light source was an UV light emitting diode (LED) with a wavelength of 365 nm at an intensity of  $20 \text{ mW/cm}^2$ . For multilayer samples, cuboid with dimensions of  $5 \times 5 \times 5 \text{ mm}$  ( $L \times W \times H$ ), each layer (comprised of 250 layers- Figure 5-1a<sub>ii</sub>) was exposed to 3 s of UV light to achieve cross-linking (SI, Chapter 5, Figure S5-1a). Here, the

top section of a printed layer refers to the area closer to the light source, while the bottom section refers to the farther one. As the 3D structure is printed LbL from bottom to top, the bottommost layer refers to the first printed layer, while the topmost layer refers to the latest printed one. Monolithic samples, cuboid with dimensions of 5 x 5 x 5 mm (L × W × H), (Figure 5-1ai) were fabricated as control samples by pouring a prepolymer solution of PEGDA and LAP without QY photoabsorber into a silicone mould with 5 mm structural height. The samples were then exposed to a UV light source from the top with the same intensity of 20 mW/cm<sup>2</sup> for 3 s (SI, Chapter 5, Figure S5-1b). The detailed protocols for preparing the samples can be found in our previous study [38].

### **5.2.2 Sample Preparation for AFM Imaging**

Both types of fabricated PEGDA hydrogel samples were cut vertically across the printed layers to prepare outer surface sections (Figure 5-1d) approximately 3 mm thick using a 100 µm thick stainless steel blade. A droplet of the prepolymer solution without QY was applied on an acrylate treated glass slide. The cut section of the sample was then placed on the glass slide and exposed to a UV light from the bottom, so it adheres to the glass slide (Figure 5-1d). The prepared samples attached to the glass slide were transferred to a 60 mm petri dish and fixed to the bottom of the dish using epoxy superglue. After 1 min, DIW was added to ensure that the samples remain hydrated. The samples were stored for 24 hrs at room temperature of 20-22 °C before AFM measurements ensure the samples are at equilibrium. The water level was kept just above the sample height to reduce the risk of damage to the AFM head. Measurements were performed while the samples were fully immersed in DIW at controlled temperature (21 °C).



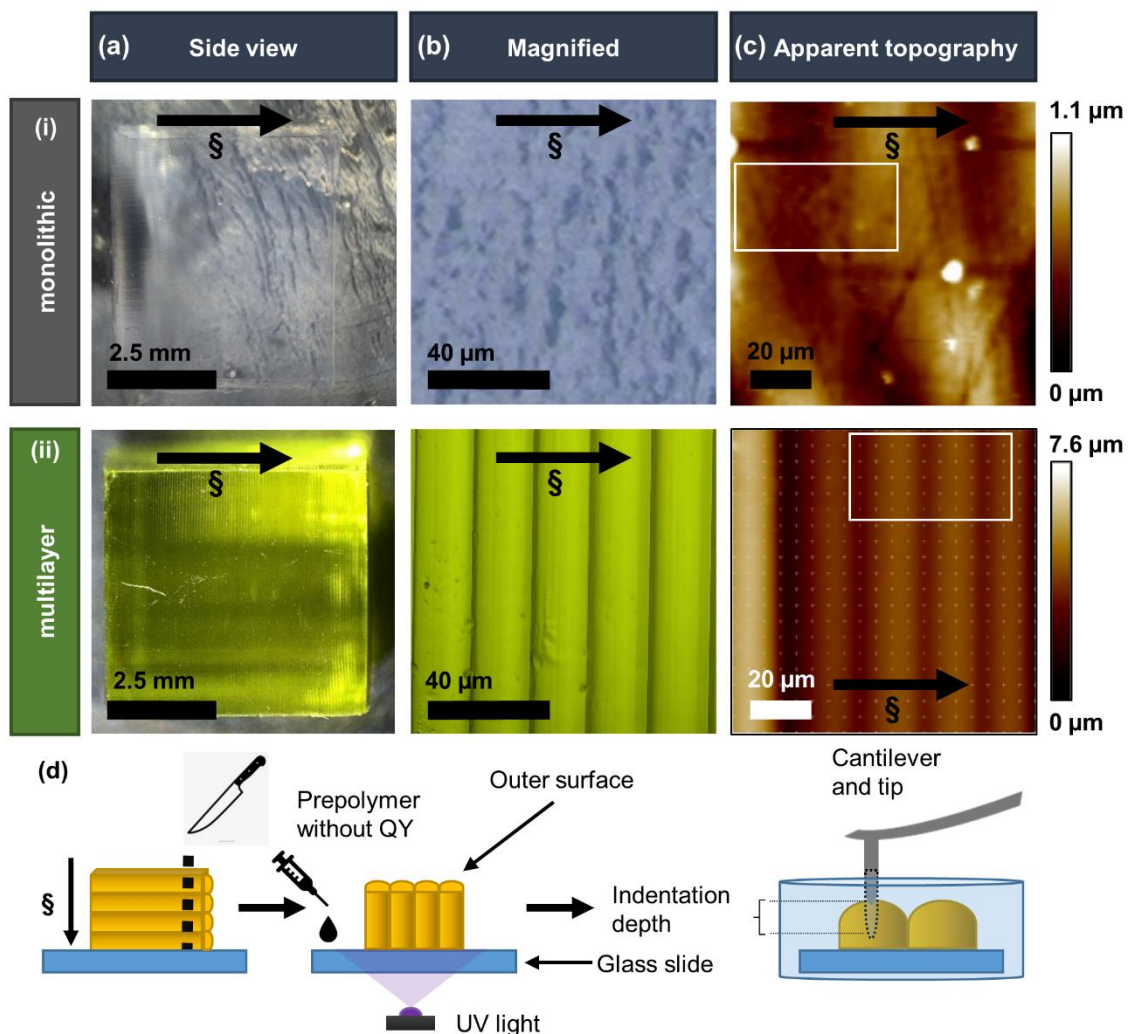


**Figure 5-1** (a) Side view, (b) magnified optical microscopy images, (c) apparent topography recorded with AFM peak force tapping mode (ramp) on outer surface of (i) monolithic, and (ii) multilayer PEGDA samples. (d) Schematic of sample preparation for outer surface section of multilayer sample. The white box areas were exclusively analysed for modulus measurements in Figure 5-2. The exact analysed indentation points can be identified in SI, Chapter 5, Figure S5-3. Direction of projection light (§) is from top to bottom of the structure.

### 5.2.3 AFM Quantitative Imaging

AFM measurements were obtained using a Bruker Dimension Icon AFM. Data analysis and image processing were conducted with NanoScope Analysis

software (version 1.85) and JPK SPM Data Processing 8.0.13. A Nature protocol for measuring  $E$  of soft culture tissue and 3D hydrogels using AFM was followed [34]. A precalibrated BioAFM spherical tip of radius of 3.46  $\mu\text{m}$  and low spring constant of 0.217 N/m was selected to induce sufficient deformation without damaging the sample while retaining their sensitivity [39]. Deflection sensitivity of 28.69 nm/V was measured by multiple indents into a silicone sample and analysing the force-distance curves. A droplet of DIW was dropped gently on the tip of the AFM probe creating a water dome to integrate with the top surface of the hydrogel and the surrounding water system when contacting the sample. A scanning area of 90  $\mu\text{m}$  x 90  $\mu\text{m}$  was measured at a predetermined location within the sample 2.5 mm from its bottom right corner and at a height of 2.5 mm from the bottom of the sample. A grid of 20 x 20 (total of 400 indents) within the 90 x 90  $\mu\text{m}$  scanning area was set with spacing between each indent set to 4.737  $\mu\text{m}$  (SI, Chapter 5, Figure S5-3).

The indentations were carried out in contact mode with a maximum load of 60 nN. The raster scan was adjusted with the tip scanned over the sample surface at speeds of 30 and 26  $\mu\text{m/s}$  and sample rate of 3,125 and 3,333 Hz for multilayer and monolithic samples, respectively. The z-length scale was fixed at 5 and 4  $\mu\text{m}$  for multilayer and monolithic samples, respectively. The load and unload profile of 164 and 154 ms for multilayer and monolithic samples, respectively, were developed.

To measure the apparent topography of the sample surface, initial scans were performed in ramp mode where the cantilever was moved rapidly along the x-axis

and slowly along the y-axis while maintaining a constant force of 60 nN.  $E$  was calculated based on the average of 6 indent points across the x-axis.

The method for measuring the elastic modulus ( $E$ ) at different heights is as follows. The variation in  $E$  across the height of the multilayer sample was assessed at three specific heights: 1.25 mm, 2.50 mm, and 3.00 mm from the sample's bottom. To achieve this, a raster scan was initially conducted over a 90 x 90 area at each height to analyse the sample's topography. Subsequently, 90 indents were made at each height using a single-line indentation function, with a spacing of 1  $\mu\text{m}$  between indents, perpendicular to the printing layers of the hydrogel. To calculate the  $E$  value at each height, the average of three adjacent indents at the peak section of the three layers was computed as illustrated in SI, Figure S5-5. Details on fitting methods of Hertz and Oliver and Pharr can be found in the supplementary information.

### 5.3 Results

Figure 5-1c shows the apparent topography for the outer surface of both monolithic and multilayer PEGDA samples. The colour code, from dark brown to white, indicates the apparent height variations within each sample from the lowest to the highest point, respectively. Figure 5-1cii shows the outer surface topography of the multilayer sample where the dark vertical lines are indicative of the interface of each printed layer and the lighter areas are indicative of the printed layers themselves. Such topography is not visible in the monolithic samples which were produced using bulk cross-linking (Figure 5-1ci).

Looking at Figure 5-2ai, random height variations in monolithic samples are visible which suggests that the sample surface is not smooth. In Figure 5-2aii, the height variations seem to be of a repeated pattern across the multilayer sample from left to right indicating the outer surface of the printed layers across the sample height. The darker areas, red boxes, are indicative of the shallow section of the sample surface close to the interfaces between two consecutive printed layers (trough) and the bright areas, blue boxes, are showing the raised areas on the sample surface (peak). Adhesion maps (Figure 5-2bi, bii) show the adhesion force between the scanning tip and the surface of both samples is less than 3.5 nN which is relatively low and insignificant in comparison with the maximum applied load of 60 nN.

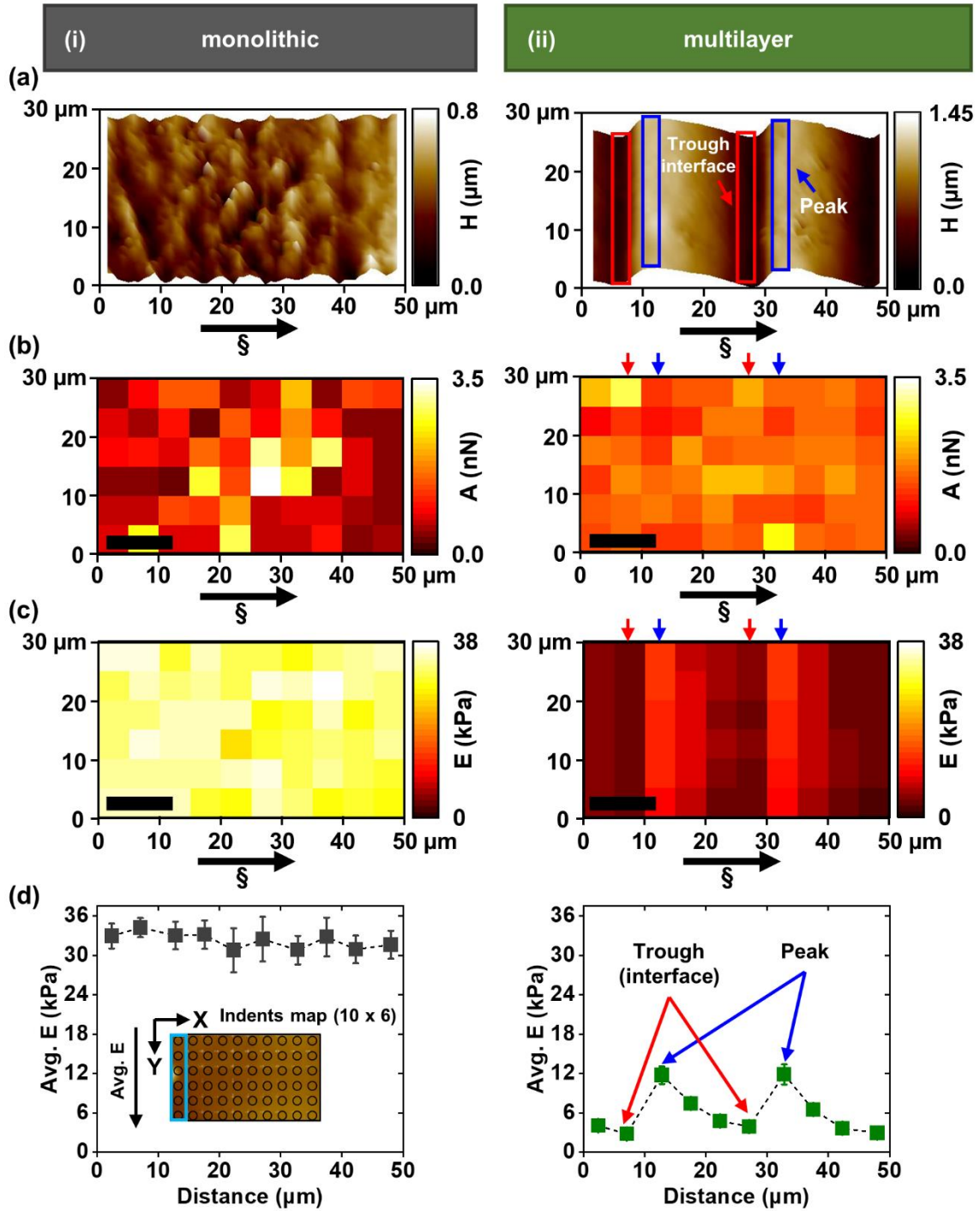


Figure 5-2 For (i) monolithic, and (ii) multilayer PEGDA samples, (a) AFM ramp images of the surface topography with indentation map  $50 \times 30 \mu\text{m}$ . The red and blue boxes indicate the peak and trough (interface) positions on the multilayer sample, (b) Adhesion map of  $10 \times 6$  indents with row and column spacing of  $4.74 \mu\text{m}$  indicating the adhesion force between the sample and AFM tip, (c)  $E$  map

showing variations in modulus in both samples, (d)  $E$  calculated by averaging 6 indents at each point across the x-axis (SI, Chapter 5, Figure S5-2b). Error bars represent standard deviation from a mean of  $n = 6$ . Some error bars are not visible as they are smaller than the data point symbols. All dashed lines are only guides for viewing. Direction of projection light ( $S$ ) is from top to bottom of the structure.  $H$ ,  $A$  and  $E$  stand for height, adhesion, and elastic modulus, respectively. Scale bars in all graphs are approximately  $10\ \mu\text{m}$ .

The results also suggests that the surface topography does not influence the measured adhesion force. Figure 5-2ci shows  $E$  for monolithic sample ranges from 26 to 37 kPa. As the sample is traversed through each layer,  $E$  gradually increases from right to left, displaying a periodic pattern (Figure 5-2cii). The results obtained using the Hertz model (Figure 5-2b and c) with an upper fitting range (SI, Chapter 5, Figure S5-4a and b), as well as the Oliver and Pharr method (SI, Chapter 5, Figure S5-4c), show a similar overall trend and are comparable. Average  $E$  increases by 4-fold from 2.8 to 11.9 kPa when comparing the bottom and the top section of the same layer in the multilayer sample (Figure 5-2dii). Such gradual variations were not visible on the calculated average  $E$  in the monolithic samples within the scanning area (Figure 5-2di). The results shown in (Figure 5-2di and dii) indicate that the monolithic sample exhibits twice the value of  $E$  compared to the multilayer sample.

In the multilayer sample,  $E$  increases by 44% across the height of the structure, going from 9.1 to 13.1 kPa when moving upwards from 1.25 to 3.00 mm at the bottom of the structure (Figure 5-3a). Conversely, the monolithic sample displays an increase in  $E$  of 120%, going from 29 to 57 kPa when moving from 1.25 to

3.00 mm at the bottom of the structure (Figure 5-3b). The data obtained from the monolithic sample reveal that the measured  $E$  values were notably higher than those of the multilayer sample, ranging between 2.6 to 4.5 times higher (Figure 5-3a and b). In particular, at a height of 3 mm, the average  $E$  for the monolithic sample was 57 kPa, compared to only 13.1 kPa for the multilayer sample.

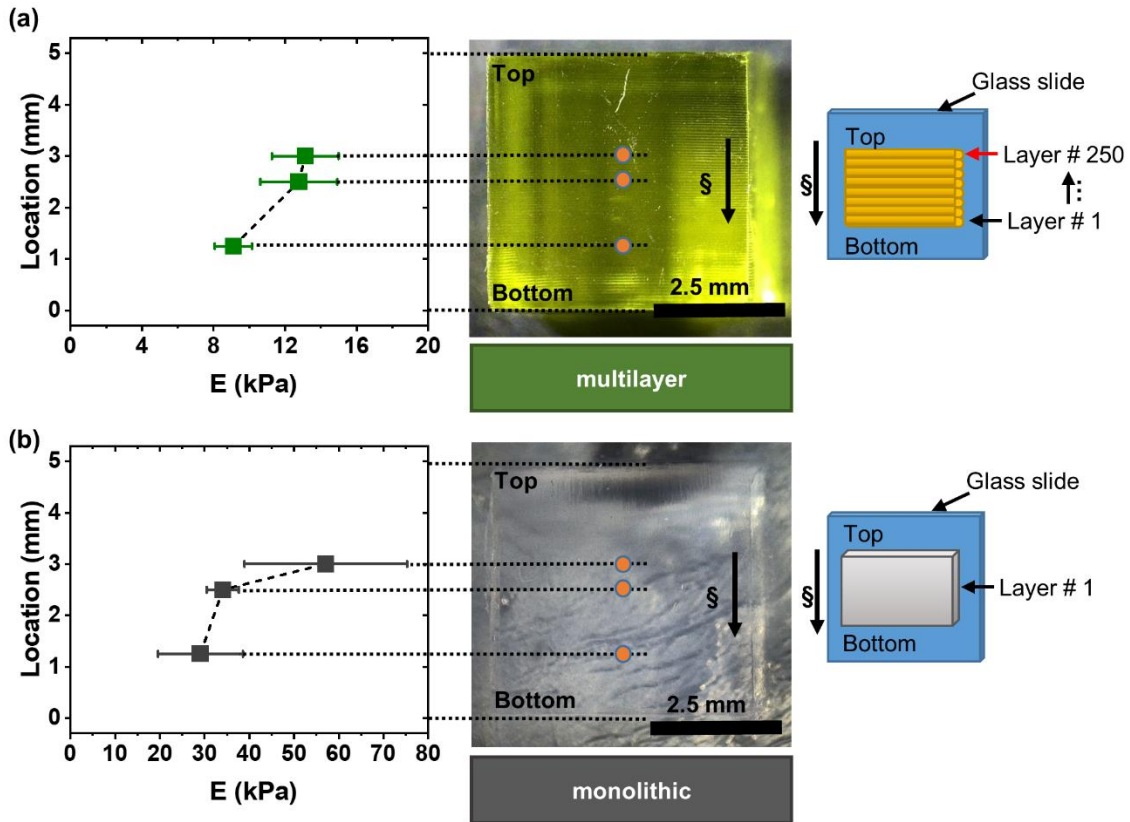


**Figure 5-3** The change in  $E$  measured at three different locations across the height on the outer surface of the (a) multilayer, and (b) monolithic PEGDA samples.  $E$  calculated for (a) by average of 3 indents on 3 layers at each height, (b) by average of 90 indents at each height (SI, Chapter 5, Figure S5-5). Error bars in (a) represent combined uncertainty of  $n = 3$  with 3 indents for each  $n$ , (b) represent standard deviation from a mean for  $n = 90$ . Direction of projection light (§) is from top to bottom of the structure. All dashed lines are for ease of viewing.

## 5.4 Discussion

The peaks and troughs observed in the surface topography of the multilayer PEGDA sample are inherent parts of the structure and reflects how the structure is printed using projection lithography (SI, Chapter 5, Figure S5-6). AFM measurements was not carried out on the cross-sectioned multilayer sample, as mechanical cutting using thin blades resulted in the introduction of unnecessary cutting artefact to the surface topography (SI, Chapter 5, Figure S5-7). As the multilayer sample structures are intended for use in tissue engineering studies, where they will be in direct contact with biological cells in their hydrated state, it is best to investigate their outer surface nanomechanical behaviour in a similar state and original surface topography to ensure the results are more representative of the actual model. While cell study is outside the scope of this current investigation, the understanding of the nanomechanical behaviour will help elucidate the cell adhesion, integration, and tissue attachment onto the 3D printed structures [13,16,18]. This will also help ensuring the measured  $E$  is not an overestimation of the real value when compared with samples measured in their dry state [26,28,30]. The gradient in  $E$  observed in each layer of the multilayer sample (Figure 5-2d) can be understood by gaining a deeper insight into the printing process. As projection lithography is a bottom-up printing approach, during the process light penetration depth governs the kinetics of cross-linking which in turn is controlled by the concentration of the photoabsorber and photoinitiator in the prepolymer solution (SI, Chapter 5, Figure S5-10) [38]. Choosing the right photoabsorber and photoinitiator is crucial for achieving optimal functionality, printing fidelity and cross-linking rate in biomedical



applications. In addition to low risk of cytotoxicity, key factors to consider when selecting a photoabsorber and photoinitiator include their absorption spectrum, molar extinction coefficient and water solubility [40]. Methyl orange and diaminobenzophenone were not practical for use because they were poorly soluble in water, despite their initial consideration for their stronger absorption at 365 nm [41]. Other photoabsorbers such as 2-hydroxy-4-methoxy-benzophenone-5-sulfonic acid (HMBS) reported to be cytotoxic [42]. Therefore, QY photoabsorber, which is a water soluble commercial food dye, shows low cytotoxicity and a good absorbance at 365 nm [41]. For selection of photoinitiator, LAP's low cytotoxicity has already been established and multiple studies in the area of tissue engineering and cell seeding have used it successfully [42]. Moreover, LAP is highly water soluble with high molar absorptivity ( $\epsilon \approx 200 \text{ M}^{-1} \text{ cm}^{-1}$ ) and has been successfully used in DLP and SLA based printing [43,44]. Alternative photoinitiators like Irgacure-2959, Eosin Y, and 2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) were found to be inadequate due to factors such as low solubility in water, insufficient absorption at a wavelength of 365 nm (absorbance range from 400 to 800 nm), and cytotoxicity, respectively (SI, Chapter 5, Section 10) [40,42]. Since UV light attenuates and decreases in intensity when it propagates through a photoabsorber containing solution, the control over photoabsorber concentration to limit the penetration of UV light into the prepolymer solution enables control over the thickness of the cross-linked layer [45]. This phenomenon causes an exposure gradient through the layer resulting in higher cross-linking at the top section of each layer, closest to the UV

light. The bottom of the layer may not even reach the full gelation point (Figure 5-4) [46].

It is critical to find the cross-linking depth, as it signifies the point at which the light intensity is inadequate to cause cross-linking that results in the prepolymer solution remaining in a liquid state. Here, the extinction coefficient of QY photoabsorber is approximated using Beer-Lambert law and then used to identify the optimal layer thickness (SI, Chapter 5, Figure S5-8) [45]. Previous investigation suggested that the optimal concentrations for QY photoabsorber and LAP were 9 mg/mL and 5 mg/mL, respectively, for the production of open channel structures [37]. For the same concentrations, we measured the cross-linking depth to be  $46 \pm 2 \mu\text{m}$  [38]. Based on this information, the layer thickness was set at approximately half of the cross-linking depth, or  $20 \mu\text{m}$ , assuming that each layer would undergo two exposures during the LbL printing process to enhance layer-layer adhesion. However, upon closer examination, it was discovered that during the printing process, only a small portion of the upper layer, closer to the interface of the previously formed layer, undergoes double exposure (SI, Chapter 5, Figures S5-9 and S5-10). This results in the creation of a highly cross-linked planar sub-layer and may also cause additional volume within the structure, leading to issues with dimensional accuracy [47]. This is likely the cause of the hump-shaped feature present at the top of each layer before every interface in the printed structure (SI, Chapter 5, Figure S5-11). The exposure gradient through the layer and the double exposure of the top section of each layer results in a gradient cross-linking density across each printed layer. This exacerbates the gradient of cross-linking degree and adversely affects the

mechanical properties across the thickness of the layer which agrees with the results in Figure 5-2 [24].

In our previous study, we found that the monolithic sample, which did not contain the QY photoabsorber in its prepolymer solution, had the highest degree of cross-linking and highest  $E$ , as indicated by both  $^1\text{H}$  NMR spectra and nanoindentation measurements. On the other hand, the multilayer sample, which had the highest concentration of QY photoabsorber, exhibited a lower degree of cross-linking as well as  $E$ . This observation suggests that a higher concentration of QY photoabsorber in the prepolymer solution leads to a reduction in the degree of cross-linking in the cross-linked hydrogel, as shown in Figure 5-2id and 2iid, and Figure 5-3a and b [48]. We have also showed the effect of cross-linking on water and sol-gel content in the monolithic and multilayer samples with equilibrium water content of 72.6% and 75%, respectively [38]. Gel fractions were measured to be 27.4% and 25%, while sol percentages were 2.5% and 5.9% for the monolithic and multilayer samples, respectively [38].

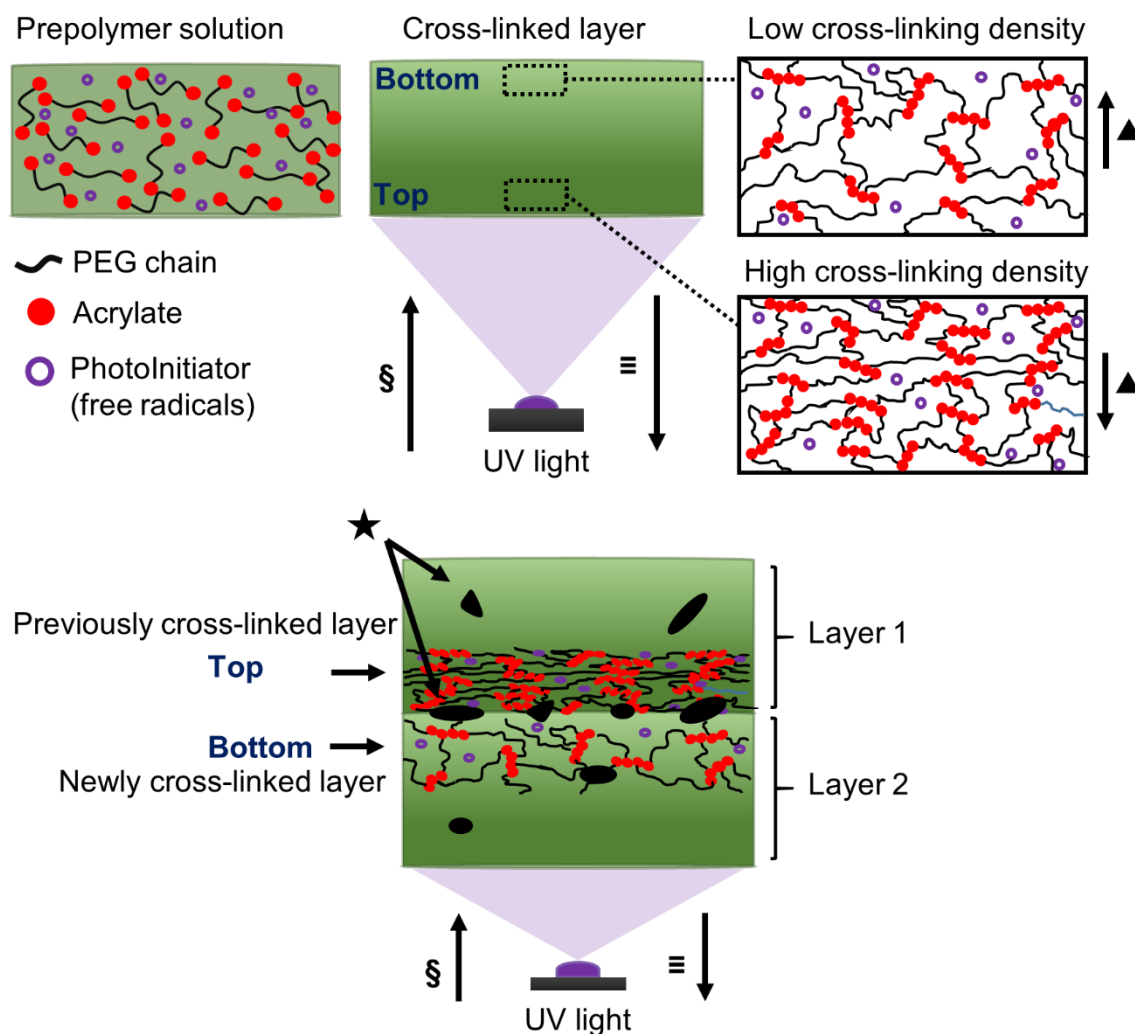


**Figure 5-4 Schematic of the prepolymer solution (top left), single cross-linked layer (top middle), two different cross-linking density at top and bottom of each layer (top right) [49], and two consecutive cross-linked layers with different amount of cross-linking at the interface (bottom middle). (§) Projection light direction, (≡) printing direction, (▲) amount of available residual free radicals at the top and bottom of a cross-linked layer, (★) empty pockets of voids swollen due to water uptake. Adapted from ref [49]. Copyright 2021 Wiley-VCH GmbH.**

As described above, the lowest  $E$  is recorded at a trough (Figure 5-2dii), which is the interface between layers. The observed tearing suggests that the interface between the layers is indeed the weakest point across the entire structure due to

its highest heterogeneity in cross-linking density. This agrees with our previous study that suggests, due to the nature of LbL cross-linking process, there are inherent structural imperfections in projection based printing processes [48]. For each layer upon cross-linking, localised areas of unreacted monomer are formed which affect the stiffness of the material at the interface. These pockets of voids or defects could result in variations in the defect density and lower  $E$  at the interface of the multilayer samples [48]. NMR characterisation carried out in our studies suggest that the presence of localised unreacted prepolymer regions is mainly caused by the insufficiency of the UV light dose of  $120 \text{ mJ/cm}^2$  to fully cross-link the exposed prepolymer solution in the presence of the QY photoabsorber with a concentration of  $9 \text{ mg/mL}$  [48]. Traces of unreacted PEGDA's absorption which corresponds to the protons of  $-\text{CH}_2\text{CH}_2\text{O}-$  and  $\text{CH}_2=\text{CH}-$  groups could be clearly observed in the NMR spectra in the multilayer samples [38,48]. A higher UV dosage may help reduce the possibility of these localised regions within the layers. However, exposing the layers to a higher dosage of UV light, e.g.  $15 \text{ s}$ , which is equivalent of  $600 \text{ mJ/cm}^2$ , may lead to overexposure. This increases the risk of tearing into individual layers and cracking within the hydrogel structure, which is observed when multilayer samples are stored at  $8$  and  $45^\circ\text{C}$  in their hydrated state (Figure 5-5a).

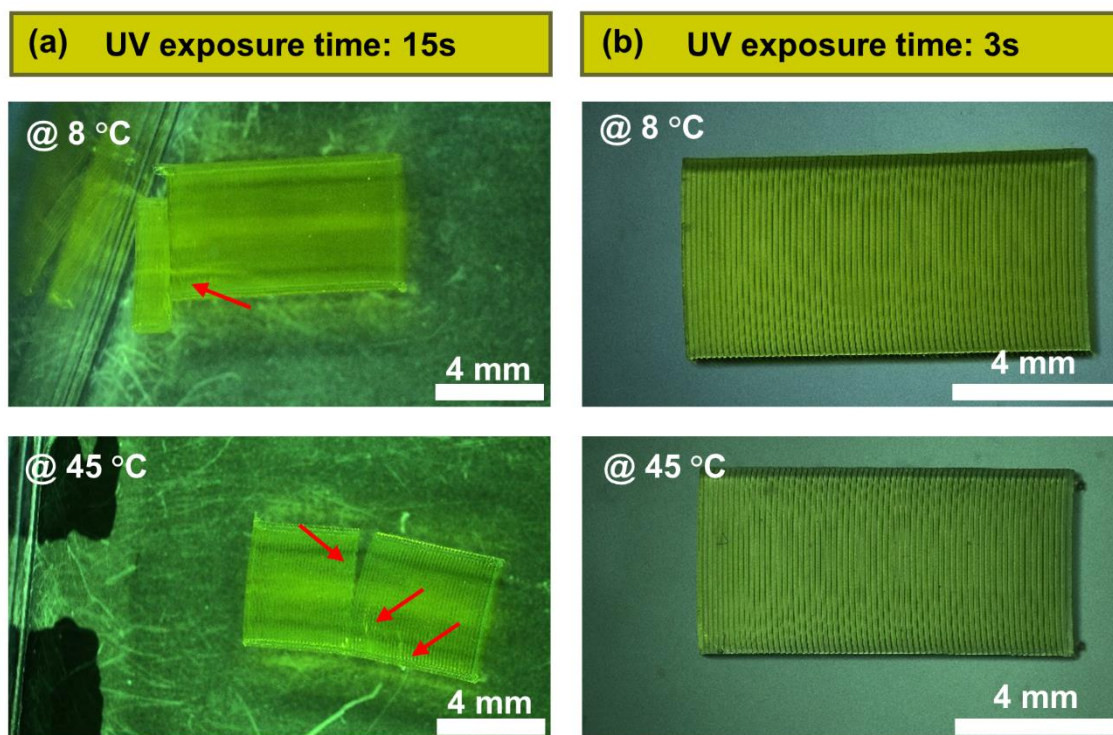


**Figure 5-5 Optical microscope images of multilayer PEGDA samples exposed to (a) 15 s, and (b) 3 s, of UV light stored at 8 and 45 °C. Layers delamination and cracking is observed in samples exposed to 15 s of UV light.**

Delamination at the interface of the multilayer sample is attributed to the lower adhesion yield stress than the cohesive yield stress, meaning layer-layer adhesion is not necessarily a 100% chemical in nature [45]. The cross-linking process occurs through free radical transfer where the LAP photoinitiator absorbs UV light to form reactive species and initiate reaction cascades, by which the PEGDA monomers react to form a cross-linked polymer [12]. The cross-linking process terminates through coupling or disproportionation, when free radicals become topologically constrained and cannot form links with the unreacted monomers and oligomers remaining in the previously cross-linked layer [45]. Therefore, the deficiency in free radicals and/or their inability to diffuse in the previously cross-linked layer and their failure to react with the unreacted polymer

and oligomers within the layer results in an inadequate cross-linking at the interface which in turns results in weak adhesion between two consecutive cross-linked layers (Figure 5-4). There is also the possibility of bubbles within the prepolymer solution that may appear on the surface of the vat and under the exposed area while the printing is in process which may result in the appearance of the voids within the multilayer samples especially at the interface. It is necessary to verify that the various layers are completely bonded by covalent cross-linking throughout the printing process. The examination of covalent cross-linking exclusively at the interface using NMR is, however, incredibly challenging due to its relatively low spatial resolution. Nonetheless, our prior research has demonstrated a definitive correlation between cross-linking and mechanical properties. Thus, we employed AFM to verify the existence of cross-linking and measure the spatial variation across the layers. Note that the observed reduction in modulus could also be attributed to the presence of voids or defects, suggesting that the layers may not be fully covalently cross-linked at the interfaces [50]. It is crucial to investigate and find the optimum amount of LAP photoinitiator, QY photoabsorber and UV dosage required to minimise the chance of appearance of these pockets of voids and increase the adhesion at the interfaces of the cross-linked layers.

In addition to a gradient in  $E$  within each layer, the printed multilayer sample also exhibits a gradient in  $E$  from the top layer to bottom layer of the structure (Figure 5-3). This strongly suggests that the mechanical properties of multilayer sample are heterogeneous across the entire height of the structure. Nonetheless, the measured gradient in  $E$  across the height of the monolithic samples is significantly

higher than that of the multilayer samples. This can be explained based on the curing reaction that occurs in the photo-cross-linking processes. Initially, when the UV light is directed from the top of the prepolymer solution, gelation occurs when the degree of cross-linking is enough to guarantee the formation of a solid structure that can result soluble or insoluble in common solvents, which has a low degree of cross-linking. This results in the coexistence of both gel and sol phases. The curing reaction continues with the same mechanism and the amount of sol phase in the system decreases until completion and formation of a insoluble hydrogel which is highly cross-linked [51]. From this point, the reaction becomes very slow controlled by the diffusion of the reactive species. It takes a much longer time for the reactive groups to find each other and form links as the bulk density of cross-linking becomes higher. This diffusion-controlled effect may lead to the formation of nonhomogeneous hydrogel structures with heterogeneous mechanical properties [51].

In the case of a monolithic sample, where the layer thickness is 5 mm, the corresponding diffusion time is considerably higher than that of the multilayer. In multilayer samples, where the layer thickness is 20  $\mu\text{m}$ , diffusion of activate groups from the top to bottom of the structure is highly unlikely. The vertical variation in  $E$  is stronger in monolithic samples compared to multilayer samples due to the fact that in monolithic samples, initiated species at the top of each layer require more time to diffuse to the bottom, causing a stronger variation.

The findings presented herein provide baseline understanding of spatial variation in elastic behaviour of 3D multilayer PEGDA within each layer, at the layer



interface, and across the entire height of the structures. One important aspect that yet to be investigated is the viscoelastic behaviour, including hysteresis, creep, and storage/loss moduli of 3D printed PEGDA hydrogels. The approach used in this study can be extrapolated to systematically investigate the effect of LbL synthesis parameters on spatiotemporal variation in mechanical properties to enable tailoring of the local and global mechanical properties of hydrogel structures. Future studies may benefit from more advanced scanning probe microscopy (SPM) techniques such as nano-DMA to fully elucidate the time dependent behaviour of hydrogels in physiologically relevant environment.

## **5.5 Conclusions**

In our study, we successfully mapped the apparent elastic modulus of the 3D printed multilayer PEGDA hydrogel sample and compared them to monolithic PEGDA hydrogel as control sample in their native hydrated state. It was observed that a gradient in elastic modulus in the printed layers of multilayer sample increases moving upward from the bottom to the top of individual layers.  $E$  of a multilayer sample at the middle of the printed structure increases 4-fold, from 2.8 to 11.9 kPa, when moving from the bottom to the top of each layer. Such variations were not observed in the monolithic samples. Experiments revealed that the LbL UV cross-linking process using projection lithography results in a cross-linking density gradient within each layer due to the spatial decay of the light intensity as the light propagates throughout the prepolymer, printing solution.

More importantly, the investigation at 3 different spatial locations across the height of both monolithic and multilayer samples shows a considerable difference

in  $E$  from the bottom to the top of the structure. Even though the variations in  $E$  in the monolithic sample were significantly higher than that of the multilayer, an increase of 44% in  $E$  for multilayer sample across its height from 9.1 to 13.1 kPa is considered large. Reducing the layer thickness in the multilayer samples and post-printing UV curing may help in improving the overall surface mechanical homogeneity of the 3D printed PEGDA structures. These findings can significantly help in understanding how biological cells and tissues interact when in contact with the surface of a heterogeneous material with mechanical property gradients and how this feature can be utilised to guide cell integration, attachment, and tissue formation for different biological applications.

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## Supplementary Information (Paper 4)

### 1. Schematic of printing setups for printing multilayer and monolithic hydrogels

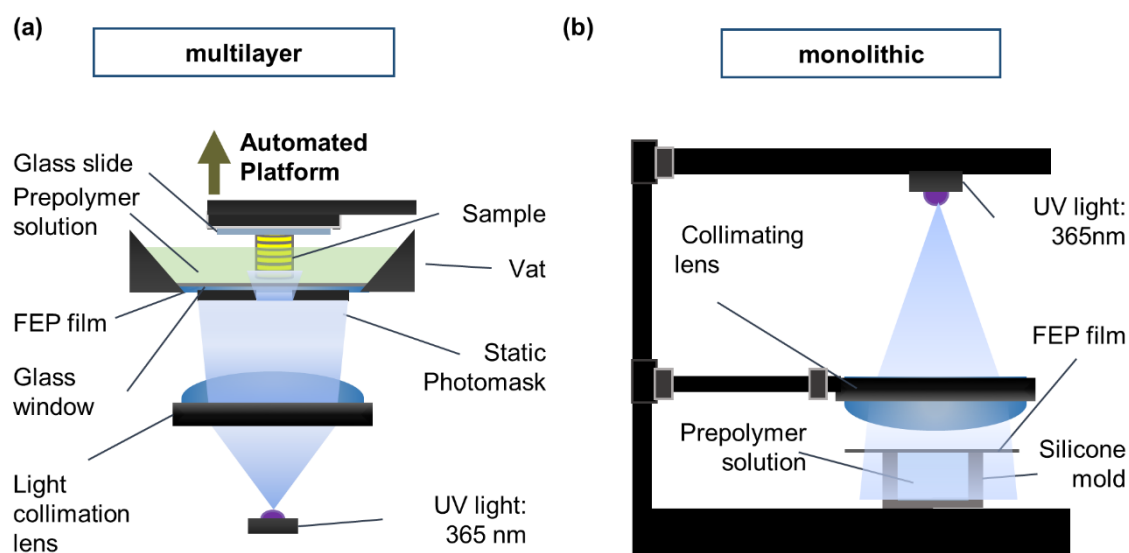


**Figure S5-1 . Schematic of (a) 3D printing projection lithography system for multilayer PEGDA hydrogels, (b) Bulk polymerisation setup for monolithic PEGDA hydrogels.**

## 2. Analysis of force-distance ( $P$ - $h$ ) curves and contact point identification

The samples were initially analysed by extracting force-distance ( $P$ - $h$ ) curves from NanoScope Analysis software (version 1.85) and JPK SPM Data Processing 8.0.13, and the elastic modulus ( $E$ ) was estimated using both Hertz model and Oliver and Pharr method by analysing the extend (loading) and retract (unloading) curves separately. For using the Hertz model, to calculate the elastic modulus ( $E$ ), a number of assumptions were made: (i) indentations are frictionless and for small strains, (ii) indentation depth is less than 1/3 of the probe radius, (iii) deformation is linearly elastic, (iv) for small fast indentations the viscoelasticity can be negligible [1,2].

JPK SPM software was used for batch processing of the ( $P$ - $h$ ) curves by following the steps: (i) smoothen the data using the Gaussian function, (ii) subtract the baseline offset by fitting range of 75% to 95%, (iii) correct for any tilt, (iv) identify contact point by adjusting the x-offset where the contact point is assumed to be the first point at which the recorded force is in a positive value, (v) Hertz model for spherical indenter was used to the extend curve [2,3].

$$P = \frac{E}{1-\vartheta^2} \left[ \frac{a^2 + R_s^2}{2} \ln \frac{R_s + a}{R_s - a} - aR_s \right] \quad (\text{S5-1})$$

$$D = \frac{a}{2} \ln \frac{R_s + a}{R_s - a} \quad (\text{S5-2})$$

$P$  is the force (unit: N),  $a$  is the radius of contact circle (unit: mm),  $\vartheta$  is the material Poisson's ratio (dimensionless),  $E$  is the elastic modulus of the material (unit: MPa), and  $R_s$  is radius of a spherical tip (unit: mm) (Eq. (S5-1)).

$D$  is the indentation depth (unit: mm) or the tip sample separation which is measured from the identified contact point to the maximum displacement. The PEGDA Poisson's ratio is assumed to be 0.5 [4]

Analysis of ( $P$ - $h$ ) curves of AFM has always been challenging especially in locating the contact point to calculate the indentation depth which is a key parameter to measure  $E$  [2,3,5]. For hard samples, it is a straightforward step where it can be easily determined by using the extend curve and looking at the clear transition point. However, for soft samples, deformation due to long range forces happens before tip-sample contact, making the contact transition difficult to define and to differentiate between the cantilever movement and sample indentation depth. This can in turn lead to large errors and deriving over/underestimated  $E$  for the sample [6]. In this study, we used a simple procedure where we defined the contact point as a point at which the recorded force crosses the y-axis for the first time. It should be noted that a few steps must be followed before identifying the contact point which is explained in the Methods and Materials section. Such an approach, even though not ideal, if used to analyse all ( $P$ - $h$ ) curves consistently can provide relatively good comparative results to understand the variation in the mechanical properties as a function of the build (printed) direction.

In Oliver and Pharr method the projected contact area of the indenter is determined based on the indentation depth. For fitting the ( $p$ - $h$ ) curves an external MATLAB script is used to fit the model to the retract curve where  $E$  is calculated following the retraction of the tip from the sample [2]. The step-by-step procedure

can be found in the nature protocol where JPK software and the MATLAB script are used to extract the  $E$  [2].

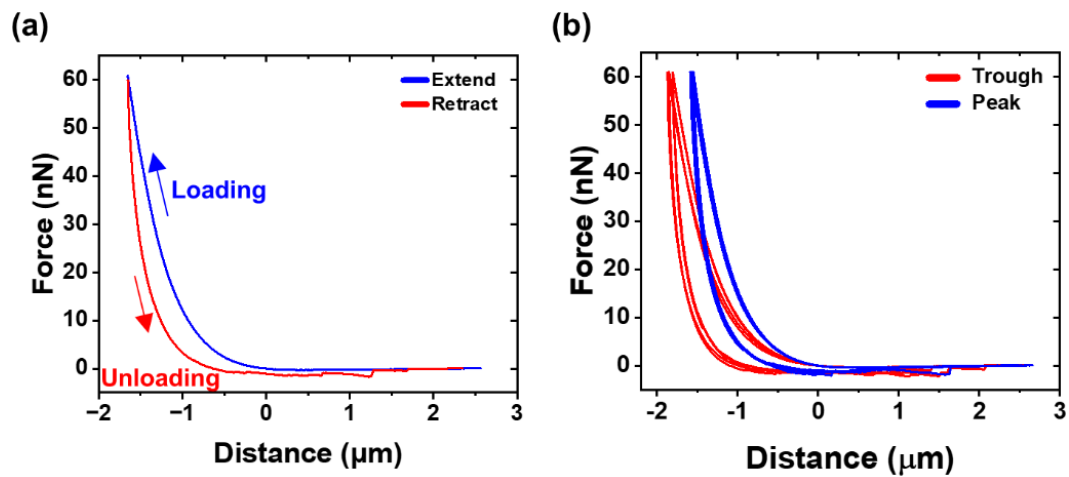


Figure S5-2 (a) A representative  $(P-h)$  curve of a multilayer PEGDA sample, indicating the extend (loading-blue colour) and the retract (unloading-red colour) sections of the curve. (b)  $(P-h)$  curves at different points across the peak and trough areas on the multilayer sample.

### 3. Indentation maps with location of every indent within a map of 90 x 90 $\mu\text{m}$

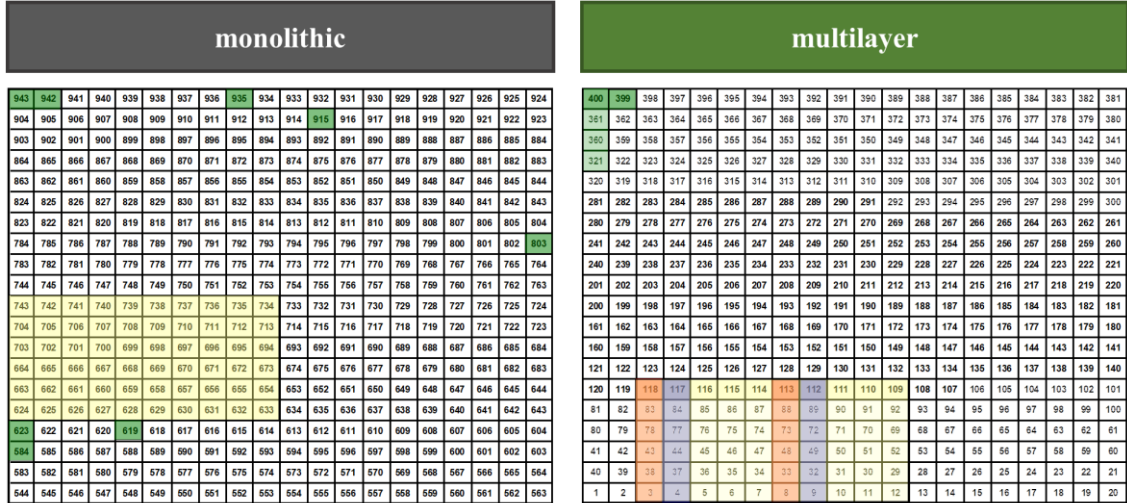


Figure S5- 3 The indentation map with location of each indent within the map of 90 x 90  $\mu\text{m}$ . The green indents are failed measurements omitted from the map. The red and blue colours represent indents on troughs and peaks, respectively. The highlighted boxes are areas exclusively analysed for modulus measurements in Figure 5-2.

#### **4. Comparison of elastic modulus maps based on two different fitting ranges (Hertz model) and a fitting based on Oliver and Pharr method**

It is a common problem with soft materials to establish a contact point and to fit the unloading slope in the ( $P$ - $h$ ) curve (Figure S5-2a). To combat this issue, two different distance ranges, from 0.0 to 1.5  $\mu\text{m}$  and from 0.5 to 2.0  $\mu\text{m}$ , were chosen to fit the data for the slope to obtain  $E$  and a comparison of these results are shown in Figures S5-4a and b. These results showed that even though the magnitude of  $E$  increased when the higher range was used, the overall pattern and trend did not change. Therefore, it can be suggested that the results are comparable and point to a distinct and clear phenomenon concerning the change in  $E$  in a single layer of the multilayer sample. The lower fitting range was used for the remaining measurements. In terms of the difference between Hertz model (Figure S5-4a and b) and Oliver and Pharr method (Figure S5-4c) it can be seen that the results are reporting a similar pattern; however, the magnitude of  $E$  has increased by 32% and 92% for both monolithic and multilayer samples, respectively when Oliver and Pharr method was used.

To measure  $E$  using the Oliver and Pharr method, the approach involves identifying a suitable multiplier that can be utilised in the script to determine the correct contact point from the differential comparison between the first point and the subsequent points on the extension curve. This process involves examining each individual curve to ensure that the fitting is correct and the contact point has been accurately identified. However, this task is tedious and requires significant data processing time. As a result, we chose to use the Hertz model as the primary

model for the study based on the assumptions outlined in the supporting information.

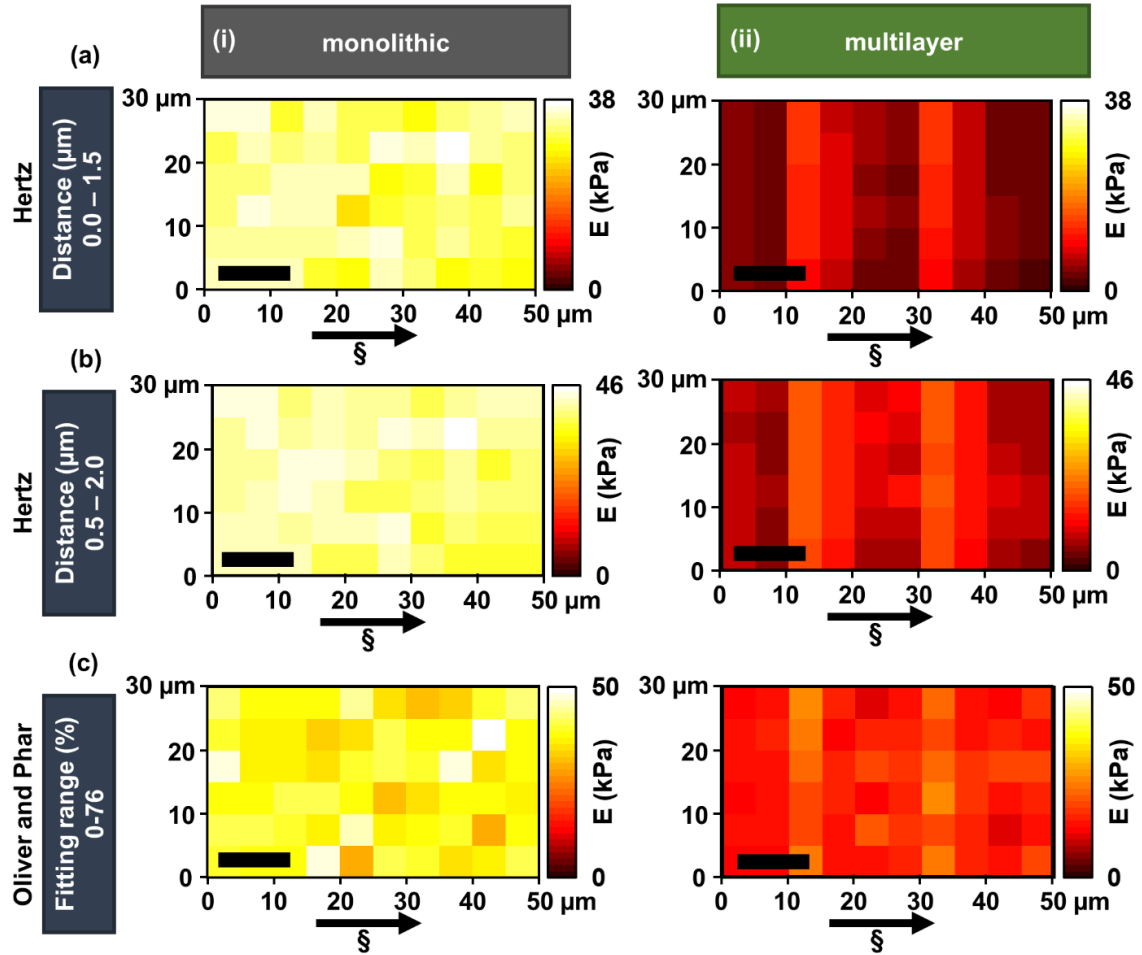


Figure S5- 4  $E$  map showing variation in modulus in both (i) monolithic, and (ii) multilayer PEGDA hydrogel samples based on two fitting ranges using Hertz model of (a) 0.0-1.5  $\mu\text{m}$ , and (b) 0.5-2.0  $\mu\text{m}$ . (c)  $E$  map using Oliver and Pharr method. Scale bars in all graphs are approximately 10  $\mu\text{m}$ .



## 5. Locations of indentation at 3 different heights on the multilayer hydrogel

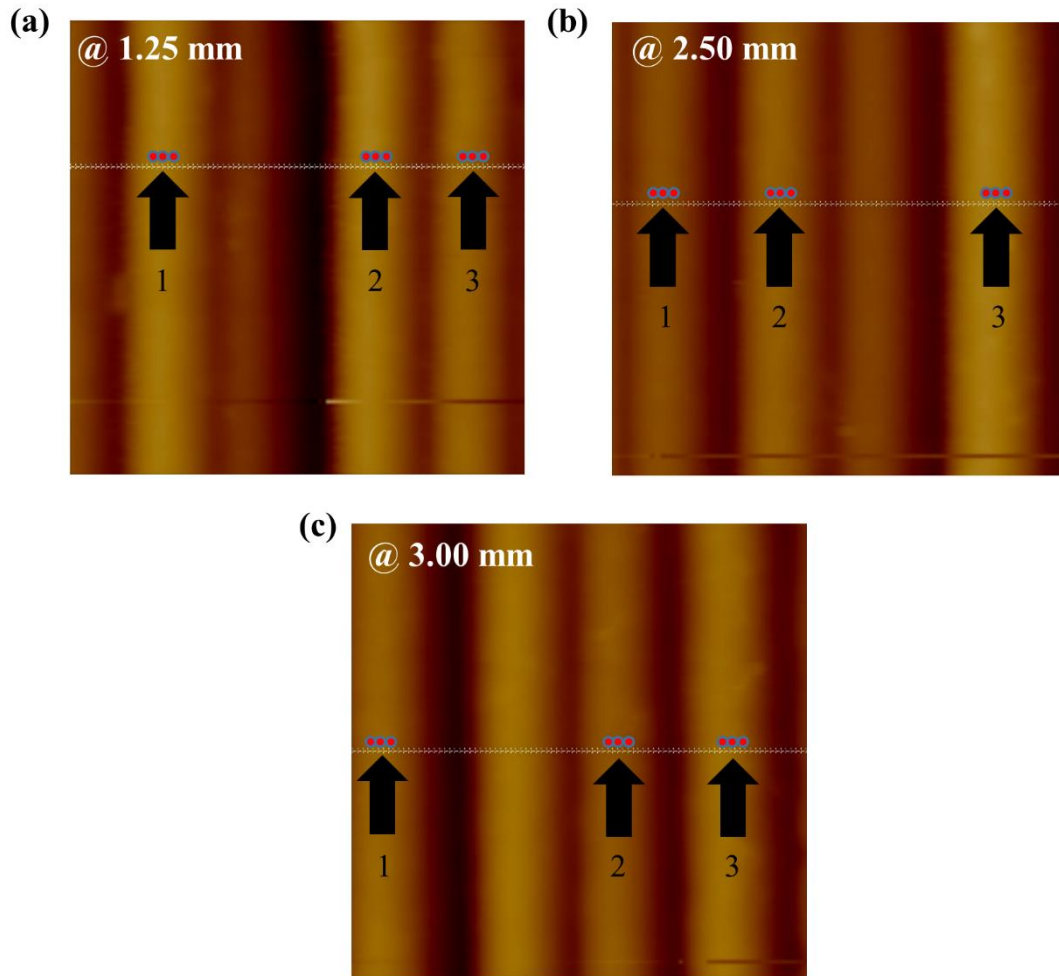


Figure S5- 5 Surface topography maps of a multilayer sample ( $90 \times 90 \mu\text{m}$ ) obtained through a raster scan at three specific heights: 1.25 mm, 2.50 mm, and 3.00 mm from the sample's bottom. White lines in each map denote the indentation locations for 90 indents conducted using the line indentation function with a  $1 \mu\text{m}$  spacing. Black arrows highlight the identified peaks on each layer, while blue circles with red dots indicate the positions of the three consecutive indents on each peak used to compute the average E at each height.

## 6. Explanations of effect of surface topography on $E$ measurements

In this study, two important arguments are addressed about the outer surface topography of the sample and the contact point. First, looking at Figure 5-2aii, it could be argued that the peak to valley (trough) depth in the multilayer sample is higher and therefore, the measured  $E$  map may be influenced by how contact is established. In the Hertz model (Eqs. (S5-1 and S5-2)) an ideal contact is assumed when the AFM tip is normal to the material's surface and the contact area follows the shape of the tip. Figure S5-6 postulates scenarios of the type of contact between the AFM tip and the sample's surface with red arrows highlighting the first contact point between the two objects. Given the size of the AFM tip (radius of 3.46  $\mu\text{m}$ ) and the printed layer thickness (20  $\mu\text{m}$ ), Figure S5-6ai shows how the contact may appear at the two points if the tip was approaching the interface between two consecutive layers, the trough. Conversely, if the tip is away from the interface, the contact scenario may differ and may occur at a single point (Figure S5-6aii). Two additional scenarios may also occur for the peak area of the printed layer where the tip may land exactly normal to the surface, the ideal situation, or on the side of the tip where the tip may slip on the surface rather than creating a well-established contact with the sample. Looking at Figure S5-5, where the exact position of the contact can be determined from the white dots on the apparent topography image, the scenarios mentioned above are plausible and cannot be ruled out.

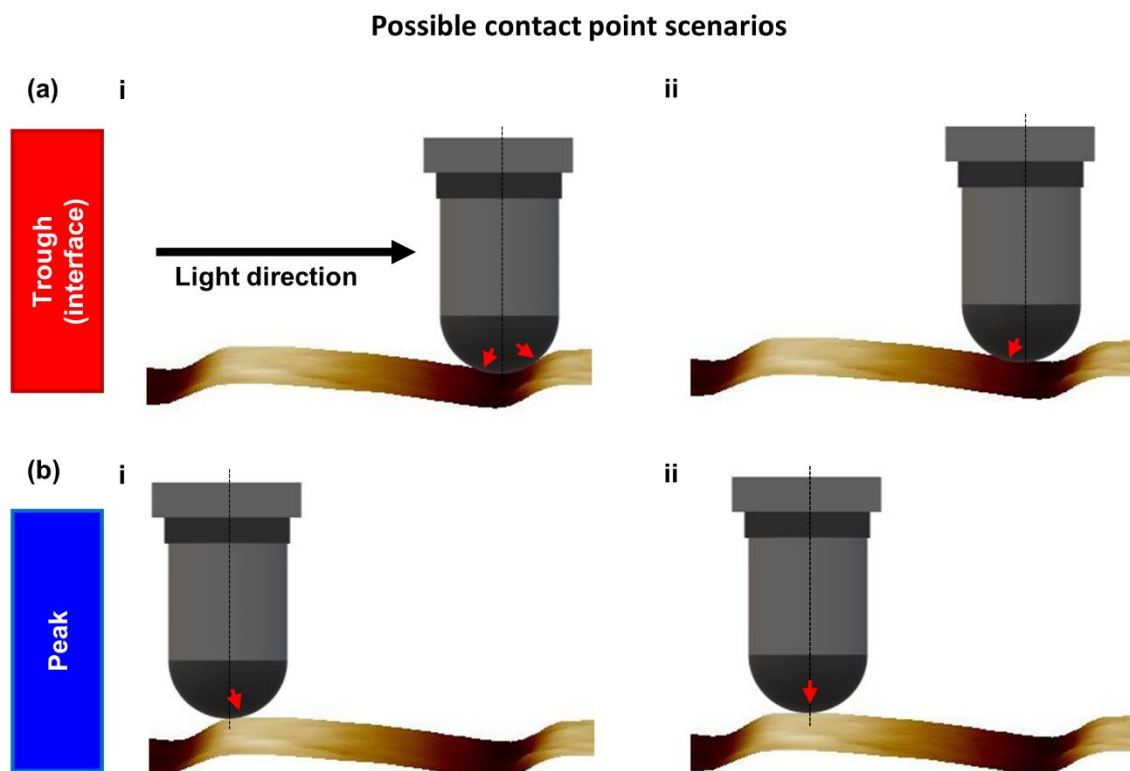


**Figure S5- 6 Schematic of different scenarios of contact between the AFM tip and the outer surface of the 3D multilayer PEGDA hydrogel at the interface. The red arrows demonstrate the contact points changing as the AFM tip moves from one area to another on the surface.**

Secondly, since the surface topography is considered rough for the AFM measurements, it could be argued that there may be variations in terms of how much material is being indented beneath the surface which may influence the estimate of  $E$ . Since the indentation depth into the sample surface is well over  $1\ \mu\text{m}$ , the effect of surface topography on the measured  $E$  may be considered negligible.

## **7. Attempting to eliminate the outer surface topography**

Attempting to eliminate the influence of the outer surface topography of the printed multilayer sample by reducing surface roughness, a cross-section of the multilayer sample was cut with 100  $\mu\text{m}$  thick stainless steel blade in its hydrated state (Figure S5-7a). However, the surface topography and roughness increased with peak to valley (trough) difference reaching maximum of 5.3 and 6.7  $\mu\text{m}$  in both multilayer and monolithic samples, respectively (Figure S5-7b), rendering it impossible to perform any sensible modulus measurements (Figure S5-7c). Another way to improve surface topography was to freeze and cryo-section the sample as it may improve the surface flatness [7]. However, such approach will completely change the hydrogel properties and its real-life condition and will lead to  $E$  measurements that are effectively not representative of the real hydrated system [8,9].

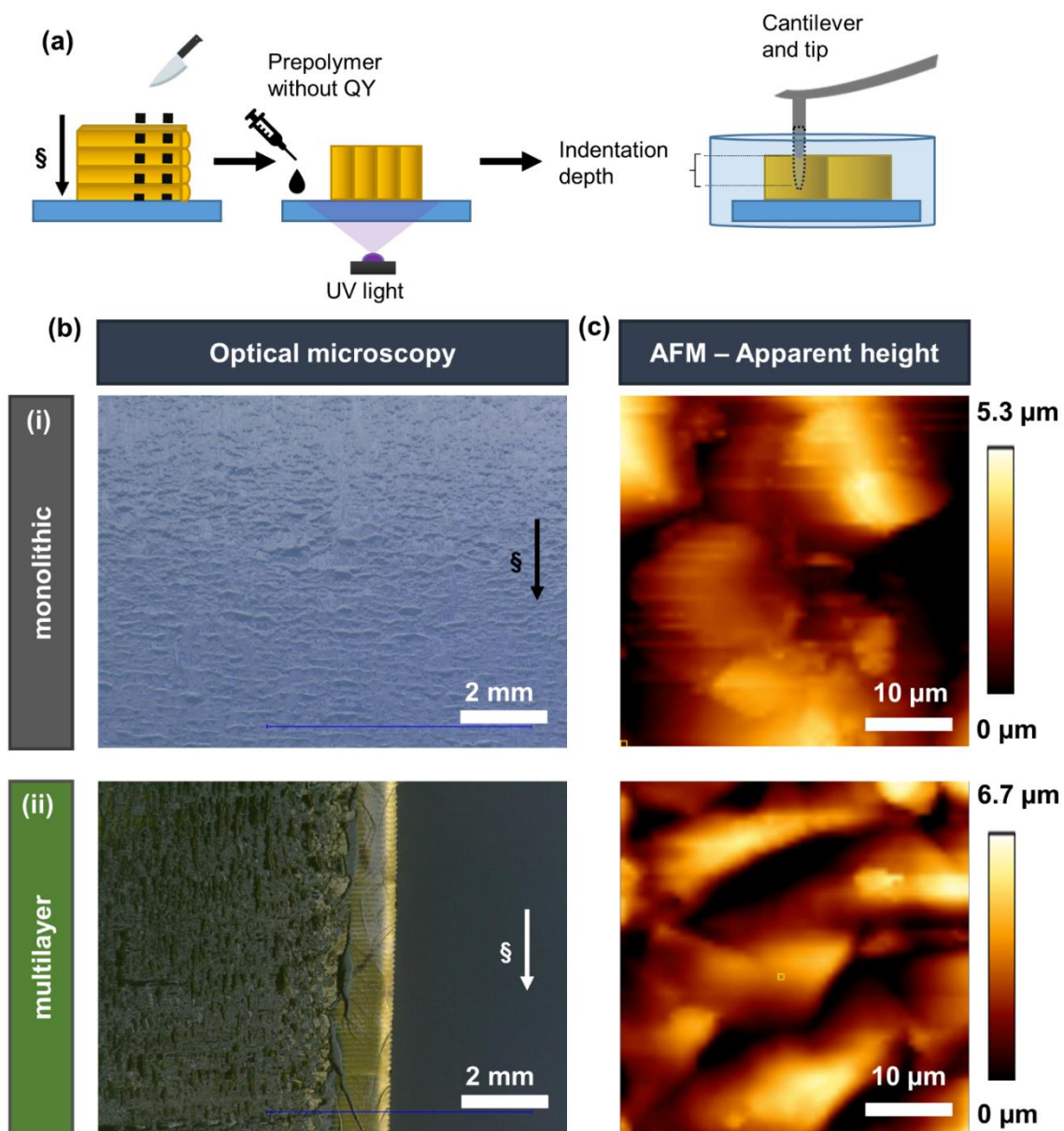


Figure S5- 7 (a) Schematic of sample preparation for cross-section of 3D multilayer PEGDA hydrogel samples. (b) Optical microscope images of cross-sections, (c) AFM apparent height for (i) monolithic, and (ii) multilayer samples.

## 8. Measuring light absorbance for prepolymer solution with fixed QY concentration

A prepolymer solution containing 9 mg/mL QY, 5 mg/mL LAP, and 200 mg/mL PEGDA was prepared. The solution was poured onto a coverslip at the bottom of a vat, and the UV light intensity was adjusted to 20.51 mW/cm<sup>2</sup>. After being exposed to UV light for 3 s, the excess solution was pipetted out from around the formed layer, and the surface of the layer was gently blotted with a paper towel. The thickness of the formed layer (known as cross-linking depth ( $C_d$ )) was measured and recorded as  $46 \pm 2$   $\mu\text{m}$ . The intensity of the UV light passing through the formed layer was measured and found to be 3.06 mW/cm<sup>2</sup>. Using (Eq. (S5-3)) the light absorbance ( $A$ , unitless) was calculated [10]:

$$A = \log_{10} \left( I_0 / I_z \right) \quad (\text{S5-3})$$

Where  $I_0 = I_{\tau, \tau = 0}$ , is the initial intensity at the bottom of the vat (unit: mW/cm<sup>2</sup>) and  $I_z = I_{\tau, \tau = z}$  is the intensity of the light as it passes through the sample of thickness  $z$  (unit: cm), the light absorbance was calculated to be 0.80 for a multi-20  $\mu\text{m}$  where the sample thickness was 20  $\mu\text{m}$ .

A combination between (Eq. (S5-3)) and Beer-lambert law (Eq. (S5-4)) can be used to calculate the extinction coefficient ( $\epsilon$ , unit:  $\text{M}^{-1} \text{cm}^{-1}$ ) [10]:

$$A = \epsilon OC \quad (\text{S5-4})$$

where ( $C$ , unit:  $\text{M}$ ) is the concentration of the photoabsorber in the prepolymer solution and ( $O$ , unit: cm) is the constant path light. The extinction coefficient was calculated to be  $5,474.6 \text{ M}^{-1} \text{cm}^{-1}$ .

## **9. Measuring cross-linking depth as light passes through a cross-linked single layer hydrogel with 20 $\mu\text{m}$ layer thickness**

Initially, single layer hydrogel with a thickness of 20  $\mu\text{m}$  and a QY photoabsorber concentration of 9 mg/mL in the prepolymer solution was printed using an intensity of 20 mW/cm<sup>2</sup>. The intensity of the UV light passing through the resulting cross-linked layer was measured to be  $3.29 \pm 0.1$  mW/cm<sup>2</sup> (as depicted in Figure S5-9). Next, a glass slide was inserted into the printing vat and the UV light intensity was adjusted to 3.29 mW/cm<sup>2</sup>. The same prepolymer solution used to print the 20  $\mu\text{m}$  layer thickness was poured onto the glass slide and exposed to the adjusted UV light for 3 s (as shown in Figure S5-9). The resulting cross-linked layer was measured using a micrometre and found to have a thickness of  $3 \pm 2$   $\mu\text{m}$ . This suggests that only the top 3  $\mu\text{m}$  of each layer in the multilayer sample were double-exposed (as illustrated in Figure S5-9), which is consistent with our previous findings and supports the formation of a highly cross-linked planar sub-layer [11]. A similar procedure for QY photoabsorber concentration of 1.8 mg/mL was used to measure the cross-linking depth as light passes through a single layer hydrogel with 150  $\mu\text{m}$  layer thickness, with adjusted UV light intensity of  $5.8 \pm 0.3$  mW/cm<sup>2</sup>, was  $80 \pm 16$   $\mu\text{m}$ .

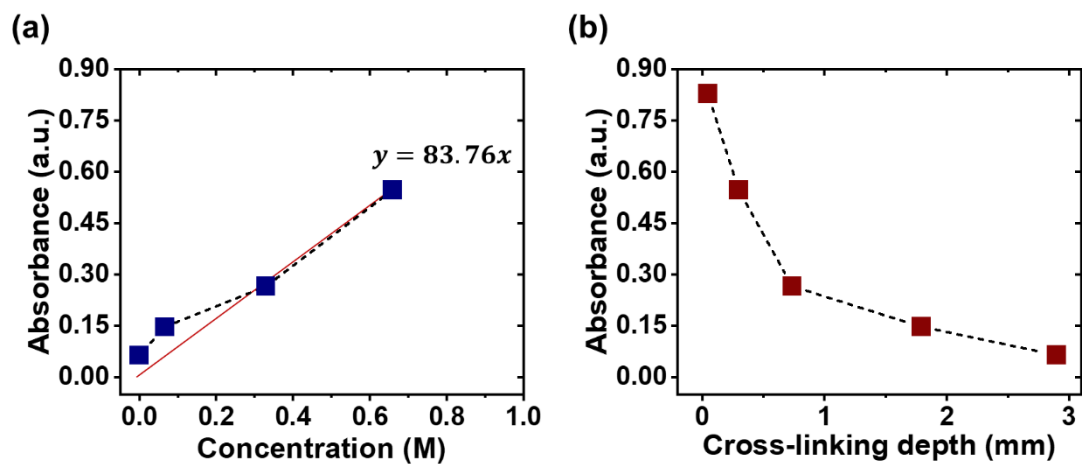


Figure S5- 8 (a) Calibration curve of absorbance versus concentration at a fixed path length. (b) Absorbance versus cross-linking depth at different QY concentrations for a multilayer PEGDA hydrogel samples.



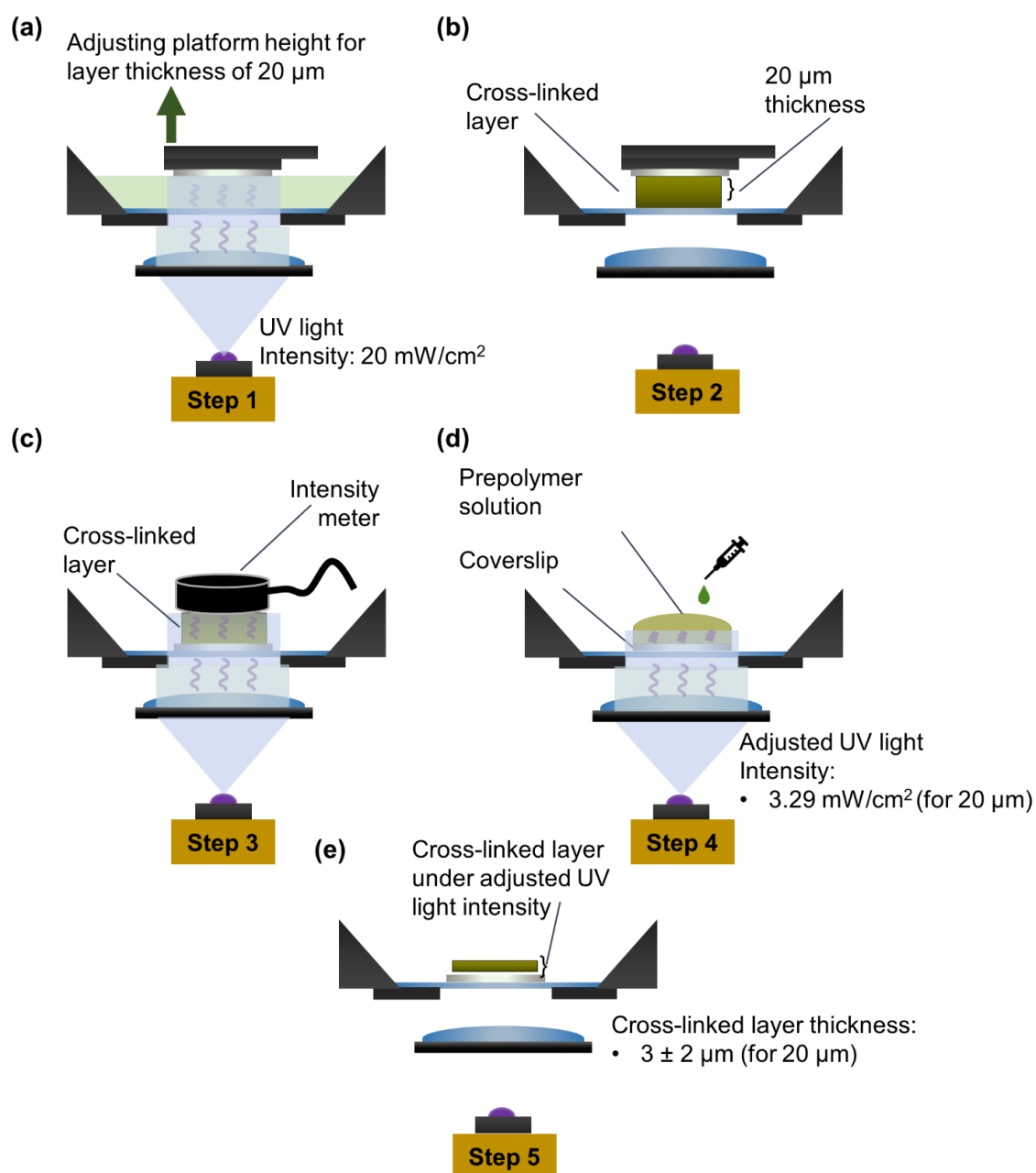


**Figure S5- 9 Schematic representation procedure for measuring the double exposed section in a 3D multilayer PEGDA hydrogel samples with layer thickness of 20  $\mu\text{m}$ .**

Based on our measurements, it appears that the intensity of the UV light passing through the prepolymer solution is insufficient to penetrate and double expose the entire previously formed layer (as shown in Figure S5-10). The QY

photoabsorber present in the prepolymer solution significantly absorbs light as it propagates through the solution during photo-cross-linking. This causes an exponential light intensity attenuation [10,12], resulting in only a small portion of the top section of the previously formed layer near the interface being double exposed.

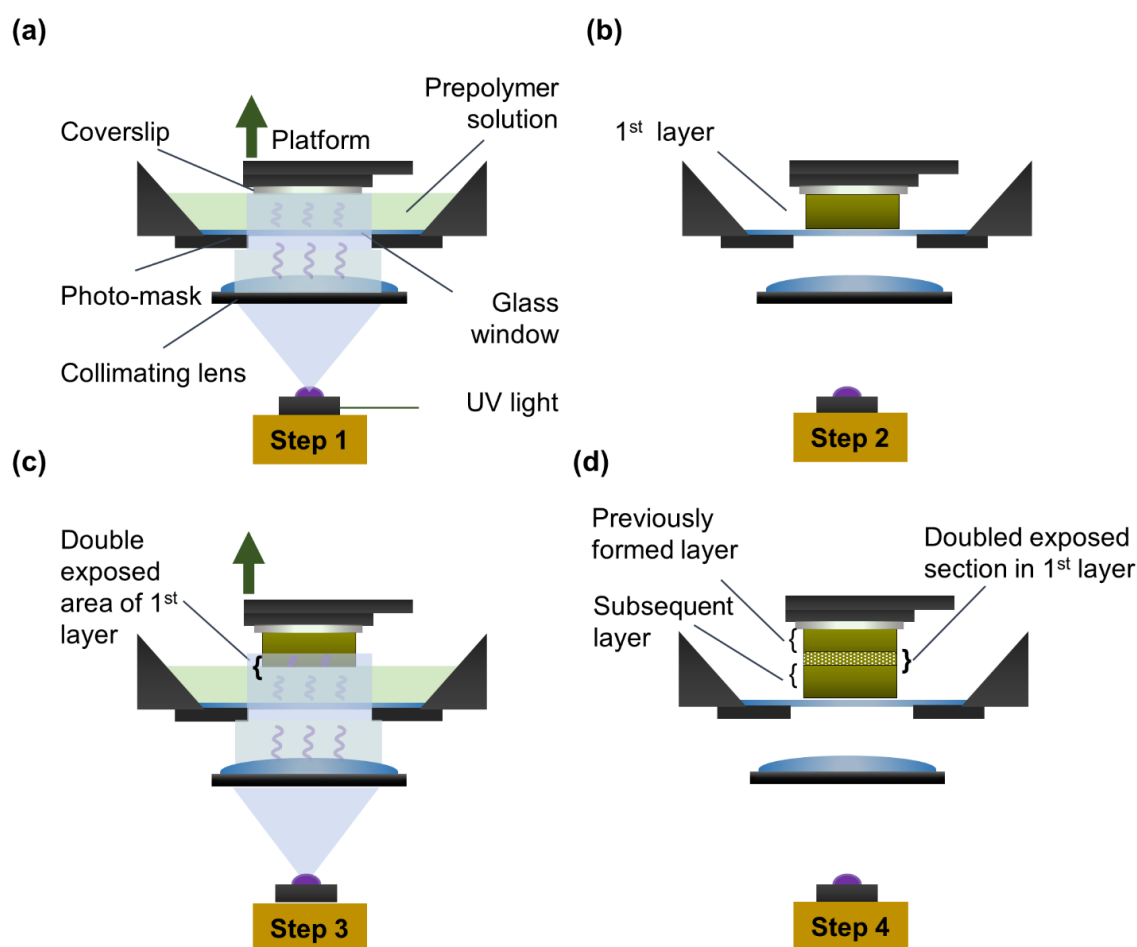


**Figure S5- 10 Schematic representation of the projection lithography system depicting the layer formation process (a-c) and (d) area within the surface of the previously formed layer which is potentially double exposed to UV light just below the interlayer interface.**

## 10. Optical microscopic images of outer surface topography of multilayer hydrogels

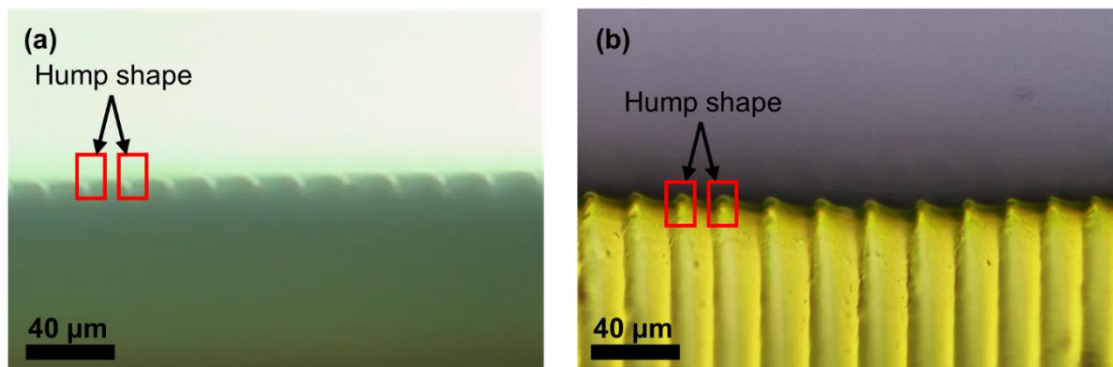


Figure S5- 11 Optical microscopic image of the printed multilayer hydrogel sample outer surface topography of a (a) cylindrical, (b) cuboid shape samples.

## 11. Alternative options to LAP photoinitiator

There is a constant search for alternatives to metal-based photoinitiators in order to examine whether they can replace LAP and lead to a more homogeneous structure. If Irgacure 2959 was soluble in water instead of methanol, it could have been a viable option for tissue engineering studies [13]. However, a more suitable photoinitiator with stable radicals such as TEMPO could be used to achieve well-controlled free-radical cross-linking. TEMPO's stable free-radical property is due to its steric bulk, which impedes the reaction of other free radicals, providing control over polymerisation kinetics [14]. While TEMPO has been shown to be cytotoxic and unsuitable for tissue engineering, alternative options could be explored to examine if they may result in more homogenous structures.

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# Chapter 6

## General Discussion

## 6.1 Discussion

Organ-on-chip devices have emerged as a promising technology for drug development and toxicity testing, offering a more accurate and ethical alternative to animal testing [1]. These devices use biomaterials as scaffolds to mimic the microenvironments of human organs, and biopolymers such as hydrogels have shown great potential for this application [2]. Hydrogels a class of biopolymers are attractive materials due to their ability to absorb and retain large amounts of water, making them similar to natural tissues [3,4].

The emergence of 3D printing has revolutionised the creation of hydrogels, leading to the development of more intricate and sophisticated organ-on-chip devices [5,6]. Thanks to layer-by-layer (LbL) fabrication techniques, it is now possible to precisely control the geometry, composition, and architecture of the hydrogels [7–9]. PEGDA, a synthetic hydrogel, has been extensively used in biomedical applications due to its biocompatibility, low immunogenicity, and ability to be cross-linked using UV light [10]. It has also shown promise in tissue engineering as it can be formed into precise three-dimensional structures, providing mechanical support while maintaining tissue-specific functions [11–13]. Photo-cross-linking has enabled the creation of complex and high-resolution structures, allowing for the precise control of scaffold formation [14,15]. Nevertheless, the 3D printing of PEGDA hydrogels still presents challenges, such as the potential impact of LbL fabrication on their physical and mechanical properties, as well as their reproducibility, repeatability, and stability over time in a physiologically relevant environments [16–21]. As such, additional research was needed to generate new understanding on fundamentals of LbL fabrication



and how to use this understanding to further advance the field of tissue engineering and organ-on-chip devices.

### **6.1.1 DLP vs In-House Projection Lithography System**

DLP printing and in-house single photomask projection lithography are two technologies that use light to create 3D printed parts using photo-cross-linkable resins [22–24]. In DLP printing, the light from a projector is directed through a digital micromirror device (DMD), also known as a DLP chip, to selectively solidify the resin into a 3D part. The DMD is made up of a matrix of thousands or even millions of small micromirrors, the size and arrangement of which determine the resolution of the DLP printer [20,25]. Beside the size of the DMD which improves shape accuracy, the uniformity of the power intensity distribution across the printing platform is another important parameter that requires optimisation [26]. Various techniques have been employed to achieve this objective, one of which is pixel blending and grayscale control method [27]. This method adjusts pixel brightness using grayscale levels and considers the Gaussian energy distribution of each pixel and its surrounding pixels to estimate the required light intensity for that particular pixel. [27]. Such technique requires extensive mathematical modelling and optimisation to achieve a high level of light intensity uniformity across the printing platform which is required for geometric accuracy in fabrication of microstructures [28,29].

Initially, a high resolution DLP system with DMD with a 10.8  $\mu\text{m}$  pixel pitch in both lateral dimensions was considered for fabricating the samples for our studies. The printing system and prepolymer solution was optimised to be able to print open channels with inner diameter of 100  $\mu\text{m}$  which required to have a

prepolymer solution that includes 5 mg/mL of LAP photoinitiator, 9 mg/mL of QY photoabsorber and 200 mg/mL of PEGDA and fixed layer thickness of 20  $\mu\text{m}$  [21,30].

Our study aimed to understand the impact of LbL fabrication on PEGDA hydrogels, resulting in the development of a reliable and repeatable in-house single photomask projection lithography system using a condenser lens and diffuser for improved uniformity of light intensity (Figure 6-1 and Table 6-1). This achieved less than 5% intensity variation over an area of 22 mm diameter in the centre of a circular printing area with a diameter of 40 mm, which is an improvement on existing DLP printers with approximately 10% variation over a rectangular printing area of 1 x 2 cm (Appendix D: Figure D-2).

|                                 | Advantages                                                                                                                                                                                                                                 | Limitations                                                                                                                                                                                                                                                                              |
|---------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| DLP                             | <ul style="list-style-type: none"> <li>• High XY resolution (10.4 <math>\mu\text{m}</math>)</li> <li>• Design agility/Complex geometry</li> </ul>                                                                                          | <ul style="list-style-type: none"> <li>• Complex system/process</li> <li>• Intensity uniformity ~ 10%</li> <li>• Limited to mm size structures</li> <li>• Extensive optics alignments</li> <li>• Limited Z-resolution (tested to 20 <math>\mu\text{m}</math> layer thickness)</li> </ul> |
| In-house projection lithography | <ul style="list-style-type: none"> <li>• High intensity uniformity ~5%</li> <li>• Simple system/process</li> <li>• High Z-resolution (tested at 5 <math>\mu\text{m}</math> layer thickness)</li> <li>• Simpler optics alignment</li> </ul> | <ul style="list-style-type: none"> <li>• Lower XY resolution (15 <math>\mu\text{m}</math>)</li> <li>• Simple shapes/Design inflexibility</li> </ul>                                                                                                                                      |

**Figure 6-1 Comparison on advantages and limitations of DLP versus in-house projection lithography.**

In LbL fabrication processes, there is a trade-off between layer thickness and printing time, with smaller layer thicknesses resulting in longer printing times. To

increase the number of samples and statistical confidence in our studies, we needed to find a way to reduce the printing time without sacrificing the accuracy and integrity of our results. We achieved this by reducing the QY photoabsorber concentration from 9 to 1.8 mg/mL, which is five times less than the original recipe, and setting the step height to 150  $\mu\text{m}$  which is half of the measured cross-linking depth of 299  $\mu\text{m}$  (Appendix D: Figure D-6) [31]. As a result, the printing time for a 5 x 5 mm cuboid with 10 mm height was reduced from approximately 4 hrs to just 33 mins. The decrease in printing time had multiple benefits. It enabled us to increase the number of samples, herein, multi-150  $\mu\text{m}$ , and improve the statistical reliability of our studies. Additionally, it expedited preliminary investigations, particularly when evaluating weight changes through NWF and volume changes through NVF using gravimetric and volumetric methods, respectively, as outlined in chapters 3 and 4 [31,32]. Building upon the results obtained, we expanded the study by examining samples with a lower layer thickness of 20  $\mu\text{m}$  to explore how this parameter affected the swelling behaviour of LbL fabricated multilayer PEGDA hydrogels.

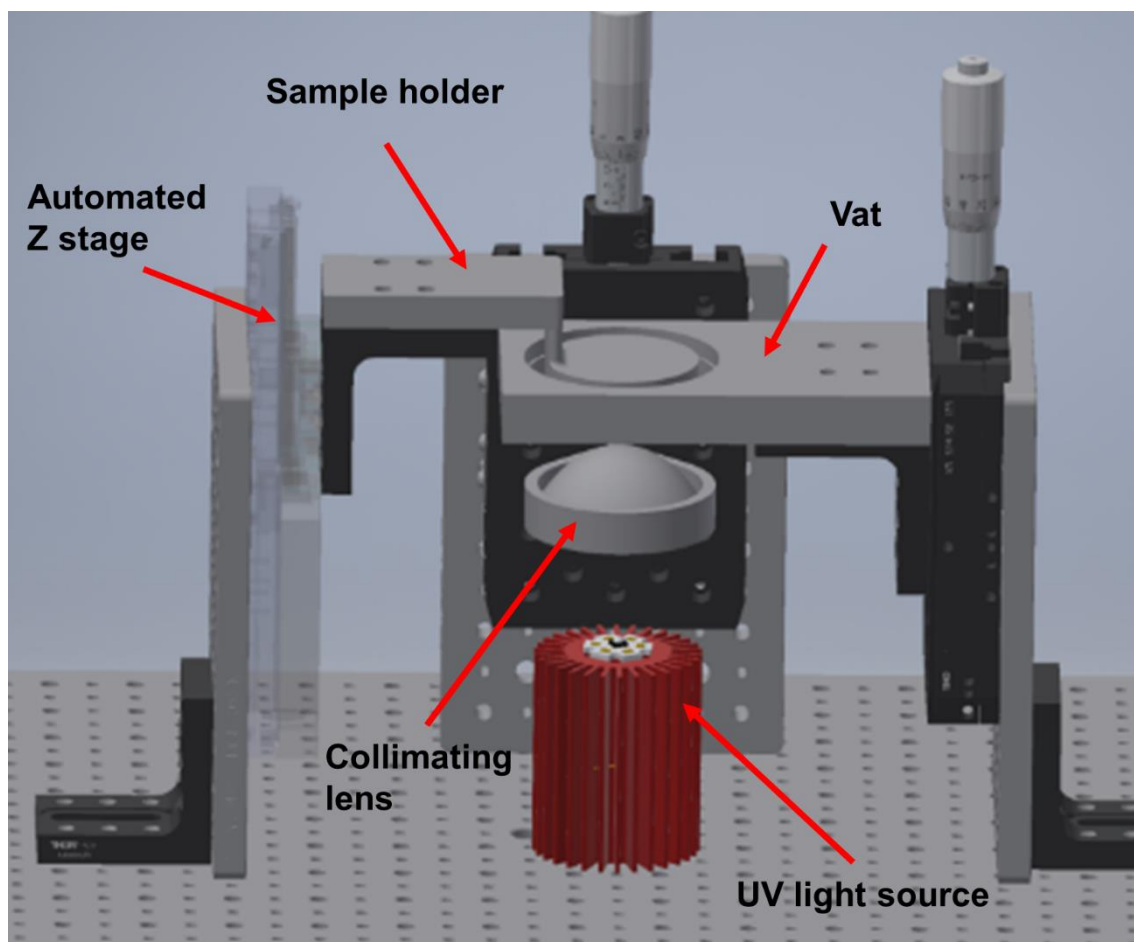


**Figure 6-2 A 3D image of the custom-made projection lithography printing system.**

In addition, our in-house projection lithography system has allowed us to deepen our understanding of how layer thickness impacts the surface nanomechanical properties of multilayer PEGDA hydrogels. Chapter 4 of our study used nanoindentation on hydrated samples to investigate how changes in layer thickness, or number of layers, could affect these properties, highlighting a research gap that had not been previously explored. Despite being unexpected, our findings showed that reducing the layer thickness in multilayer PEGDA hydrogels leads to a decrease in measured  $E$ , providing a novel understanding of LbL fabricated hydrogels. However, further research is necessary, including indentation on single-layer samples with varying thickness, to differentiate the

impact of layer thickness from the number of layers on  $E$ . Additionally, it is important to examine samples fabricated using DLP printers to determine whether the creation of voids at the interface is a result of the printing setup or a genuine phenomenon where LbL printing results in internal defects at the interfaces and within the cross-linked layer, with the defect density increasing as the number of layers increases.

Regarding the study discussed in chapter 5 that examines the spatial variation in nanomechanical properties of multilayer, multi-20  $\mu\text{m}$  hydrogels within each layer and at the interface, it is important to investigate whether these variations are consistent in the DLP printing system specifically or are specific to the in-house projection lithography printing system. Moreover, it is possible to expand the study to ascertain the minimum layer thickness required to attain the least spatial variation in cross-linking density and nanomechanical behaviour within the layers, particularly given that the in-house projection lithography system can print structures with a layer thickness of 5  $\mu\text{m}$ .

The understanding acquired from these investigations can assist in selecting suitable printing parameters and optimising them for specific applications in organ-on-chip devices, by adjusting factors such as light intensity, exposure time, layer thickness, and other relevant parameters. This iterative optimisation process can be repeated until the desired physicochemical and mechanical properties are achieved.

**Table 6-1 List of main components of the in-house projection lithography system**

| #  | Component            | Description: Use                                                                         |
|----|----------------------|------------------------------------------------------------------------------------------|
| 1  | Automated stage      | z- 50 mm motorised translation stage: moving the sample holder                           |
| 2  | UV LED               | LZ1-10UV0R-0000: 365 nm wavelength                                                       |
| 3  | Heat sink            | 50 mm diameter, 60 mm length Heatsink: disseminating heat                                |
| 4  | Collimating lens     | 50 mm Aspheric condenser lens w/ diffuser: Light collimation                             |
| 5  | Sample holder        | 24 x 24 mm sample holder: Holding the glass cover that the sample printed on             |
| 6  | Vat                  | 55 mm diameter with 9 mm height vat: container with clear window for prepolymer solution |
| 7  | B270 Window          | 50 mm diameter: bottom screen of vat                                                     |
| 8  | FEP film             | 0.15 mm thick: non-stick film attached to screen                                         |
| 9  | Micrometre stage Y   | 25 mm travel: holding and adjusting vat position                                         |
| 10 | Micrometre stage XYZ | 25 mm travel: alignment of collimating lens                                              |

### 6.1.2 Dimensional Stability of 3D PEGDA Hydrogels

In Chapter 3, we investigated the dimensional stability of 3D multilayer PEGDA hydrogels by storing them at 4 different temperatures (8, 20, 37, and 45 °C) for a period of 7 days. The physical dimensions of the hydrogels were monitored throughout the defined period. We found that the PEGDA hydrogels exhibited different responses to low temperatures (8 °C) compared to increased temperatures (37 and 45 °C). The volume change was significant, ranging from +15% to -12%, depending on the composition and layer thickness. This change was attributed to the changes in hydrogen bond strength at different temperatures and the hydrophilic to hydrophobic transition of the backbone oxyethylene chain, which caused the hydrogen bonds to break and the water molecules to be expelled from the network chain, resulting in shrinkage.

In Chapter 4, we conducted a 30-day study to investigate the behaviour of the PEGDA hydrogels when stored in water and CCM at nominal temperatures of 8, 20, and 37 °C. Initially, similar behaviour was observed, where the multilayer PEGDA hydrogels reached equilibrium mass and volume within 24-48 hrs. However, after 30 days of storage, a gradual increase was observed, with a maximum of 7% increase in weight and volume for samples stored in both solutions at all temperatures. Although not statistically significant for some temperatures, this increase shows a trend that requires careful consideration. The literature presents conflicting findings regarding UV cross-linked PEGDA samples with varying molecular weights [16,33]. In one study, PEGDA hydrogels with Mn 3.4k and 10k molecular weight exhibited minor, nonsignificant increases in swelling over 6 weeks in PBS. However, a subsequent linear regression analysis revealed a gradual swelling trend for both PEGDA hydrogel formulations, indicative of slow hydrolytic degradation [16]. Conversely, another study involving PEGDA hydrogels with Mn 0.7k molecular weight displayed reduced swelling over 28 days in water [33]. These results challenge the perception of PEGDA as a biostable control. It's crucial to consider this finding, especially when using PEGDA in long-term tissue studies, where the stability of PEGDA hydrogels is essential for supporting or securing living tissue. Changes in the dimensions of multilayer PEGDA hydrogels supporting contractile tissue can significantly affect the measured tissue force, potentially leading to misinterpretation of study outcomes.

Chapter 3 revealed another significant discovery regarding the multilayer PEGDA hydrogels' anisotropic properties, indicating that the axial dimension's

transformation was approximately 58% greater than that of the lateral dimension. This transformation occurred due to the multilayer hydrogels' thermal response to high (i.e., 45 °C) or low (i.e., 8 °C) temperatures. This was attributed to the formation of highly cross-linked planar sublayers at the interface of each layer within the hydrogel structure that leads to a more stable structure along the direction of the sublayers, i.e. lateral direction, while maintaining compliance in the direction normal to the sublayers, i.e. axial direction. It is vital for this anisotropic behaviour to be considered when there is a volume change especially in contractile tissue studies where beam bending theory is used to calculate the measured force of a living tissue based on the deflection of cantilever support structures [21,34]. In beam bending theory there is a reverse correlation between the aspect ratio (AR), diameter and length of the support cantilever, and the deflection force (DF) where an increase in AR leads to reduction in DF [21]. Figure 6-3 illustrates the relationship between AR of a cantilever post and the DF required to deflect its free end to a known distance. It demonstrates that a rise in aspect ratio, caused by anisotropic swelling, leads to a reduction in the necessary deflection force.



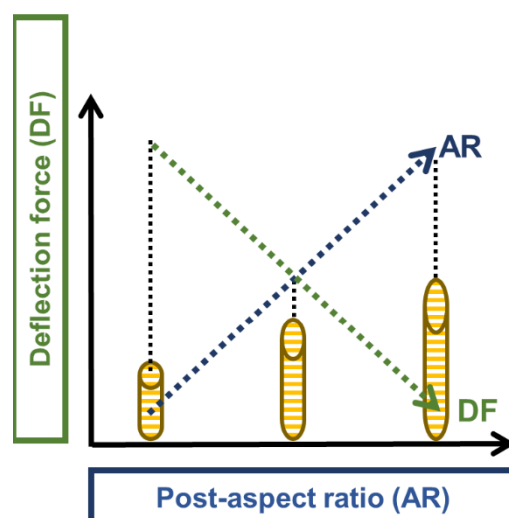


**Figure 6-3 Schematic showing the effect of dimensional stability of 3D multilayer PEGDA hydrogel cantilevers (posts) translated as aspect ratio (AR) where the aspect ratio is the length of the cantilever ( $L$ )/diameter ( $d$ ) of the cantilever, ( $x$ ) is the distance from the fixed end to the point of force application. The deflection force (DF) is the force required to deflect the cantilever to a known distance.**

From the above discussion, it is evident that understanding the dimensional stability of 3D multilayer PEGDA hydrogels is of utmost importance. This is particularly relevant considering the growing use of these hydrogels as extracellular matrix (ECM) and support scaffolds in various organ-on-chip devices.

### 6.1.3 Cross-Contamination and Bacterial Growth

Another possible reason for the mass increase in samples stored in DIW could be attributed to cross-contamination. Despite the recorded resistivity of 18.2 M $\Omega$ .cm at the source, impurities in the DIW may have caused the mass increase in samples after 30 days. Energy Dispersive Spectroscopy (EDS) analysis of dried hydrogel samples uncovered traces of Na and Cl on their surface (Figure

6-4). The salt found on the surface of the dried hydrogels could suggest contamination in the DIW. This contamination may be related to salt buildup in the pipes that connect the source and the tap.

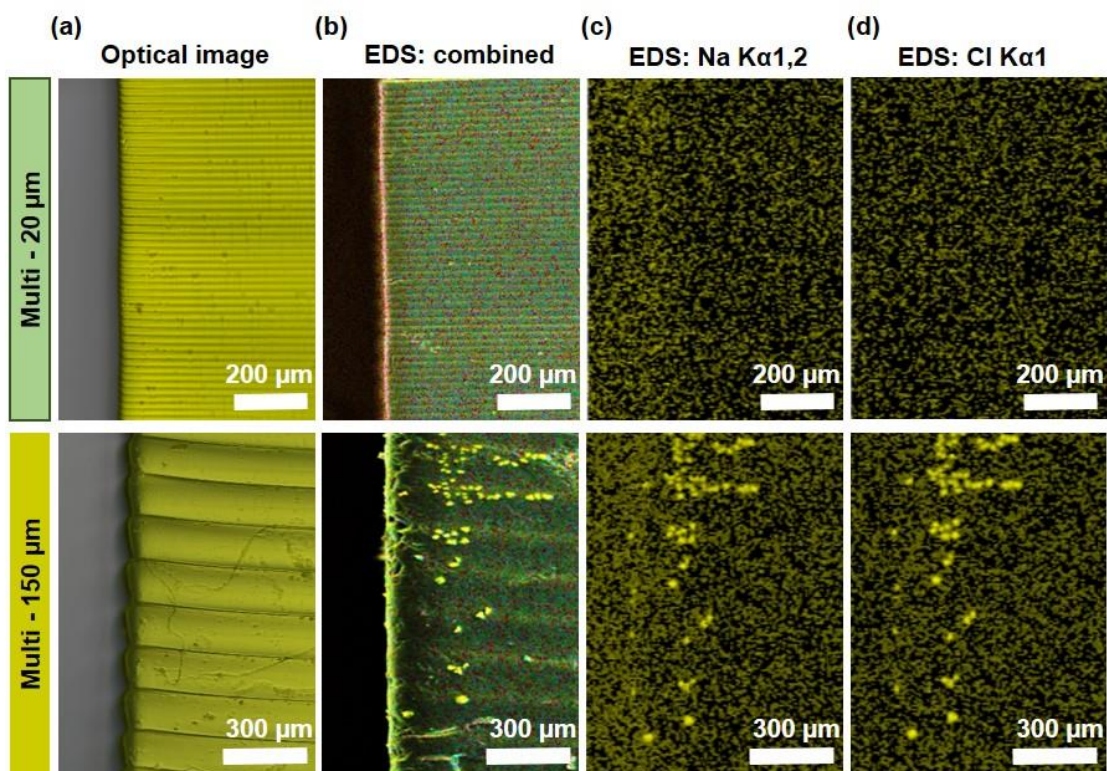


**Figure 6-4 (a) Side view optical microscopy image of the multilayer, multi-20μm (top) and multi-150μm (bottom) PEGDA hydrogels, along with Energy Dispersive Spectroscopy (EDS) of (b) combined all detected elements, (c) element Na, and (d) element Cl.**

In samples stored in CCM, precipitation and suspected bacterial growth were observed, which may have been caused by not replacing the solution during the experiment (Figure 6-5). This may have led to infiltration of the polymer network, causing increased mass and volume in the hydrogels. However, it should be noted that the sample size was small ( $n=2$ ), and future studies should aim to increase the number of samples before drawing conclusions. As mentioned in the

previous section, although bulk cross-linked PEGDA hydrogels are considered non-degradable, a 6-week study in PBS showed a slight increase in swelling ratio of 5-10% but within the standard deviation, indicating no degradation [16]. However, when PEGDA was incorporated with or in contact with other materials like GelMA and HA, hydrolytic activity was observed after a few days to weeks of storage in PBS [35–37].

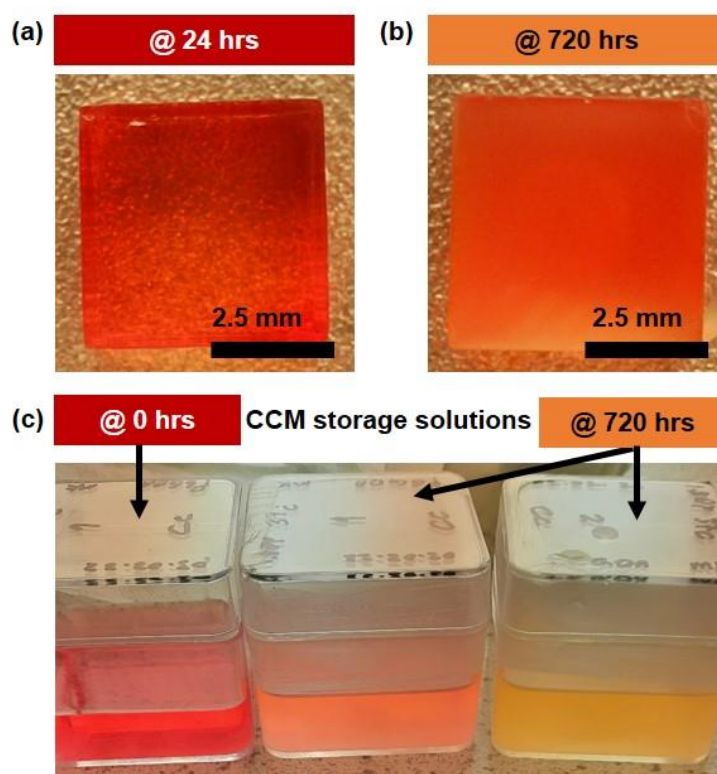


**Figure 6-5** Top view, optical microscopy images of multilayer, multi-20µm (a) stored in CCM for 24 hrs, has a bright red colour, very clear and see-through, (b) stored in CCM for 720 hrs, has an orange colour, and looks opaque. (c) CCM storage solutions imaged @ 0 hrs and 720 hrs. The change in colour from red to orange indicates bacterial growth within the solution.

Conducting additional long-term studies at different temperatures and in different storage solutions with relevant pH levels and ionic strengths can help determine

not only the dimensional stability of the 3D printed PEGDA hydrogels, but also establish and implement a standardised protocols for, synthesis, storage, transport, use, and shelf life of the 3D printed hydrogels across all laboratories. This will also provide important information for the development and application of organ-on-chip devices that use 3D printed PEGDA hydrogels as support scaffolds.

#### **6.1.4 Chemical Analysis of the 3D PEGDA Hydrogels**

In chapter 3, the chemical analysis of 3D multilayer PEGDA hydrogels aimed to determine the degree of cross-linking by changing the concentration of the photoabsorbing agent (QY) from 9 to 1.8 mg/mL and by adjusting the printing parameters such as layer thickness from 20  $\mu\text{m}$  to 150  $\mu\text{m}$ . The analysis was qualitative, attempting to establish the degree of cross-linking by comparing  $^1\text{H}$  NMR spectra of various samples (Figure 3-4).

In chapter 4, it was reported that increasing the UV exposure time from 3 s (120  $\text{mJ}/\text{cm}^2$  UV dosage) to 15 s (600  $\text{mJ}/\text{cm}^2$  UV dosage) was not sufficient to fully cross-link the entire exposed layer in the presence of high concentration of QY photoabsorber (9 mg/mL) for the multilayer hydrogel, multi-20  $\mu\text{m}$ -15 s. Traces of unreacted PEGDA's absorption which corresponds to the protons of  $-\text{CH}_2\text{CH}_2\text{O}-$  and  $\text{CH}_2=\text{CH}-$  groups could still be clearly observed in the NMR spectra (Figure 4-6g). By reducing the amount of QY photoabsorber from 9 mg/mL to 1.8 mg/mL, less absorption of these protons was observed, leading to a higher degree of cross-linking in the multilayer, multi- 150  $\mu\text{m}$ -15 s, hydrogels (Figure 4-6d). However, increasing the UV dosage also raises the risk of overexposure and

delamination between the layers, resulting in cracking and tearing of the hydrogel structure (Figure 4-4 and Figure 5-5).

Although the  $^1\text{H}$  NMR spectra of multilayer structures with varying QY photoabsorber concentration were analysed in the same study, the spectra of two other systems with constant QY concentration but different layer thicknesses were not examined. It is essential to study these multilayer systems, such as multi-37.5  $\mu\text{m}$ -3 s, multi-150  $\mu\text{m}$ -3 s, and multi-300  $\mu\text{m}$ -3 s, to determine the effect of layer thickness on cross-linking. It is assumed that samples with thinner layers will have a higher degree of cross-linking due to multiple passes of light, but it is unclear if the light can penetrate all the newly formed layers and reach the previously cross-linked layers. It is worth noting that this method may not provide a comprehensive understanding of how each parameter affects cross-linking in the layered structures, especially with changes in layer thickness. However, the results demonstrate that the concentration of QY photoabsorber and UV light dosage are crucial factors that can impact the degree of cross-linking in 3D multilayer PEGDA hydrogels.

#### **6.1.5 Effect of Number of Layers and Layer Thickness on Mechanical Behaviour of 3D PEGDA Hydrogel**

The results from the nanoindentation test conducted in Chapter 4 raise important questions about the effect of layer thickness on the behaviour of 3D PEGDA hydrogels. The unexpected finding that greater compliance was observed as the number of layers in a fixed height structure increased was attributed to the increased number of interfaces and higher defect density within the 3D printed structures (Figure 4-5). SEM images further revealed the presence of localised

regions of unreacted prepolymer, which appeared as pockets of voids and were believed to be the result of variations in defect density and lower modulus of elasticity at the interfaces within the multilayer hydrogels (Figure S4-4).

These findings have significant implications for the mechanical properties and potential applications of 3D printed hydrogels. Further investigations, such as conducting in-situ compression tests with fixed photoabsorber concentrations and varying layer thicknesses, may help to better understand the effect of layer thickness on the mechanical properties of these structures. It is also important to consider the size effect and how changes in layer thickness could potentially impact the mechanical behaviour of the structures.

In order to ensure long-term dimensional and mechanical stability for the intended application, it is crucial to carefully consider the selection of printing parameters such as layer thickness, UV exposure time, and the choice of molecular weight, monomer concentration, and amount of photoinitiator and photoabsorber in the prepolymer solution. These parameters must be optimised for not only the best printing resolution and accuracy, but also the long-term stability of the structures. The mechanical stability of 3D multilayer hydrogels is of utmost importance for their potential applications in areas such as tissue engineering, drug delivery systems, and soft robotics. A careful consideration of these parameters and their impact on the mechanical properties of the structures is necessary in order to realise the full potential of these materials. In the example of the 3D PEGDA hydrogel cantilever (Figure 6-6) it is suggested that as the degree of cross-linking (DOC) increases due to higher UV exposure or lower photoabsorber concentration, the force needed to deflect the cantilever (DF) to a specific

distance also increases (Figure 6-6a). An increase in the DOC of the cantilever results in increased stiffness, leading to an actual stiffness greater than the nominal stiffness. Therefore, for the same applied force a lower deflection may be exhibited in the higher cross-linked cantilevers. This concept can be applied to living tissue, where higher cross-linked cantilevers may be less deflective compared to lower cross-linked cantilevers (Figure 6-6b). This reduced deflection can lead to misinterpretation of the measured contractile force (CF) and the overall performance of the living tissue.

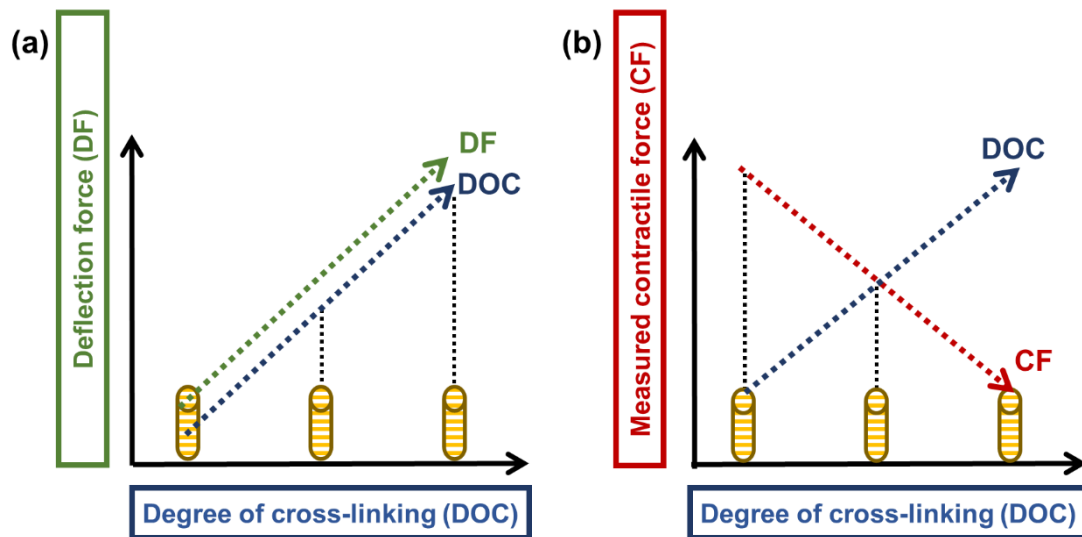


**Figure 6-6 Schematic showing the effect of degree of cross-linking (DOC) in 3D PEGDA cantilevers on (a) the force required to deflect the cantilever (DF) to a known distance and (b) the measured contractile force (CF) exerted by the tissue on the cantilever.**

#### **6.1.6 Implications of the Heterogeneity in Elastic Modulus of 3D PEGDA Hydrogel**

In the context of the study on the mechanical properties of 3D printed PEGDA hydrogels (in chapter 5) the fact that AFM results showed heterogeneity in the

elastic modulus across each printed layer and across the entire structure can be utilised in several ways. Firstly, the correlation between the degree of cross-linking and the material stiffness can be used to understand the effect of the surface nanomechanical behaviour of the hydrogels on cell adhesion. Cell adhesion is greatly influenced by the substrate's nanomechanical properties, which is critical for cell integration, migration, and attachment, ultimately affecting tissue formation and attachment to the substrate [38–41].

Furthermore, the sample preparation method, measurement strategy and quantification method used (in chapter 5) in mapping the surface nanomechanical properties of multilayer PEGDA hydrogel in a physiological relevant condition opens a pathway to spatially modulate the local stiffness of 3D printed multilayer biostructures. This new understanding into the surface nanomechanical behaviour of the 3D printed hydrogels can also be utilised in the contractile tissue studies using PEGDA cantilevers. There are two important components for successful tissue studies: tissue attachment on the cantilevers and its position on the cantilevers. By finding the ideal stiffness where cells best attach to the PEGDA hydrogels, cantilevers with similar stiffness can be fabricated, resulting in a robust attachment between cells and the cantilevers and higher success rates in tissue formation [21,42,43]. Additionally, directing cell attachment to a defined area on the cantilevers can help in the reproducibility of the tissues in a well-defined and well-positioned microenvironment.

This knowledge can help simplify the hydrogel cantilevers, eliminating the need for additional volume on top, mechanical cues, or markers, and enhance the contractile force measurements [21]. The ideal stiffness of the hydrogels can be



used to direct the cells to the top section of the cantilevers, which is also used to track the cantilever's deflection as a result of the contractile force exerted by the muscle tissue strip.

To attain consistent mechanical properties through uniform cross-linking, post-curing of 3D printed PEGDA hydrogel may be required. The hydrogel must be illuminated from all sides, including the top, bottom, and sides, to achieve homogeneous cross-linking [44]. Prior to post-curing, the samples should be washed multiple times to eliminate any trapped photoabsorber within the structure. It is recommended that the hydrogel turns transparent before post-curing to ensure that all parts of the structure receive sufficient light for complete cross-linking [45]. If the photoabsorber is not forced out, UV post-curing could result in a "candy shell" outcome where only a thin layer, comparable to the cross-linking depth of the specific photoabsorber concentration, is fully cross-linked on the exterior surface of the structure. Meanwhile, a gradient may occur where the centre of the structure may not even benefit from the post-curing process at all [44]. Moreover, during post-curing, caution should be exercised to avoid overexposure, which can damage the sample and alter its mechanical properties from those of the not post-cured 3D PEGDA hydrogel. In such cases, the physicochemical and mechanical properties of the post-cured 3D printed hydrogel must be thoroughly characterised. Additionally, printing parameters, such as UV light exposure, layer thickness, and prepolymer solution composition, may require adjustment to suit the desired application.

### **6.1.7 Nanomechanical Stability of 3D PEGDA Hydrogel**

In Chapter 4, it was found that the surface nanomechanical characteristics of the 3D PEGDA hydrogel showed stability within a month-long test, with observed changes within the standard error of the measurements (Figure 4-5b). This stability is crucial in the context of organ-on-chip devices, particularly when monitoring contractile forces in muscle tissue [46]. In order for such devices to be used for accurate and reliable long-term monitoring, it is important to confirm the stability of the mechanical properties of the supporting hydrogel structures [47]. This is essential for understanding and predicting the behaviour of the muscle tissue over time, as well as for troubleshooting when unexpected changes in behaviour occur. In the example of the contractile tissue studies, the stability of the mechanical properties of the hydrogel can help to differentiate between changes in the muscle tissue and changes in the support structures, such as the 3D printed PEGDA cantilevers. This new understanding is valuable not just for this specific study, but also for other organ-on-chip devices that rely on similar hydrogel support structures to monitor and study the behaviour of various tissues and organs.

### **6.1.8 Research Limitation and Remaining Research Gaps**

The research limitations and remaining research gaps in this study acknowledge the need for further research to expand our knowledge of physicochemical and mechanical properties of 3D multilayer PEGDA hydrogels. The study has focused on 3D printed PEGDA hydrogels fabricated using an in-house projection lithography system and monolithic samples fabricated using bulk cross-linking were used as control samples. Therefore, other LbL printing techniques such as

DLP or SLA need to be investigated to understand if there are any differences in the physicochemical and mechanical properties of the printed hydrogels depending on the fabrication process.

In terms of materials, the prepolymer solution was mostly kept constant, and only one variable, QY photoabsorber concentration, was changed. Therefore, the behaviour of multilayer PEGDA with different molecular weights and concentrations should be analysed. Alternatives to metal-based photoinitiators, such as LAP, should also be examined to find whether they can replace LAP and lead to a more homogeneous structure. The long-term stability of these hydrogels needs to be thoroughly studied, and longer than 30 days studies spanning months are required to establish the shelf life of the multilayer PEGDA after printing.

The current study used a constant CCM for all measurements, whereas changing the CCM every 12 hrs could mimic the same procedure while the multilayer hydrogel is used in tissue engineering and organ-on-chip devices. The behaviour of these hydrogels under different environments, such as temperature and CCM, needs to be thoroughly studied. Measuring the thermal response at more temperatures may help in identifying the critical gel transition temperature (CGTT) at which the hydrogel's backbone chain changes from being hydrophilic to hydrophobic.

Further investigation with a high number of cycles or frequency needs to be undertaken to fully understand the effect of temperature cycles on the degradation of the hydrogels. The degree of cross-linking of 3D multilayer

hydrogels should be quantified, and the mechanical behaviour of these hydrogels in their physiological environment should be established. Investigating the viscoelastic properties of 3D PEGDA hydrogels using nanoDMA is also necessary, as they demonstrate viscoelastic behaviour beyond their elastic properties.

Sophisticated experiments utilising techniques such as synchrotron X-ray may be pursued to understand the potential influence of residual stress that could develop during the printing of each layer. Investigating the cause of variation in cross-linking and mechanical properties across the entire LbL fabricated structure and changes that may be happening to the prepolymer solution while the structure is built would be useful. In-situ compression testing may be beneficial to understand the effect of layer thickness or number of layers on the bulk mechanical properties of the multilayer PEGDA hydrogels.

Increasing the sample size for nanoindentation and AFM measurements would increase confidence in the results, and environmental SEM may be required to look at the cross-section of hydrated samples to identify any voids and defects in their hydrated state in multilayer PEGDA hydrogels. Investigation of defects on the dried hydrogels should also include more samples to increase confidence in the acquired data. Finally, it is imperative to explore the potential of cutting-edge printing techniques like continuous liquid interface production (CLIP), fast hydrogel stereolithography printing (FLOAT) and computed axial lithography (CAL), which aim to completely eliminate the interface problem and promise improved resolution. These techniques must also be thoroughly studied to ensure uniformity in cross-linking and mechanical properties [48–50].

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# **Chapter 7**

## **Overall Conclusions, Recommendations for Future Work**

## 7.1 Overall Conclusions

The aim of this work has been to investigate and develop understanding around physicochemical and mechanical characteristics of 3D multilayer PEGDA hydrogels for contractile force measurements in muscle tissues fabricated using custom-made projection lithography printer. The study focused on gaining insight on how 3D PEGDA hydrogels behave at relevant physiological conditions and what are the effects of temperature, storing solution and printing parameters such as layer thickness, number of layers, UV light dosage and QY photoabsorber concentration have on the physical, chemical and mechanical behaviour of the hydrogels. A set of findings were published as journal papers and passed to the industrial partner in support of the thought and design process for future tissue contractile measurement device.

There are six key findings that require the attention of the reader.

1. The dimensional stability when the 3D printed structures are put in incubation conditions (temperature of 37 °C) has to be taken into account. It is suggested that printed structures are stored at 37 °C at least 24 hrs before cell seeding is done. This will allow the hydrogel to reach both gravimetric and volumetric equilibrium before cells are seeded.
2. The anisotropic dimensional change due to change in temperature from 20 to 37 °C has to be taken into account when designing the structures, as changes to the micro cantilever dimensions may well affect the amount force exerted by the tissue and the contractile performance together.

3. Based on the aging experiment and looking at the results from nanoindentation, the results strongly suggest that the hydrogels are mechanically stable within 30 days as the change observed is statistically insignificant. This finding is significantly important as cell culture and tissue studies are done within a 30 day period requiring dimensionally and mechanically stable support structures, so they do not interfere with the muscle tissue contractile force measurements.
4. The surface nanomechanical properties of the hydrogels are highly influenced by the number of layers and layer thickness as it was observed that an increase in number of layers (or reduction in layer thickness) from 1 to 8 and further to 60 results in a reduction in elastic modulus from 5.53 to 1.69 and further to 0.67 MPa, respectively. This reduction is attributed to mesoscale imperfections that appears as pockets of voids at the interfaces of the multilayer hydrogels attributed to localised regions of unreacted prepolymer resulting in variations in defect density in the samples examined.
5. Higher dosage of UV exposure ( $600 \text{ mJ/cm}^2$ ) leads to an increase in the degree of cross-linking suggesting that the initial UV dosage ( $120 \text{ mJ/cm}^2$ ) is insufficient for complete cross-linking. Therefore, post printing curing may become necessary with consideration of how it may affect overall stiffness of the 3D printed hydrogels.
6. The AFM measurements indicate that the multilayer PEGDA hydrogels produced by 3D printing exhibit mechanical inhomogeneity. Specifically, the  $E$  increases 4-fold, from 2.8 to 11.9 kPa, when moving from the bottom to the top of each layer. The inhomogeneity is also observed throughout the height

of the structure, with a 44% increase in  $E$  from 9.1 kPa at the bottom to 13.1 kPa at the top. These measurements suggest that the layers may not be fully integrated and fused together, as the interfaces appear to be the weakest points in the structure. The reported  $E$  at the interfaces (trough) was around  $3.2 \pm 0.7$  kPa, while furthest away from the interface (peak) it was reported to be  $11.8 \pm 1.4$  kPa.

## 7.2 Recommendations for Future Work

Future work for this PhD thesis should focus on several areas that will build upon the foundation laid for the development of organ-on-chip devices and the utilisation of 3D printed UV cross-linked PEGDA hydrogels.

1. Elimination of the interfaces: The objective is to eliminate interfaces in multilayer hydrogels by utilising new techniques such as CLIP and FLOAT, rather than physical layers. These techniques involve continuous printing where the sample holder rises slowly to enable the resin to flow underneath and maintain contact with the bottom of the sample. Similar geometries can be printed using this method, and the resulting samples can be analysed for their physical, chemical, and mechanical properties. Subsequently, the printing parameters can be optimised for use in organ-on-chip devices.
2. Analyse the physical, chemical, and mechanical properties of the printed samples using techniques such as ESEM imaging, FTIR, SAXS, in-situ compression testing, photo-rheology, photo-DSC and AFM.
3. Dimensional stability: Further investigation of the dimensional stability of 3D printed PEGDA hydrogels over a longer period of time, including studies on the effects of storage environment and potential bacterial growth.



4. Incorporation of functional materials: Developing and incorporating other functional materials into the hydrogels, such as nanoparticles and cells, to enhance the functionality and mimicry of human organs in organ-on-chip devices.
5. Study of layer thickness: Detailed analysis of the layered 3D printed PEGDA structures and the impact of layer thickness on cell adhesion, proliferation, and tissue formation. Understanding the relationship between surface elastic modulus and cell viability, as well as the optimal thickness for enhanced tissue adhesion, is important for improving the design of 3D structures for tissue engineering applications.
6. Nanomechanical characteristics: Obtaining more data for statistical significance on the nanomechanical characteristics of the 3D PEGDA hydrogels, including the effect of changing the prepolymer solution midway through printing, post-printing curing, and varying the layer thickness within the same structure.
7. Permeability and porosity: Investigating other characteristics of the PEGDA hydrogels, such as permeability and how the porosity within the structures changes by varying the printing parameters.
8. Increased sample size: Increasing the sample size in all experiments to remove any bias in the results and eliminate the batch-to-batch variations within the samples that may have had an influence on the obtained results.

Overall, this PhD thesis provides a solid foundation for further research and development in the field of organ-on-chip devices and the utilisation of 3D printed UV cross-linked PEGDA hydrogels.

### **7.3 Impact for Industry and Recommendations for the Sponsoring Company**

Action plan for the sponsor company includes the following:

1. Comparison and evaluation of different printing technologies:
  - Identify the two printing technologies that need to be compared, DLP and custom-made projection lithography.
  - Print samples with similar geometries using both methods.
  - Based on the results, optimise the printing parameters for use in organ-on-chip devices.
2. Dimensional stability of 3D printed PEGDA hydrogels:
  - Store the 3D printed hydrogels in different environments, such as controlled temperature and humidity, for a longer period of time.
  - Monitor the dimensional stability of the hydrogels over time using techniques such as AFM, nanoindentation and compression testing.
3. Incorporation of functional materials:
  - Identify the functional materials to be incorporated into the hydrogels, such as peg functionalised magnetic iron oxide nanoparticles and cells.
  - Determine the optimal methods for incorporating these materials into the hydrogels, such as physical mixing or chemical conjugation.
  - Analyse the effects of the functional materials on the hydrogels using techniques such as SEM imaging, X-ray diffraction, tensile testing, and FTIR spectroscopy.
  - Evaluate the ability of the hydrogels to mimic the functionality of human organs in organ-on-chip devices.

4. Impact of layer thickness on cell adhesion and tissue formation:
  - Print 3D structures with varying layer thicknesses using the 3D printing technology optimised in step 1.
  - Seed the structures with cells and monitor cell adhesion and proliferation over time.
  - Evaluate the ability of the cells to form tissue using techniques such as SEM imaging and histology.
  - Compare the results with the layer thickness to determine the optimal thickness for cell adhesion and tissue formation.
5. Surface elastic modulus and cell viability:
  - Print 3D structures with varying surface elastic moduli using the 3D printing technology optimised in step 1.
  - Seed the structures with cells and monitor cell viability over time.
  - Evaluate the relationship between the surface elastic modulus and cell viability.
  - Determine the optimal surface elastic modulus for cell viability and tissue formation.
6. Increase sample size and eliminate batch-to-batch variations:
  - Increase the number of samples for each experiment to increase the statistical confidence of the results.
  - Eliminate batch-to-batch variations by using standardised methods for sample preparation and analysis.
  - Consider repeating the experiments multiple times to confirm the results.

7. On-line/off-line measurement of mechanical characteristics of 3D printed PEGDA hydrogels for quality control

- Select a suitable measuring system from the recommended document in Appendix D to characterise bulk mechanical behaviour of the hydrogel and monitor the results for least 4 weeks
- Select 4-5 locations within the well plate to insert the monitoring samples and them in defined intervals.

It is recommended that the sponsor company considers the necessary resources, including personnel, equipment, and funding, to carry out these experiments. A timeline should also be established to ensure that the experiments are completed in a timely and efficient manner.

## APPENDICES

### Appendix A – Contributions from the PhD Candidate to the Papers

| Paper # | Candidate's contribution to papers                                                                                                                   | Additional author's contributions to papers                                                                                                                                                                                                                                                                                              |
|---------|------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1       | The candidate conducted the literature review and identified the gaps within the conducted study. The candidate was the primary author of the paper. | R.Zhang, S.Wilson, SA.Impey, Goel.S, Al.Aria – Supervised the research, and contributed to review the technical content of the paper.                                                                                                                                                                                                    |
| 2       | The candidate conducted the experimental work and analysis/optical imaging. The candidate was the primary author of the paper.                       | A.Afsar, conducted DSC. E.Dossi, conducted NMR and supervised and contributed to technical content. R.Zhang, S.Wilson, SA.Impey, Goel.S, Al.Aria – Supervised the research, and contributed to the technical content of the paper.                                                                                                       |
| 3       | The candidate conducted the experimental work and analysis/optical imaging. The candidate was the primary author of the paper.                       | C.J.Williams, conducted the nanoindentation. C.Micallef and F.Duarte-Martinez, conducted SEM imaging. A.Afsar, conducted DSC. E.Dossi, conducted NMR and supervised and contributed to technical content. R.Zhang, S.Wilson, SA.Impey, Goel.S, Al.Aria – Supervised the research, and contributed to the technical content of the paper. |

| <b>Paper #</b> | <b>Candidate's contribution to papers</b>                                                                                      | <b>Additional author's contributions to papers</b>                                                                                                                                                                                    |
|----------------|--------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 4              | The candidate conducted the experimental work and analysis/optical imaging. The candidate was the primary author of the paper. | V.Panchal and A. Dulebo, conducted the AFM measurements. S. Hawi, conducted data processing. R.Zhang, S.Wilson, E.Dossi, S.Goel., SA.Impey, Al.Aria – Supervised the research, and contributed to the technical content of the paper. |

## Appendix B – Overview of Papers

### B.1 Paper One

#### ***Additive Manufacturing and Physicomechanical Characteristics of PEGDA Hydrogels: Recent Advances and Perspective for Tissue Engineering***

|                                                    |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |
|----------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Question the paper set out to answer</b>        | <p>For 3D printed PEGDA hydrogels:</p> <ul style="list-style-type: none"> <li>• What are the current state-of-the-art printing techniques used?</li> <li>• What are the current understanding of the impact of 3D printing on the thermal, dimensional, chemical and mechanical behaviour?</li> <li>• What are the current applications within the tissue engineering domain?</li> <li>• What are the current gaps within the research community that require addressing?</li> </ul>                                                |
| <b>Data generated</b>                              | <p>In depth understanding of:</p> <ul style="list-style-type: none"> <li>• Current printing techniques and their advantages and limitations</li> <li>• Summary tables on swelling and mechanical behaviour of PEGDA hydrogels with focus on 3D printed PEGDA.</li> <li>• Research gaps in 3D printed PEGDA hydrogels</li> </ul>                                                                                                                                                                                                     |
| <b>Analytical investigations</b>                   | <ul style="list-style-type: none"> <li>• N/A</li> </ul>                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
| <b>Key findings and contributions to knowledge</b> | <p>For 3D multilayer PEGDA hydrogels:</p> <ul style="list-style-type: none"> <li>• 4 main printing techniques of fabrication</li> <li>• New state-of-the-art printing including CLIP and FLOAT that eliminate the interface effect in 3D printing hydrogels.</li> <li>• Research gaps including, thermal response, reversibility, isotropic behaviour, dimensional stability, layer-by-layer effect, postprinting curing, nanomechanical interface effect, printing parameters such as layer thickness where identified.</li> </ul> |

## B.2 Paper Two

### *Thermal response of multilayer UV cross-linked PEGDA hydrogels*

|                                                    |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
|----------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Question the paper set out to answer</b>        | <p>For 3D multilayer PEGDA hydrogels:</p> <ul style="list-style-type: none"> <li>• Does changing the storing temperature between 8, 20, 37 and 45 °C, influence the weight and dimensional stability?</li> <li>• Is the dimensional change isotropic or anisotropic?</li> <li>• What is effect of QY photoabsorber concentration on degree of cross-linking in the hydrogels?</li> </ul>                                                                                                                                                                                                                                                                                   |
| <b>Experimental testing and data generated</b>     | <p>Experimental studies were carried out by:</p> <ul style="list-style-type: none"> <li>• Making multilayer samples with 1.8 and 9 mg/mL of QY photoabsorber in the prepolymer solutions and used layer thicknesses 150 and 20 µm, respectively. Monolithic hydrogels were fabricated as controlled samples.</li> <li>• Gravimetric and volumetric methods were used to measure weight and volume change in the stored hydrogels for 7 days.</li> </ul>                                                                                                                                                                                                                    |
| <b>Analytical investigations</b>                   | <ul style="list-style-type: none"> <li>• Swelling test by monitoring weight and dimensions change of the hydrogels at different temperatures.</li> <li>• NMR for chemical analysis</li> <li>• SEM imaging and DSC analysis of samples.</li> </ul>                                                                                                                                                                                                                                                                                                                                                                                                                          |
| <b>Key findings and contributions to knowledge</b> | <p>For 3D multilayer PEGDA hydrogels:</p> <ul style="list-style-type: none"> <li>• It was observed that the volume increased to about 10% at a temperature of 8 °C, while their volume decreased by 8% and 12% when stored at 37 and 45 °C, respectively.</li> <li>• A reversibility behaviour at temperatures of 8–45 °C was observed.</li> <li>• An anisotropic characteristic were observed where the axial dimensional change was about 43% higher than the lateral dimension.</li> <li>• These findings suggest that differences in the degree of polymerisation and application temperatures have to be considered in the design and fabrication process.</li> </ul> |



## B.3 Paper Three

### *Nanoindentation Response of 3D Printed PEGDA Hydrogels in a Hydrated Environment*

|                                                    |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
|----------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Question the paper set out to answer</b>        | <p>For 3D multilayer PEGDA hydrogels:</p> <ul style="list-style-type: none"> <li>• How increasing number of layers and layers thickness can affect the nanomechanical and creep behaviour?</li> <li>• How does increasing the UV light dosage affect the degree of cross-linking and nanomechanical behaviour of the hydrogel samples?</li> <li>• Is there any aging effect observed for samples stored in their physiological environment for the duration of 720 hrs (30 days)?</li> </ul>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
| <b>Experimental testing and data generated</b>     | <p>Experimental studies were carried out by:</p> <ul style="list-style-type: none"> <li>• Varying the UV light dosage, number of layers and photoabsorber concentrations.</li> </ul>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |
| <b>Analytical investigations</b>                   | <ul style="list-style-type: none"> <li>• Nanoindentation for nanomechanical testing</li> <li>• NMR for chemical analysis</li> <li>• Swelling test by monitoring weight and dimension change of the hydrogels at different temperatures.</li> <li>• SEM imaging and DSC analysis of samples.</li> </ul>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| <b>Key findings and contributions to knowledge</b> | <p>For 3D multilayer PEGDA hydrogels:</p> <ul style="list-style-type: none"> <li>• Reduction of elastic modulus (<math>E</math>) for samples composed of 4 layers and other composed of 8 layers can be as high as -33%, while reducing the layers from 32 to 8 increases the <math>E</math> by +218%.</li> <li>• The creep index was also observed to be increasing with increasing number of layers.</li> <li>• Increasing the UV light dosage from 3 s, (120 mJ/cm<sup>2</sup>) to 15 s (600 mJ/cm<sup>2</sup>) can increase the <math>E</math> of 3D multilayer multi-20 <math>\mu</math>m by 96%.</li> <li>• Increased UV light exposure yields a higher conversion of the unsaturated chemical bonds and completion of cross-linking.</li> <li>• Samples showed heterogeneity and lower <math>E</math> due to interfacial defects, pockets of voids, presence of unreacted prepolymer and residue of LAP photoinitiator and QY photoabsorber within their polymer network.</li> <li>• No aging effect was observed for 30 days meaning the samples are nanomechanically stable.</li> <li>• The CCM storage solution did not have any adverse effect on the nanomechanical behaviour and thermal response of samples at 8, 20 and 37 °C.</li> </ul> |

## B.4 Paper Four

### *Mechanical Behaviour of 3D Printed Poly(ethylene glycol) Diacrylate Hydrogels in Hydrated Conditions Investigated Using Atomic Force Microscopy*

|                                                    |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
|----------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Question the paper set out to answer</b>        | For 3D multilayer PEGDA hydrogels: <ul style="list-style-type: none"><li>• What is the influence of layer-by-layer fabrication compared to monolithic PEGDA hydrogel in their native hydrated state?</li><li>• Are there variations in elastic modulus across the entire structure height?</li></ul>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |
| <b>Experimental testing and data generated</b>     | Experimental studies were carried out by: <ul style="list-style-type: none"><li>• Making multilayer samples and monolithic samples with and in absence of photoabsorber in the prepolymer solution and measuring the elastic modulus.</li></ul>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
| <b>Analytical investigations</b>                   | <ul style="list-style-type: none"><li>• AFM for nanomechanical testing on the outer surface of the printed hydrogels.</li></ul>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
| <b>Key findings and contributions to knowledge</b> | For 3D multilayer PEGDA hydrogels: <ul style="list-style-type: none"><li>• It was observed that the gradient in the elastic modulus in the printed layers of PEGDA hydrogel increases as moving upwards from the bottom to the top of every individual layer.</li><li>• The increase in <math>E</math> for a 3D multilayer hydrogel in each layer, at the middle of the printed structure, increased by 4-fold compared to the bottom section of the layer to the top section of the same layer.</li><li>• Our experiments revealed that layer-by-layer UV cross-linking process using projection lithography resulted in a gradient cross-linking density within each layer that is due to the spatial decay of the light intensity as the light propagates throughout the prepolymer, printing solution.</li><li>• There is a considerable difference in values from bottom to top of the structure. Even though the variations in <math>E</math> in the monolithic PEGDA sample were significantly higher than that of the multilayer sample, an increase of 44% in <math>E</math> for multilayer hydrogel across its height was considered quite high given it was assumed that the structure is mechanically homogeneous.</li></ul> |

## Appendix C – Other External Disseminations

### C.1 Conference Contributions

- *A system to reliably and reproducibly characterise the mechanical behaviour of a 3D manufactured hydrogel post structure for cardiac tissue engineering applications (poster presentation)*

Mohammad Hakim Khalili, Susan A. Impey, Saurav Goel

2019 EPSRC Centre for Doctoral Training in Ultra Precision Engineering Conference, Cranfield, UK, 17 June 2019.

- *Physicochemical and mechanical behaviour of micron size 3D printed PEGDA hydrogel cantilevers for cardiomyocyte study (oral presentation-winning best 3<sup>rd</sup> year presentation)*

Mohammad Hakim Khalili, Susan A. Impey, Sandra Wilson, Adrianus Indrat Aria, Saurav Goel

2021 EPSRC Centre for Doctoral Training in Ultra Precision Engineering Conference, Cranfield, UK, 2nd June 2021.

- *Physicochemical and mechanical behaviour of 3D printed PEGDA hydrogel structures for engineered contractile tissue studies (oral presentation)*

Mohammad Hakim Khalili, Rujing Zhang, Sandra Wilson, Saurav Goel, Adrianus Indrat Aria, Susan A. Impey

EUROMAT 2021 European Congress and Exhibition on Advanced Materials and Processes, Graz, Austria, 12-16. September 2021.

### C.2 Competition

- *Physicochemical and mechanical behaviour of 3D printed PEGDA hydrogels for engineered contractile tissue studies (oral presentation- winning people's choice vote)*

Mohammad Hakim Khalili

Cranfield Doctoral Network 3MT: 3 Minutes Thesis Competition 2022, Cranfield, UK, 17 June 2022.

## **Appendix D – In-House Projection Lithography System**

The custom-made projection lithography setup for 3D printing of PEGDA hydrogel samples is a complex and highly specialised machine. It is designed to produce high-quality, precise, and repeatable samples for various scientific experiments and applications (Figure D-1). The heart of the machine is the light exposure unit, which consists of a high-power UV LED (LuxiGen, LZ1-00UV0R, LED ENGINE) with a single wavelength of 365 nm which is mounted on a heat sink (ILA-HSINK-STAR-50X60MM-BLK-K, RS). The LED is capable of emitting intense and consistent light, making it ideal for photopolymerisation of the PEGDA hydrogel resin. The LED is controlled by custom LabView code written by the in-house scientists, which synchronises the exposure light with the movement of the fabrication stage. This automated process allows for greater control and flexibility in adjusting various experimental parameters such as layer thickness and UV exposure time. The fabrication stage is a motorised linear stage with 50 mm travel stepper motors (KMTS50E/M, Thorlabs, US) that moves vertically, allowing for precise control of the structure's height and thickness. The movement of the stage is controlled by a K-Cube DC Servo Controller (KDC101, Thorlabs, US) and is synchronised with the light exposure, ensuring that each layer is exposed to the UV light for the correct amount of time.

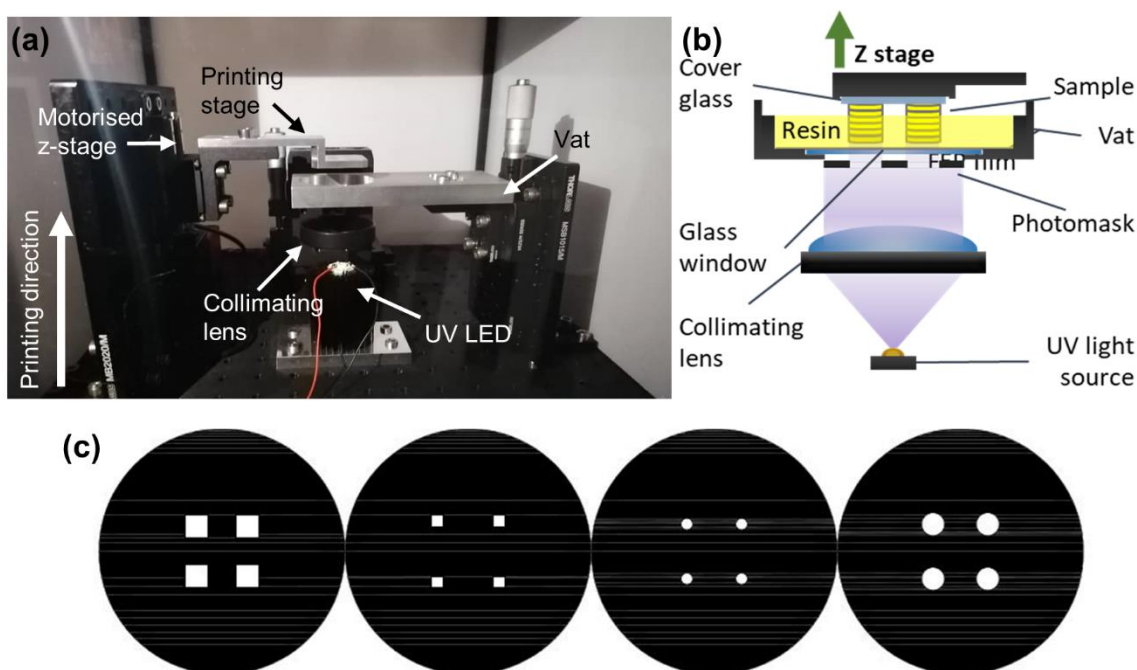


**Figure D-1 (a) Photography and (b) schematic of the custom-made projection lithography printing setup. (c) Examples of the photomasks used for fabricating cuboid and cylinder shape hydrogels.**

The photoresist masks (Laser Photoplot Film, 10k dpi resolution, JDphotodata) made from Idealine CPF2 film with 0.18 mm thickness which has a photographic emulsion coated onto one side. The photoresist mask used in the process are designed using Autodesk AutoCAD, a popular and versatile software for computer-aided design. The masks are used to control the exposure of the resin to the UV light, resulting in a well-defined and controlled pattern of hydrogel. The resin used in the process is an aqueous mixture of PEGDA, LAP, and QY, which are carefully mixed to the correct concentration before being added to the vat. The vat is covered with a non-adhesive fluorinated ethylene propylene (FEP) foil, which facilitates the release of each printed layer from the vat and helps to maintain the integrity of the structure.

After setting up the experimental parameters, the process begins with the addition of the resin to the vat and the projection of the mask onto the resin. The fabrication stage is then raised by a set step height for each additional layer exposure. After printing is complete, the object is washed with DIW and detached from the cover glass using a razor blade. The finished object is then immersed in either DIW or CCM, depending on the intended experiment.

### **D.1 Pre-Treatment of Glass Slides**

The process of fabricating 3D PEGDA hydrogel samples using custom-made projection lithography involves several steps to ensure the successful printing of the hydrogel onto the desired substrate. Prior to printing, the microscope slides (25 x 25 x 1 mm squares VMR) are treated to secure the printed PEGDA to the glass support. The treatment involves cleaning the glass surface with a plasma treatment (50W with 3 repeats of 2.5 mins in a barrel etcher) to remove organic contaminants, followed by soaking in a 2% v/v 3-(trimethoxysilyl)propyl methacrylate solution in 98% v/v ethanol/water for 10 mins. The slides are then washed with pure ethanol and dried at 105 °C for 10 mins on a hot plate. These pre-treated slides provide an ideal substrate for the printing process, enabling easy handling of the printed hydrogel samples.

### **D.2 Intensity Uniformity across Printing Platform**

To ensure uniform cross-linking in the printed hydrogel samples, it was important to maintain a uniform intensity of UV light across the printing platform. The printing area was determined to have a diameter of 22 mm (Figure D-2). An aspheric condenser lens with diffuser (ACL5040U-DG15-A, Thorlabs) was

installed between the LED light and the printing vat to collimate the light and prevent the reflection of the internal structure of the LED from affecting the hydrogel structure. The position of the lens was adjusted using a XYZ travel translation stage (PT3, Thorlabs) to ensure proper alignment. The intensity of the UV light was measured at the bottom of the vat using a calibrated hand-held intensity meter (Digital UV AB Light meter, UV513AB, GENERAL) set at a wavelength of 365 nm. A uniform intensity with less than 5% variation between the centre and the edges of the printing area was crucial for uniform cross-linking of the hydrogel and uniformity in the printed sample.

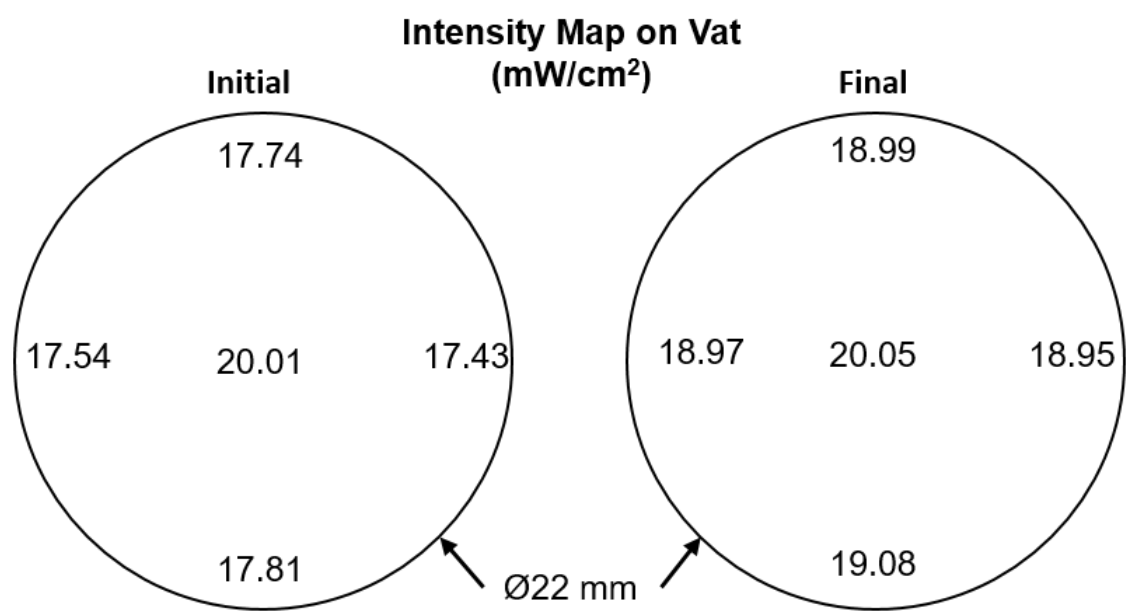


**Figure D-2 Light intensity measurements using intensity meter to achieve highest uniformity across the centre of the printing platform.**

### **D.3 Applying Non-Adhesive Layer to the Vat Screen**

To avoid having the printed hydrogel sample stick to the bottom of the printing vat instead of the cover slip, a non-adhesive layer or film was necessary. Initially, a 2 mm thick layer of PDMS was applied to the bottom of the vat, but this resulted

in hazy spots on the PDMS layer after printing, causing failure in the subsequent prints (Figure D-3c). To remedy this, Direct Reactive Ion Etching (DRIE) was attempted using a thin layer of carbon tetrafluoride ( $\text{CF}_4$ ), but this did not adhere to the glass surface and came off during washing (Figure D-3d). Next, a 150  $\mu\text{m}$  thick layer of FEP film was secured to the ottom of the vat, subsequently resulted in smooth release of each formed layer and improved attachment between layers and eliminated leaks, too (Figure D-3a,b).

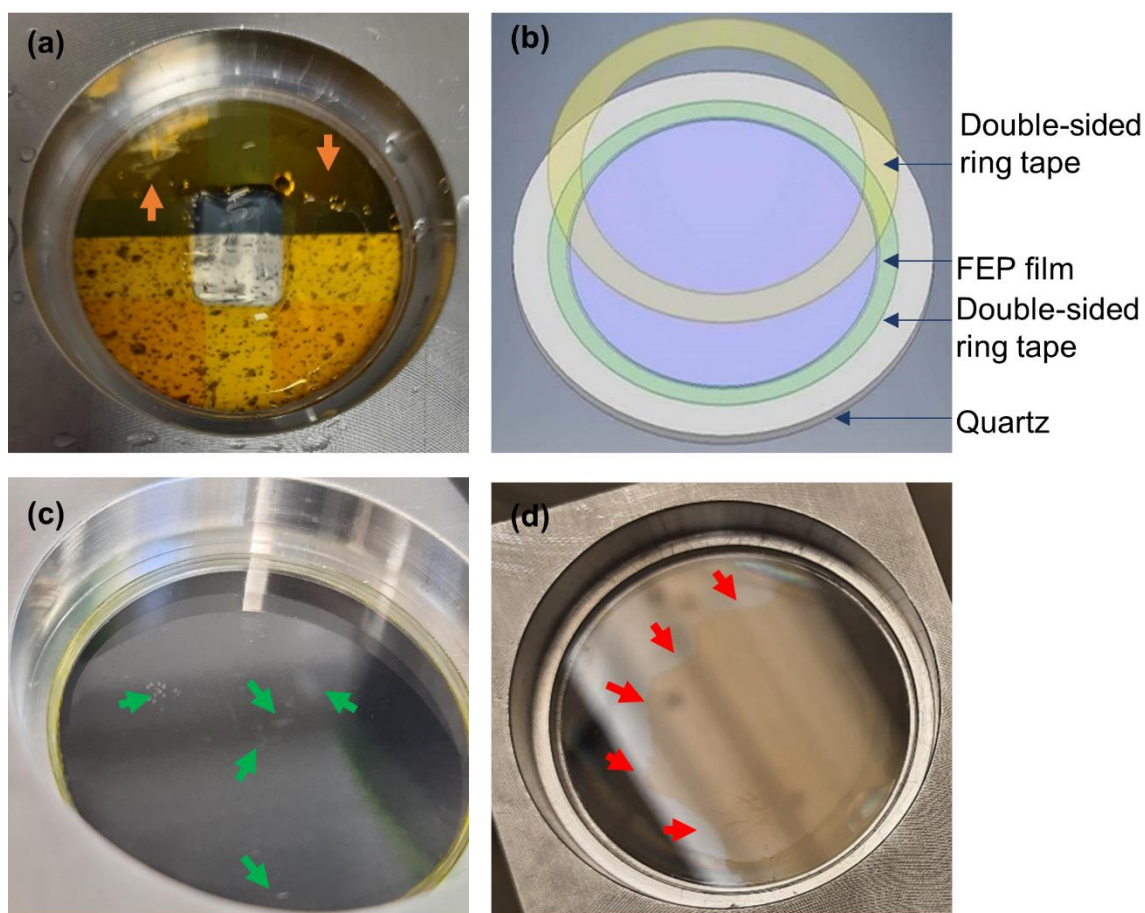


**Figure D-3 Unsuccessful attempts to apply a non-stick surface to the screen of the vat. (a) Fixing FEP film with Kapton tape resulted in leaks (orange arrows) beneath the Kapton tape during printing, (b) securing the FEP film by using ring shaped double sided tapes with different diameters. (c) Applying a 2 mm thick PDMS layer on the screen, white hazy spots (green arrows) appeared after single print of 15**



mins. (d) Applying a thin layer of CF<sub>4</sub> (red arrows) on the screen using DRIE, which was washed out after cleaning.

The final solution involved modifying the vat to include a ring-shaped vat cap with a silicone O-ring that mechanically secured the FEP film between the vat cap and the vat's screen, resulting in a finely bow-shaped film at the bottom of the vat that improved attachment between printed layers and eliminated leaks (Figure D-4).

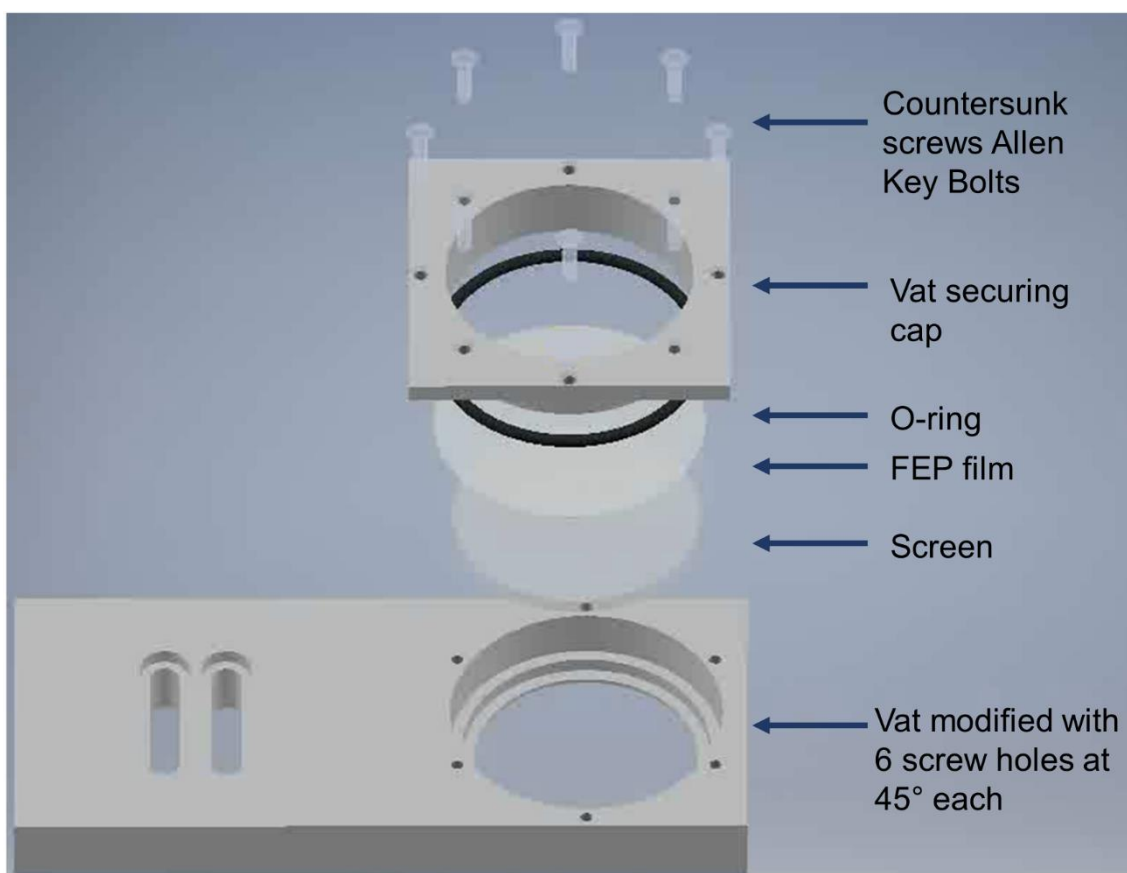


**Figure D-4 Schematic of the modified vat with O-ring fitting for securing the FEP in place.**

#### **D.4 Components of the Prepolymer Solution (Resin)**

A water soluble poly(ethylene glycol) diacrylate (PEGDA; Merck 455008, Mn 700 g/mol) with low molecular weight was selected due to its ability to create

mechanically stable hydrogels with low cytotoxicity, as found in multiple research studies. Additionally, this PEGDA is more cost-effective for large-scale production using SLA, as it is cheaper compared to its high molecular weight analogs. The Lithium phenyl-2,4,6-trimethylbenzoylphosphine photoinitiator (LAP; Merck 900889,  $\geq 95\%$ ) is a water-soluble and cytocompatible photoinitiator that weakly absorbs in the visible range (400-420 nm) and has been widely used for cell encapsulation in photo-polymerisable hydrogels. It is chosen as the photoinitiator for this study and is commercially available. The Quinoline Yellow photoabsorber (QY; Merck 309052), a commonly used food dye, was selected for its high absorption at 405 nm, excellent water solubility, and low cytotoxicity. It was used to control light penetration and improve the optical Z resolution of the printer. The concentrations of the pre-polymer solution components (200 mg/mL PEGDA, 5 mg/mL LAP, and 9 mg/mL QY) were optimised in a previous study to fabricate 3D-printed perfusable microfluidic channels with an internal diameter of 100  $\mu\text{m}$ . This was done to meet the desired printing resolution for the internal open channel cavities.

## **D.5 QY Photoabsorber UV-VIS Attenuation Study**

Printing a centimeter-long PEGDA hydrogel is a long process, taking around two hrs with the existing setup, so the concentration of the QY photoabsorber was reduced and layer thickness increased. The transmission at 365 nm of diluted QY concentrations (from 9 mg/mL to 0.09 mg/mL) was measured. The UV-Vis spectra showed little difference between the transmissions of 9, 4.5, 1.8 and 1.35 mg/mL, suggesting that the system's resolution might not be sufficient to detect differences at those concentrations (Figure D-5). Therefore, pre-polymer

solutions with various QY concentrations were prepared and used to determine the curing depth at each concentration.

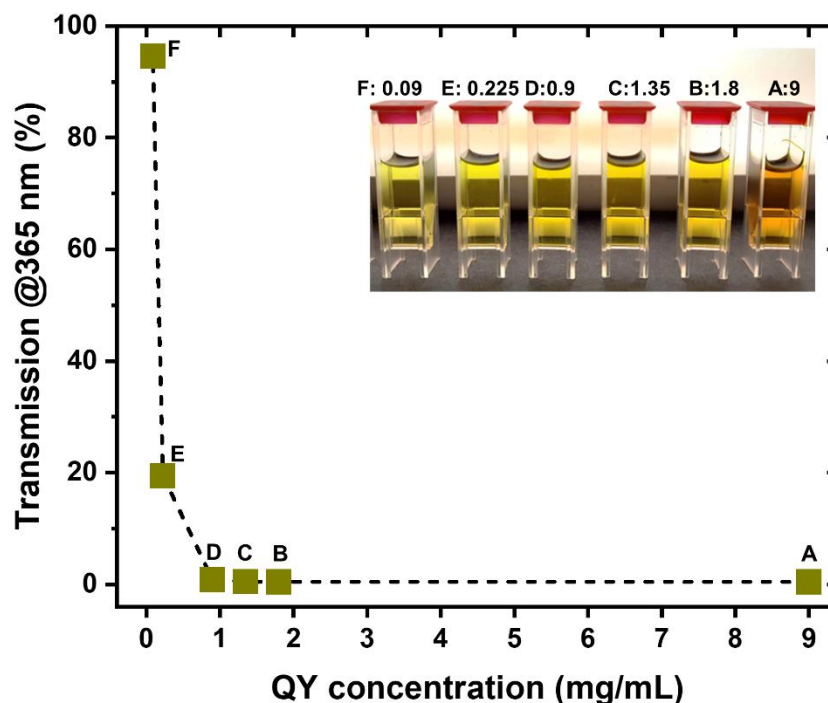


**Figure D-5** Transmission data captured through UV-VIS spectroscopy for PEGDA solutions with various QY photoabsorber concentrations on a background of PEGDA solution.

## D.6 Layer Thickness Calibration (Axial Resolution)

Preparation of the prepolymer solutions with varying concentrations of the QY photoabsorber was carried out. A coverslip glass was treated and placed at the bottom of the vat. The prepared prepolymer solution was then poured into the vat, filling nearly half of its volume. The prepolymer solution was exposed to UV light with a dose of  $120 \text{ mJ/cm}^2$  for 3 seconds. The resulting cross-linked hydrogel layer was covalently bonded to the coverslip glass. The thickness of the cross-linked layer was measured using digital vernier caliper, serving as an indicator of the cross-linking depth for each concentration (Figure D-6).

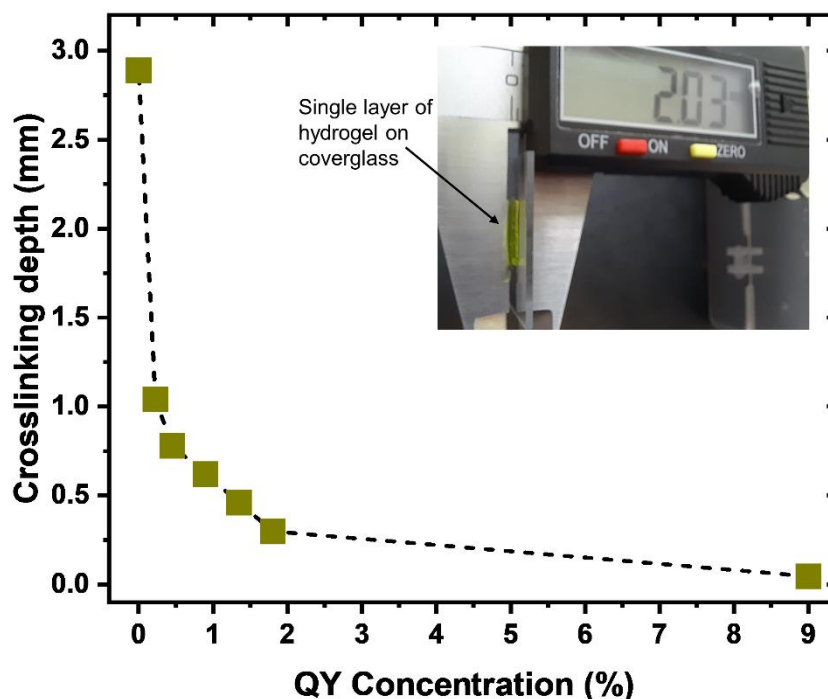


**Figure D-6** Cross-linking depth measured for different concentrations of QY photoabsorber measured physically by a digital Vernier calliper showing increased depth as QY concentration reduces.

## **D.7 Measuring Intensity Attenuation on Different Layer Thicknesses**

Understanding how the intensity attenuate as the light passes through a single cross-linked layer of PEGDA hydrogel with different QY concentration is important as it forms the bases for understanding how deep the light can travel in an already cross-linked layer in the printing process. This will form the basic understanding of double exposure in every layer and how far the light can penetrate within the formed layer. In order to do this, initially solutions with different QY concentrations were prepared. The solutions were poured onto a coverglass and exposed to 3 s of UV light. Then, the intensity was measured through the exposed layer by placing the intensity meter on top of the cross-linked layer and exposing it to 3s of UV light (Figure D-7).

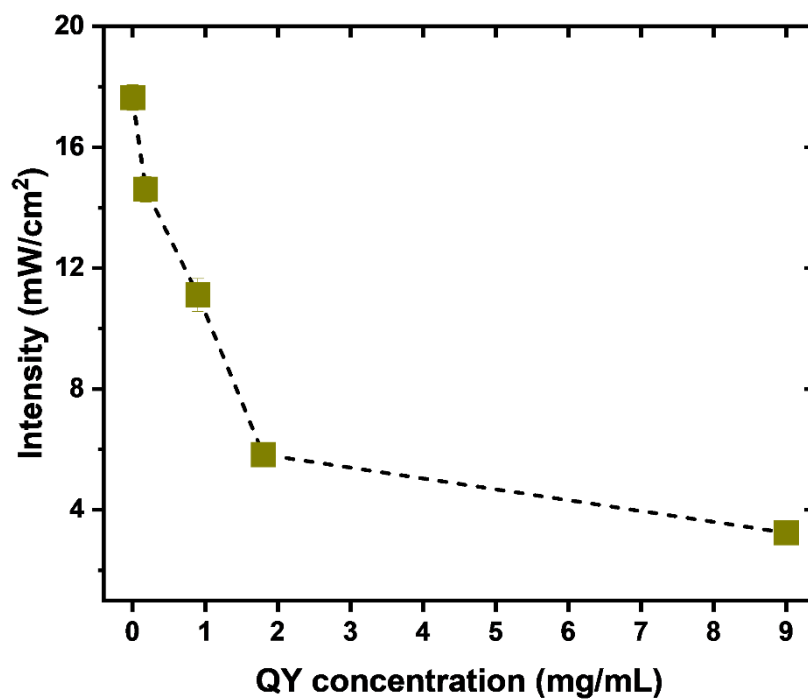


Figure D-7 Light intensity measured through single layer hydrogels prepared with different concentrations of QY photoabsorber measured by the intensity meter, showing increased intensity as the QY concentration reduces.

## **Appendix E – Measurement Devices for Mechanical Characterisation of 3D PEGDA Microcantilevers**

The production of 3D printed microcantilevers made of PEGDA hydrogel is performed using DLP technology. These microcantilevers serve as a platform for testing the contraction force generated by engineered contractile tissues. The microcantilevers are pulled inward by the contracting tissue, and their deflection can be monitored and measured to determine the applied force using the Euler-Bernoulli equation, assuming small deflection and a constant elastic modulus [1].

To accurately quantify the contraction force, it is crucial to understand the mechanical properties of the microcantilever. In particular, the elastic modulus of the material, which governs the stiffness of the structure, must be studied in detail. The elastic modulus of the PEGDA hydrogel can be influenced by various physical and chemical factors, such as the shape and dimensions of the cantilever, and the concentration of the backbone material in the hydrogel. Environmental changes such as temperature, humidity, and pH level can also affect the elastic properties of the hydrogel.

The target shape of the microcantilever is cylindrical, with a diameter of 150-200  $\mu\text{m}$  and a height of 800-1000  $\mu\text{m}$ . The aim is to establish a reliable and reproducible system for characterising the mechanical behaviour of 3D printed PEGDA hydrogel microcantilevers. A variety of measurement tools and techniques will need to be identified, evaluated, and tested to determine their suitability for the intended application.

One significant challenge in this endeavor is the need to keep the microcantilever hydrated to prevent degradation and disintegration. This restriction limits the number of measurement techniques and technologies that can be used, but also presents an interesting challenge to overcome. Several techniques and instruments are being investigated, including Optical Coherence Tomography, Laser Doppler Vibrometry, Force Sensing Techniques, and Atomic Force Microscopy.

### **E.1 Optical Coherence Tomography**

Optical Coherence Tomography (OCT) is an optical imaging technique that provides real-time video rate images in 2D or 3D of biological tissues in a microscale (Figure E-1) [2]. It uses the power of light, Infrared light, to penetrate through the sample and create light interference which form an image of the structure. The technique is same as in Michelson interferometer with a difference that the fixed mirror is replaced by a sample. By varying the position of the scanning mirror, it is possible to determine the depth from where the reflection originates and determine the thickness of the sample. The 2D image is constructed by moving the beam across the sample and acquiring data. And by translating the beam in two directions over the sample's surface a 3D image can be acquired [3].

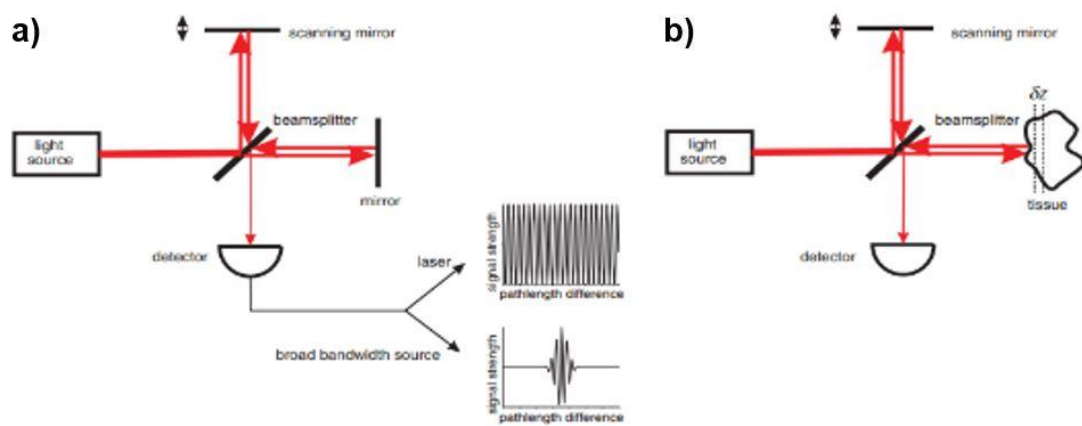


**Figure E-1 a) Michelson interferometer. b) Michelson interferometer with the fixed mirror replaced by a sample [3].**

The possibility of imaging a 3D printed hydrogel beam structure using OCT was investigated. The beam structure is composed of two cylindrical cantilevers with dimension of 150  $\mu\text{m}$  diameter and 850  $\mu\text{m}$  height. The beam itself is 1764  $\mu\text{m}$  length, 175  $\mu\text{m}$  width and 75  $\mu\text{m}$  thickness (Figure E-2).

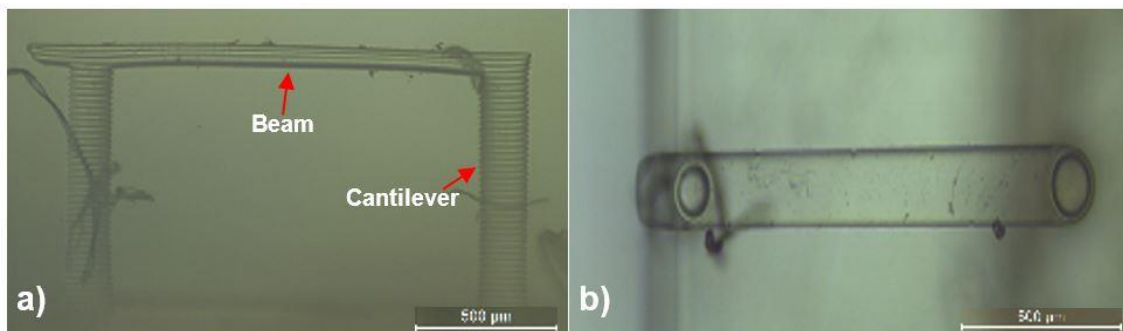


**Figure E-2 Optical microscopic image of 3D printed beam structure in DI water, (a) side-view, (b) top-view.**

The wavelength of the laser is 890 nm with approximately 100 nm bandwidth. The scanning rate is 8 frames per second and the frequency is 20 kHz. For every image 10 images are averaged to increase the accuracy of the system. Figure E-3 shows a sketch of the OCT system while scanning the beam structure.



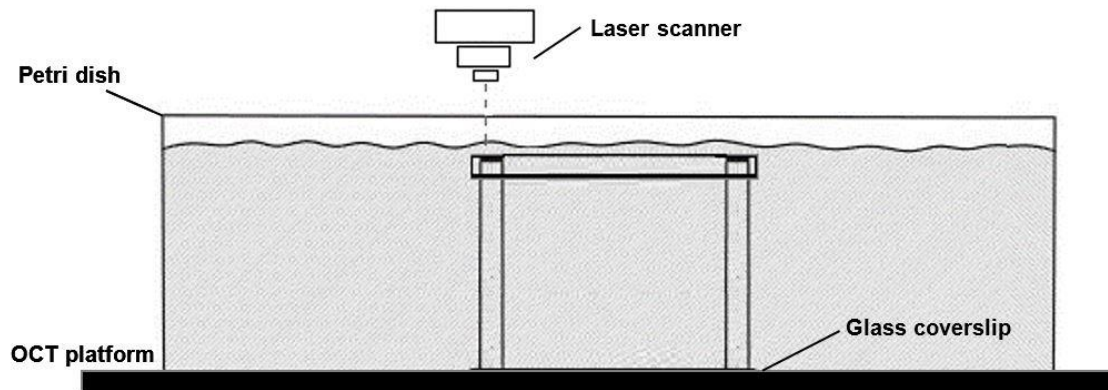


**Figure E-3 Sketch of the OCT system setup for imaging hydrogel beam structure.**

The presence of water effects the intensity of the laser light, by absorbing some of the light. To overcome this issue, a laser with longer coherence light, which can be tuned to have much straighter shape to improve depth of field, is required. On the other hand, longer coherence light will reduce the system resolution. Therefore, it is a trade-off between depth of field and resolution which should be considered. To reduce the effect of light absorption by water, without exposing the sample to air, the water level was reduced until it reached the surface of the beam. This has helped in locating the sample more easily and getting clearer images from the system. Figure E-4a is generated when the light is focused on top of the sample which resulted in not having a clear detail of the bottom part of the structure. Due to light interference with the water, petri dish and the coverslip multiple reflection of the structure is appearing in the captured image. In Figure E-4b shows that the captured image does not really show the true dimension of the beam structure. This might be due to misalignment of the laser beam with the structure which results in not having the normal incident angle [4]. Notice in the image Figure E-4c there are multiple white spots on the beam structure which can only confirm that the sample is nonhomogeneous.

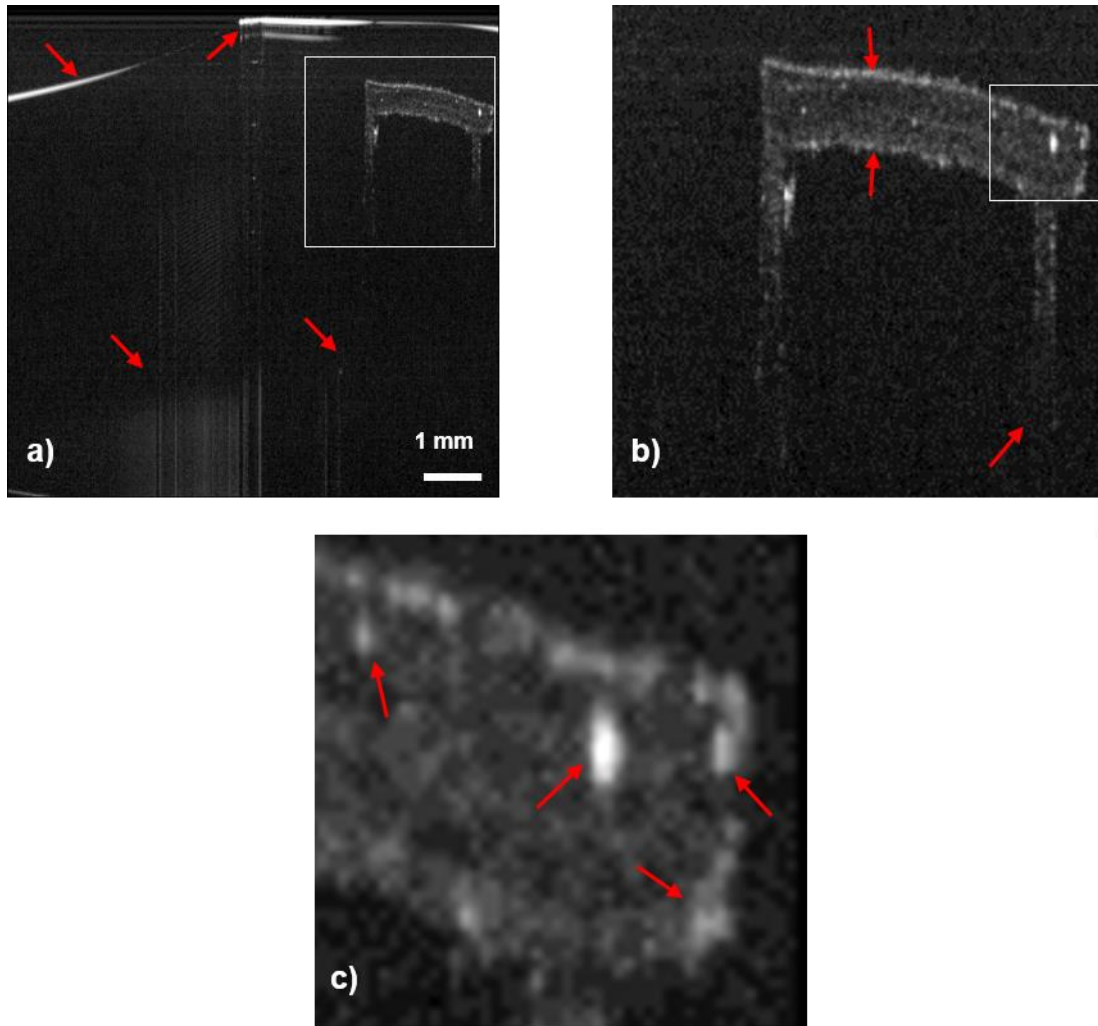


**Figure E-4 Images acquired from the OCT of hydrogel beam structure in water. (a) Multiple reflections of light at different interfaces. (b) The width of the beam is stretched in one direction and squeezed on the other direction. The cantilever is also looking stretched. (c) White spots at multiple places can only confirm material inhomogeneity.**

*Advantages:*

From the acquired images, it is obvious that the system can provide some information about the internal architecture of the hydrogel such as volume porosity, pore size quantification and possibly some information about the 3D layer interfaces and connectivity. It can also be used to image the cantilever

structure and provide deflection measurements while the cantilever is poked or vibrated in a non-contact mechanism and live.

#### *Disadvantages:*

The real-time video rate resolution of the system is between 8-10  $\mu\text{m}$  in the axial and transverse direction [2,5,6]. Imaging resolution of 2  $\mu\text{m}$  is also reported in the literature [7]. However, such resolution could potentially be a limiting factor in its ability to capture features than 1  $\mu\text{m}$  such as small pores and scaffold interconnectivity. For imaging cantilever structures while poked or under vibration, a high frame rate is required to be able to capture the smallest cantilever deflections. To use the system for quantitative characterisation that will also require few modifications to the existing system such as new laser source and new data acquisition programme. It will also require optimisation of depth of focus, which is governed by the focal spot size for transverse resolution, and coherence length of the light source for axial resolution [7]. That will enable measurement of volumetric porosity, interconnectivity and pore size. Another issue is that due to similarity in Refractive Index (RI) of both water and the hydrogel sample, it is challenging to locate and focus the laser light. That also creates a challenge for the laser light to be able to distinguish between both. Therefore, a way to change the RI of either hydrogel or water will be required.

## **E.2 Laser Doppler Vibrometry**

Laser Doppler Vibrometry (LDV) or Laser Point Vibrometry (LPV) is another technique which has been investigated for structural vibrations measurements. The vibration measurements are characterised in terms of its modal properties

which are the temporal (natural frequencies) and spatial (mode shapes) [8]. So basically, performing a modal analysis using LDV system. This is possible by applying and measuring an excitation force on the structure, and then measuring the response at various points. By doing so, the natural frequencies of the structure can be extracted from the response measurement at a single point. For identifying the modal shapes, the response is measured rapidly with spatial resolution limited to the laser beam diameter and the time required to capture each time frequency response function (FRF). This can then be used to fit the data to a polynomial curve to get the order of the deflection, or mode shape [8]. Using the measured resonance frequency elastic property, elastic modulus of a structure can be derived.

### **E.2.1 LDV Polytech**

For this experiment, a Polytech PSV-500 was used to only measure the resonance frequency at the first mode. The scanning head consists of a HeNe laser source with a wavelength of 633 nm [9]. The spot size is reported to be 50  $\mu\text{m}$ . The excitation source was a loudspeaker which was generating white noise and placed as close as possible to the petri dish containing the 3D printed cantilever, with 150  $\mu\text{m}$  diameter and 850  $\mu\text{m}$  height. The cantilevers also had a 3D printed protective wall around them to reduce the chance of breakage during transport (Figure E-5).

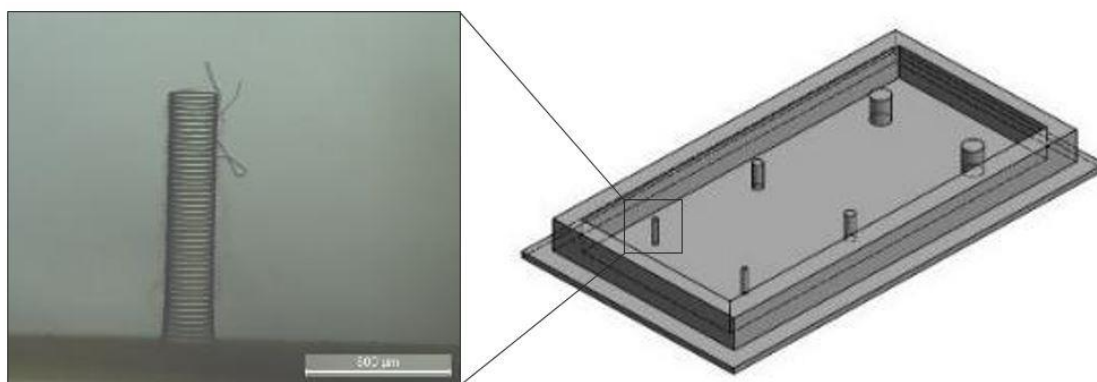


**Figure E-5 Drawing and microscopic image of the 3D printed hydrogel cantilevers.**

Due to similarity in RI of both water and the sample, it was challenging to locate and focus the laser light on the cantilever. Hence, it was decided to shine the laser on the 3D printed wall rather than the cantilever as it is bigger and can be found by the scanning head. To do so, the laser beam was directed through the petri dish's side wall and focused perpendicularly on to the 3D printed wall.

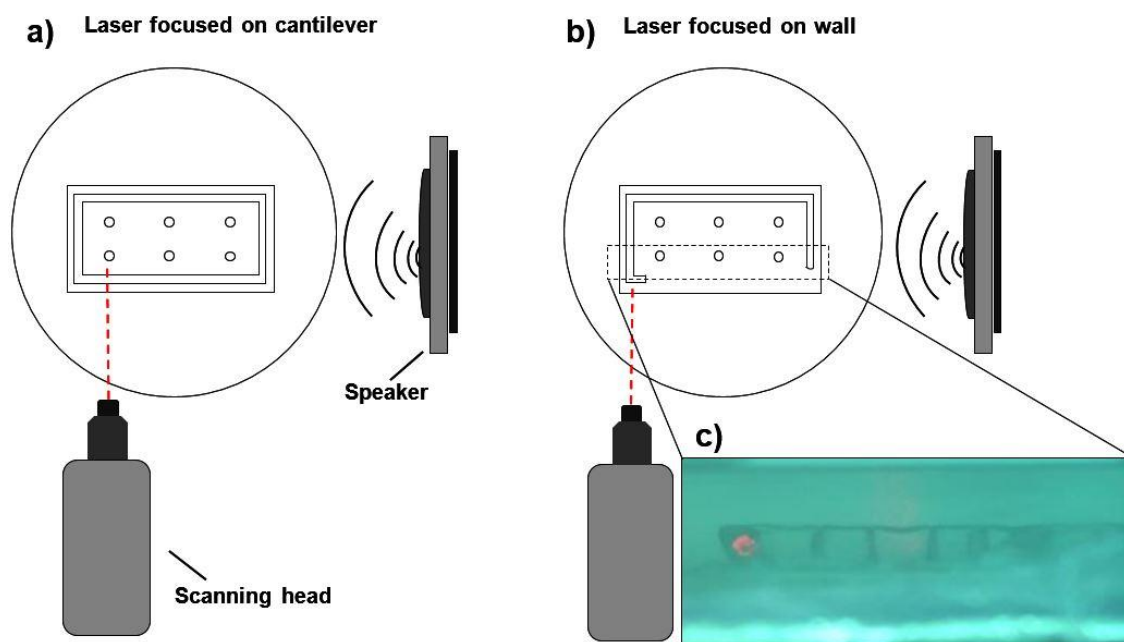


**Figure E-6 Illustration of LDV setup for measuring cantilever resonance frequency.**

The laser beam is focused on a) the cantilever. b) the wall. c) Image taken using

**the scanning head showing the side view of the sample while the laser beam is focused on the wall.**

The result showed that the frequency of the first resonance mode occurred at 971 Hz (Figure E-6). Such a high resonance frequency for the first mode for such small cantilever made of polymeric gel is surely doubtful. A thin layer of poly(vinyl alcohol)-poly(sodium acrylate) composite hydrogel (PVA-PAA gel) with thickness of 0.41 mm and elastic modulus of 1.16 MPa showed a resonance frequency of 1-2 Hz [10]. And a 2 mm thin polyacrylamide droplet shape gel with shear modulus of 0.055 kPa had a resonance frequency of between 51-182 Hz, first to third mode [11].

Therefore, it was concluded that the recorded frequency response from the system does not represent the resonance frequency of the hydrogel. One potential reason for this could be that passing the light through the petri dish wall has affected the results. And the light might have been detecting the vibrations generated from the petri dish rather than the sample. Having a sample which has a very much similar RI to the water and been transparent adds to the complexity of the problem.

### **E.2.2 LDV SmarAct PICOSCALE**

SmarAct PICOSCALE vibrometer was used to investigate the resonance frequency of cantilever structures. The measurements consist of investigating the mechanical behaviour of printed hydrogel cantilevers. A thin rectangular layer (14 x 8 x 0.2 mm) of hydrogel is first printed on a glass coverslip (with dimension of 20 x 20 mm). Then six cantilevers are printed on top which have exact same

height but different diameter (150, 300 and 600  $\mu\text{m}$ ). There is a hydrogel wall printed around the cantilevers to minimise the risk of water flow damaging the cantilevers during transport (Figure E-5).

All measurements were performed with a standard PICOSCALE Vibrometer and standard sensor head ( $<5\text{ }\mu\text{m}$  optical resolution) with laser wavelength of 1550 nm. Initially, all measurements were performed in water. The amount of water in the dish was adjusted to allow the investigation of the sample: roughly 5 mm is required between sample and sensor head. At first, a reflective tape was added next to the sample to allow a fast adjustment of the device. This also allowed to find a focal plane roughly similar to where the sample was. Next a preliminary image on top of the sample allowed to identify roughly the region of interest.

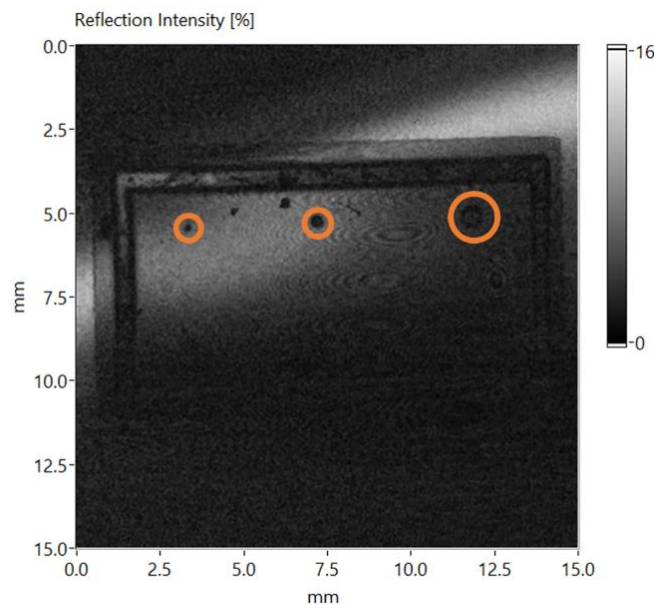


**Figure E-7 Zoom on the image taken at 1200  $\mu\text{m}$ . Three black areas, the cantilevers, can be seen, red circles. The cantilevers appear dark either because the image taken is not recorded at the optimal focal plane, or because they are invisible for the laser.**

The cantilever appears completely dark and a Z-position versus Reflection curve did not show any interfaces visible (Figure E-7). Considering that the cantilevers are supposed to be 850  $\mu\text{m}$  long and knowing that the coverslip is located at approx. 1200  $\mu\text{m}$ , it is possible that the top of the cantilever was too close to the water surface, which dominates the response, to be seen. The system was adjusted while the sample was immersed in water. The adjustment was performed directly at the water surface. Because the hydrogel is 80% water, it will reflect at least as much as the water itself so readjusting the system is later not necessary. After successful adjustment, the water is slowly removed using pipette until the sample stick out from the liquid. Then, coarse imaging (low resolution) is performed to locate the cantilevers. Later, the proper focal plane is found after performing an automatic routine (recording the laser beam intensity while scanning in the z direction). Finally, the cantilevers are imaged again with higher resolution. The scan area is 1x1 mm with 500x500 pixels.

After imaging, the laser beam is positioned at the centre of the cantilever top surface (Figure E-8). Vibrations using a piezoelectric shaker are induced while the displacement signal from the cantilevers are recorded. The excitation signal was a linear chirp of constant amplitude (maximum amplitude in this case) and frequency range from 1 Hz to 5 kHz. Only the high-resolution images are shown, left. The corresponding vibration spectrum is displayed on the right.



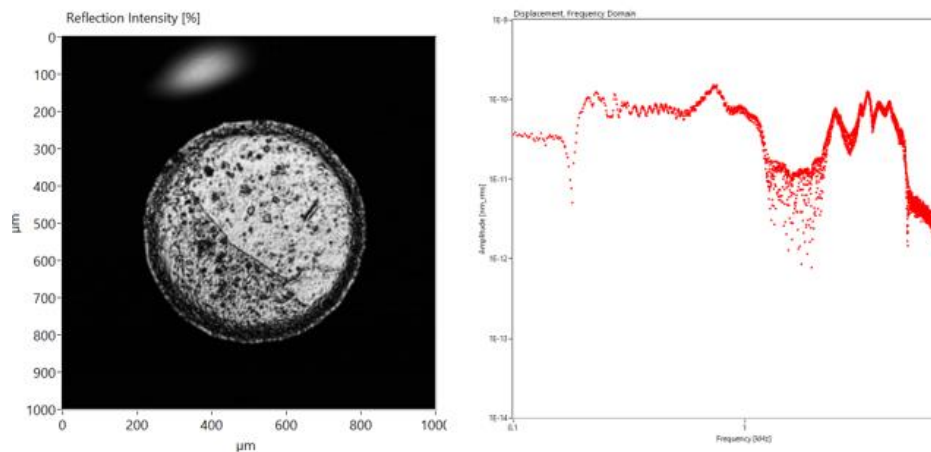


**Figure E-8 Left, High resolution image of the 600  $\mu\text{m}$  diameter cantilever. Right, corresponding vibration spectrum.**

*Advantages:*

The LDV can be used to measure the resonance frequency of hydrogel and subsequently extract the elastic properties of the hydrogel. It can be used to investigate the mechanical properties in a non-contact way. And it can be used in different shapes including cantilevers or beam structures. And it can possibly be used to measure the mechanical response of a structure under different environmental and mechanical (vibrational) conditions and investigate their effect in short and long term non-destructively. It can also be used for measurements on micron size structures.

*Disadvantages:*

RI similarity between water and hydrogel creates a big challenge for this type of measurement. It has proven to be challenging to resolve and distinguish between the sample and the water. And either water elimination or change in refractive index is required. To overcome the issue of RI similarities, nanoparticles such as

gold or silver particles could be used in the gel mix and tested. The nanoparticles can potentially change the RI of the gel and create a good contrast to perform the measurements in water. If it works for larger samples with basic shapes such as a disk or cube, then it can be extended and used on cantilever shapes to find the resonance frequency and elastic properties thereafter.

Another issue is the complexity in modelling systems with presence of water element. Creating a representative model that matches or correlate with experimental data is challenging.

The use of correct contact mechanisms, boundary conditions and understanding viscoelasticity parameters of the candidate material is very important. For estimation using analytical solutions it is also important to use equations that are representing the soft viscoelastic system rather than using a simple equation for solid beams.

### **E.3 Micromanipulator Force/Deflection Measurements**

Kleindiek micromanipulator (MM3A-LS) with force measurement sensor (FMS) was used to investigate the forces generated on the cantilever structures by means of linear displacement. The measurements consist of investigating the force acting on the 3D printed hydrogel cantilevers. A thin rectangular layer (14 x 8 x 0.2 mm) of hydrogel is first printed on a glass coverslip (with dimension of 20 x 20 mm). Then six cantilevers are printed on top which have exact same height but different diameter (150, 300 and 600  $\mu\text{m}$ ) (Figure E-5).

All measurements were performed with an FMS mounted on a MM3A-LS micromanipulator. The sensitivity of the force sensor is 200  $\mu\text{N/mV}$ . The

resolution of the MM3A-LS micromanipulator in the linear motion is less than 0.5 nm. Initially, all measurements were performed in water. The amount of water in the dish was adjusted to allow the investigation of the sample, roughly 10 mm.

At first, a sharp glass tube was mounted on the force sensor to act as contact tip with the hydrogel cantilever. The glass tube was not perpendicular to the hydrogel cantilever. Also, it was not possible to know the effective height where the cantilever and tip are in contact. The sample was placed under microscope for positioning and to make sure the cantilever and tip are just touching before resetting the force sensor initial reading (Figure E-9).

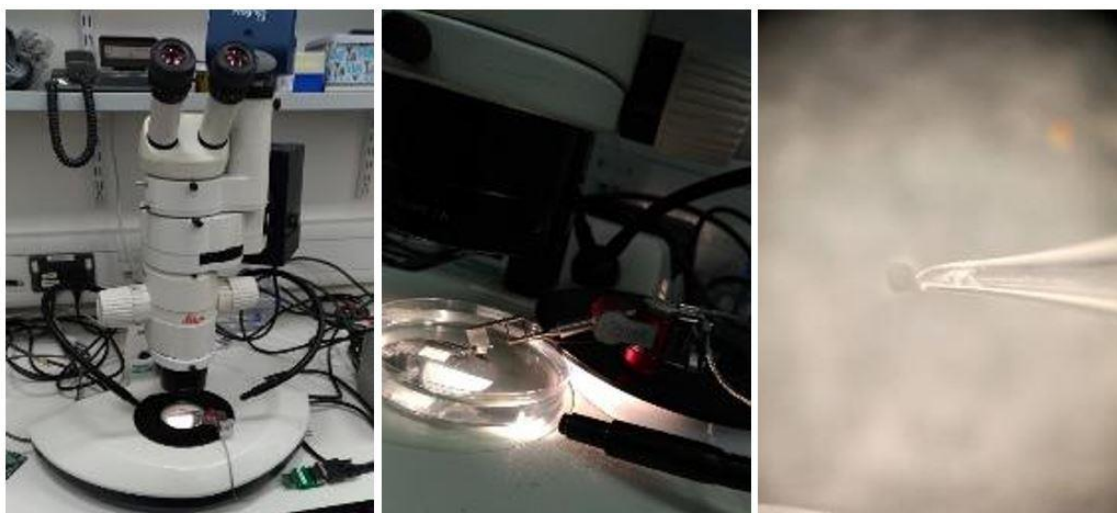


**Figure E-9 Experimental setup for measuring forces on hydrogel microcantilevers.**

Each cantilever was displaced by the same amount as its diameter, i.e. cantilever with 150  $\mu\text{m}$  diameter was pushed by 150  $\mu\text{m}$ . The exerted force was recorded throughout the displacement.

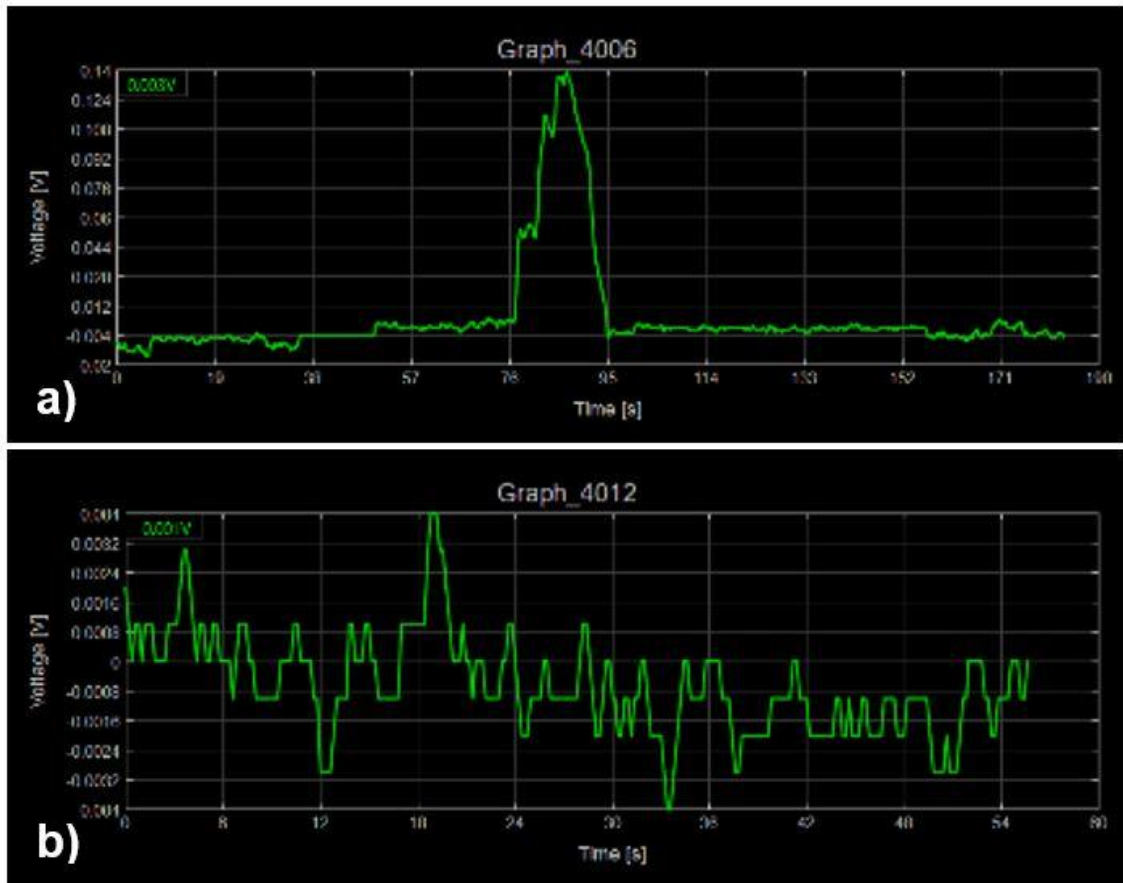


**Figure E-10 a) Force graph for the cantilever with 600  $\mu\text{m}$  diameter. b) Force graph for the cantilever with 150  $\mu\text{m}$  diameter.**

Figure E-10 compares two force tracking graphs for 600  $\mu\text{m}$  and 150  $\mu\text{m}$  diameter cantilevers. It shows how there is a distinctive peak on the larger sample where the force curve is clearly showing when the cantilever is touched by the tip, moved and released. However, such features are missing in the second graph due to the sensitivity of the force sensor which cannot pick any forces less than 200  $\mu\text{N}$ . Therefore, the graph only shows force fluctuations which are most probably noise.

**Table E-1 Force measurement acquired from the system compared with theoretical values obtained from the beam bending equations (Eqs. (6-1 and 6-2)).**

|                                                                                     |           |              |            |              |                  |
|-------------------------------------------------------------------------------------|-----------|--------------|------------|--------------|------------------|
|    |           |              |            |              |                  |
| Diameter                                                                            | Ø 600 µm  |              |            |              |                  |
| Length                                                                              | L: 850 µm |              |            |              |                  |
| File name                                                                           | Reading # | (V)          | (mN)       | (µN)         | Theoretical (µN) |
| Graph_000                                                                           | 1         | 0.177        | 35.4       | 35400        | 10631.15         |
| Graph_3002                                                                          | 2         | 0.133        | 26.6       | 26600        | 10631.15         |
| Graph_4006                                                                          | 3         | 0.138        | 27.6       | 27600        | 10631.15         |
|                                                                                     | Average   | <b>0.149</b> | <b>30</b>  | <b>29867</b> | <b>10631.15</b>  |
|                                                                                     |           |              |            |              |                  |
|    |           |              |            |              |                  |
| Diameter                                                                            | Ø 300 µm  |              |            |              |                  |
| Length                                                                              | L: 850 µm |              |            |              |                  |
| File name                                                                           | Reading # | (V)          | (mN)       | (µN)         | Theoretical (µN) |
| Graph_4007                                                                          | 1         | 0.021        | 4.2        | 4200         | 332.22           |
| Graph_4008                                                                          | 2         | 0.026        | 5.2        | 5200         | 332.22           |
| Graph_4010                                                                          | 3         | 0.029        | 5.8        | 5800         | 332.22           |
|                                                                                     | Average   | <b>0.025</b> | <b>5</b>   | <b>5067</b>  | <b>332.22</b>    |
|                                                                                     |           |              |            |              |                  |
|  |           |              |            |              |                  |
| Diameter                                                                            | Ø 150 µm  |              |            |              |                  |
| Length                                                                              | L: 850 µm |              |            |              |                  |
| File name                                                                           | Reading # | (V)          | (mN)       | (µN)         | Theoretical (µN) |
| Graph_4011                                                                          | 1         | 0.009        | 1.8        | 1800         | 10.38            |
| Graph_4012                                                                          | 2         | 0.004        | 0.8        | 800          | 10.38            |
| Graph_4013                                                                          | 3         | 0.005        | 1          | 1000         | 10.38            |
|                                                                                     | Average   | <b>0.006</b> | <b>1.2</b> | <b>1200</b>  | <b>10.38</b>     |

It is obvious that the force values are far away from being equal. Surely there are various reasons for that including error in measurements, wrong effective length input or elastic modulus value. Also using a simple beam bending equation might not be representative of the system in place. It could also be possible that the force sensor is not calibrated (Table E-1).

To be able to use the system, it is recommended to use larger cantilevers so that the force sensor can capture the forces while in contact. Working with larger cantilevers will help in distinguishing between noise and the forces applied and provide more repeatable results using this system. It is recommended to have a curved shape probe tip so that it improves contact area with the cantilever.

*Advantages:*

Contact mechanism using probe to push the cantilever and measure the force is probably one of the simplest approaches. The cantilever is pushed to a certain point and force is measured using a force sensor. The elastic modulus can be derived from the beam bending equation. The deflection and force readout can be monitored while environmental changes are taken place and their effect is recorded.

*Disadvantages:*

The sensitivity of the sensor is limited to 200  $\mu\text{m}$  which means it is not suitable for cantilevers with less than 300  $\mu\text{m}$  diameter. There will be a challenge to create a probe tip that can have a good contact with the cantilever and hold it while displacing it. Repeatability will be challenging as the position of the probe tip has to be exactly at the same place every time which requires precise positioning stages in place.

The use of Euler–Bernoulli beam bending equations might not be ideal since it is only valid of small deflections. Moreover, it does simplify the nature of a complex viscoelastic system which creates a very large variations between experimental and analytical answers. Additionally, strain rate is also something that must be

considered as it does affect the force response of the cantilever which is also not considered in Euler–Bernoulli beam bending equations.

#### **E.4 Conclusions**

Based on the studies carried out, the advantages and disadvantages of each technique was discussed at the end of each section. A table that uses the Multiple Criteria Decision Approach (MCDA) was constructed. The table ranks all three techniques based on their advantages and disadvantages while taking into account several important criteria such as: cost, availability of the instrument, ability to measure elasticity, setup preparation (complexity), dependency on others, the need for software development, system resolution and being destructive or non-destructive.

The technique with the highest points should be considered as most viable option to proceed with and focus on delivering results with. In this case, the LDV has the highest points (9 points), while FMS scored 5 points and OCT scored only 2 points. Therefore, it is more sensible to choose LDV as the candidate system to carry out the experiments for measuring the mechanical properties of hydrogel cantilevers.

**Table E-2 MCDA for selecting the most viable technique for measuring mechanical properties of hydrogel cantilevers.**

| POTENTIAL TECHNIQUES  |               | OCT                      | LDV  | FMS  |
|-----------------------|---------------|--------------------------|------|------|
| SCORE                 |               | 6                        | 8    | 6    |
| Detailed Scores:      |               | 5.50                     | 8.25 | 5.50 |
| <i>Criteria</i>       | <i>Weight</i> | <i>Raw Rankings Data</i> |      |      |
| Measuring elasticity  | 20%           | 0                        | 10   | 10   |
| Setup complexity      | 15%           | 0                        | 10   | 5    |
| Mobility of technique | 15%           | 10                       | 0    | 10   |
| Software development  | 5%            | 0                        | 5    | 5    |
| Resolution            | 10%           | 5                        | 10   | 0    |
| Non/Destructive       | 15%           | 10                       | 10   | 0    |
| Repeatability         | 20%           | 10                       | 10   | 5    |

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