

The Development of a Soft Tissue Mimicking Hydrogel: Mechanical Characterisation and 3D Printing

A Thesis

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by

Zhengchu Tan

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Declaration of Originality

The work in this thesis is entirely original and the author's own. All else is appropriately referenced. Work done in collaboration with others is specifically referenced. References to work achieved in which the author played a supervisory role will be acknowledged in the text.

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Abstract

Accurate tissue phantoms are difficult to design due to the complex hyperelastic, viscoelastic and biphasic properties of real soft tissues. The aim of this work is to demonstrate the tissue mimicking ability of a composite hydrogel (CH), constituting of poly(vinyl alcohol) (PVA) and phytigel (PHY), as a soft tissue phantom over a range mechanical properties, for a variety of biomedical and tissue engineering applications. Its compressive stress-strain behaviour, relaxation response, tensile impact stresses and surgical needle-tissue interactions were mapped and characterised with respect to its constituent hydrogel formulation. The mechanical characterisation of biological tissues was also investigated and the results were used as the ground truth for mimicking.

The best mimicking hydrogel compositions were determined by combining the most relevant mechanical properties for each desired application. This thesis demonstrates the use of the tissue mimicking composite hydrogel formulations as tissue phantoms for various surgical procedures, including convection enhanced drug delivery, and traumatic brain injury studies. To expand the applications of the CH, a preliminary biological evaluation of the hydrogel was performed using human dermal fibroblasts. Cell seeded on the collagen-coated composite hydrogel showed good attachment and viability.

Finally, a novel fabrication method with the aim of creating samples that replicate the anisotropic properties of biological tissues was developed. A cryogenic 3D printing method utilising the liquid to solid phase change of the composite hydrogel ink was achieved by rapidly cooling the ink solution below its freezing point. The setup was able to successfully create complex 3D brain mimicking material. The method was validated by showing that the mechanical and microstructural properties of the 3D printed material was well matched to its cast-moulded equivalent. This greatly widens the applications of the CH as a mechanically accurate tool for *in-vitro* testing and also demonstrates promise for future mechanobiology and tissue engineering studies.

List of Publications

Journal Papers

1. Tan, Z., Dini, D., Rodriguez y Baena, F. & Forte, A. E. Composite hydrogel: A high fidelity soft tissue mimic for surgery. *Materials & Design* 160, 886–894 (2018).
2. Tan, Z. et al. Cryogenic 3D Printing of Super Soft Hydrogels. *Scientific Reports* 7, 1–11 (2017).
3. Leibinger, A. et al. Soft Tissue Phantoms for Realistic Needle Insertion: A Comparative Study. *Annals of Biomedical Engineering* 44, 2442–2452 (2016).

Conferences

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- 2019 **International Tribology Conference, Sendai, Japan**
Z. Tan, F. Rodriguez y Baena, D. Dini, Frictional Behaviour of 3D Printed Catheters for Neurosurgical Procedures
- 2019 **Society for Image Guided Neurointerventions Conference, Baltimore, USA**
Z. Tan, F. Rodriguez y Baena, D. Dini, EDEN2020: A Bio-Inspired Robotic Platform for Neurosurgery.
- 2018 **International Conference on Biotribology, Montreal Canada**
Z. Tan, A. Bernardini, E. Porte, A. Forte, F. Rodriguez y Baena, D. Dini, Needle-tissue interactions during convection-enhanced drug delivery in neurosurgery.
- 2018 **World Biomechanics Conference, Dublin, Ireland**
Z. Tan, C. Parisi, L. Di Silvio, D. Dini, A.E. Forte, Cryogenic 3D printing of brain mimicking hydrogels.
- 2017 **World Tribology Congress, Beijing, China**
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- 2016 **European Robotics Conference, Edinburgh, UK**
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1. Introduction

Over the past half a century, the research topic of hydrogels has been an exponentially growing scientific and commercial field due to the ever expanding list of tuneable mechanical properties that allow them to be used for an increasing range of applications[1–3]. In the 1960s, the first reference to hydrogels appeared in the context of using polyhydroxyethylmethacrylate (pHEMA) as a biomaterial[3]. The material was described as a three dimensional network of hydrophilic polymers, and this has become the modern definition of the word ‘hydrogel’. Through the development of hydrogels over the decades, the definition now encompasses natural and synthetic polymer networks that crosslink through chemical or physical processes[4].

One of the most typical and exploitable properties of hydrogels is their ability to express different material properties when their crosslinking conditions are affected. Factors that affect the number of crosslinks formed, and therefore the resulting polymer network, are the polymer concentration, molecular weight, crosslinking method and crosslinker concentration, to name just a few. These will affect microstructural properties such as network morphology and pore size, and also macromechanical properties, such as hyperelasticity, viscoelasticity, mechanical hysteresis and permeability. Furthermore, the hydrogel hydroxyl group can be readily functionalised to allow hydrogels to react under chemical and biological stimuli[5]. Therefore, the versatility and flexibility of hydrogels has allowed them to achieve a wide range of mechanical, chemical and biological properties, and specialised hydrogel formulations can be developed for specific applications. This brings us to the topic of the thesis: the exploration of hydrogels as a mechanically accurate tissue mimicking material.

Without even taking into account the complex biological functions of the simplest tissues, mimicking the mechanical properties of biological tissues using synthetic materials is still an ambitious task. On the macroscopic scale, the mechanical stress-strain behaviour of biological tissues is often characterised and efforts towards characterising the fracture behaviour and friction between biological tissues and other materials have also been reported. On the microscale, one can also mimic the extra cellular matrix (ECM) by matching the average pore size. However, this is often a contentious subject as tissue mimicking becomes even more complex when evaluating the accuracy of the microstructure. This is because biological tissues have an extremely complex physical structure. It not only includes the ECM but also its substructures, such as veins, arteries, bronchi in lungs, which all effect the mechanical properties of the tissue. Alongside stiffness anisotropy, the tissue permeability may also be directional, for example interstitial fluids may flow in a preferential direction related to the

alignment of the internal tissue structures. Although it is widely known that the internal structures affect the mechanical directionality, little substantial developments to try to characterise these effects through rigorous mechanical testing protocols have been made. Without sufficient ground truth evidence, there have been little efforts to try to replicate or mimic the anisotropic effects of these structures.

The slow development in mimicking the intricate geometries of real biological tissues and their internal structures was mainly due to the lack of a fabrication method that could achieve these complex geometries and anisotropy. Although various hydrogels have been used as tissue mimicking materials for decades, these samples were almost always homogeneous and isotropic. This is because, previously, the fabrication of hydrogel samples was limited to what could be achieved by simple cast-moulded processes. However, in recent years, with the advancement of technologies such as electrospinning and 3D printing, the possibility of creating hydrogels with complex geometries has been unlocked. Additionally, these processes can be used to create materials with directionality and anisotropy by spinning fibres and printing material in a certain alignment. This development in science and technology brings an added dimension to the realm of hydrogels as it allows materials to mimic the directionality of tissues. As a result, this is a rapidly growing subject in the tissue engineering field as it is known that cells respond to the mechanical properties of the substrate they grow on[6]. As such, the finale of this work is the development of a 3D printing system that is able to create samples with complex geometries, even in 3D, and to begin to explore the possibilities of creating samples with imposed directionality.

In summary, the scope of this work is to develop a tunable material that is able to mimic the mechanical properties of a variety of soft biological tissues. The material can be used as a surgical phantom for path planning, surgical training and as a high fidelity *in-vitro* model for the development of novel surgical techniques. It can also be used to study how traumatic brain injury loads cause brain damage and disease progression. Finally, the accuracy of the phantom is further developed by creating a novel fabrication method that is able to replicate complex biological structures.

Hence, the thesis will begin with a detailed literature review covering the four main research areas of this work, which are summarised as follows. The first main results chapter, 3, will report the characterisation of a selection of biological tissues that display different mechanical behaviours, also taking into account the intricacies of their individual internal anatomical structures. Subsequently, Chapter 4 will detail a range of mechanical characteristics of the composite hydrogel, including its mechanical response to applied loads at varying initial strain

rates, relaxation response and fracture behaviour. The next chapter, 5, will tie together the work presented in Chapters 3 and 4, by utilising the mapping results to determine tissue mimicking compositions for each biological tissue investigated and demonstrate how these phantoms can be used for important bioengineering applications. Lastly, the final chapter, 6, will reveal a new 3D printing technique can be used to produce complex biological geometries that mimic the softest tissues in our bodies.

2. Literature Review

The work must firstly begin with an exploration into the characterisation of biological tissues and their synthetic tissue mimicking counterparts already achieved thus far. The characterisation of biological tissues is significantly complex as it involves a multi-faceted and multi-disciplinary collaboration between the fields of biochemistry and mechanical engineering. This section will focus on a review of the mechanical properties of soft tissues and how this can affect their physiological properties. Although the focus is limited in this way, the mechanical behaviour expressed by soft tissues is still wildly complex and dissimilar to traditional engineering materials that can be accurately defined using classical material models. The understanding of biological tissues therefore requires the development of constitutive models, which will also be briefly reviewed here. In addition, this section will review the range of the mechanical properties used as tissue mimicking parameters for specific dedicated applications.

Section 2.1 begins with background knowledge on the constitutive laws used to characterise the mechanical behaviour of biological tissues. Section 2.2, covers the range of mechanical and biological characterisations that have been achieved of hydrogels. Then section 2.3 will further investigate the specific use of hydrogels as surgical tissue phantom. In order to replicate the complex geometries and structures that exist in nature, a 3D printing method has been proposed and developed and so Section 2.4 will review the literature surrounding the introduction of 3D printing as a tissue scaffold fabrication method.

2.1. Mechanical Characterisation of Biological Tissues

Investigations into the mechanical characterisation of biological tissues began in the 1950s, although the tensile properties of skin were reported earlier by Howes et al.[7,8] in 1929. Despite the advancement of testing machines and systems in the past 70 years, most historical literature results on the material characterisation on the brain remain relevant for comparison to modern results.

The most common mechanical characterisation is the tensile and compressive material properties. The tests involve subjecting the tissue to tensile or compressive loads in one direction and measuring the resulting deformation. A testing machine can apply either a force or displacement load to the specimen. It is usually the case that a displacement load is applied and the force from the material is measured using a load cell. From these measurements, the

stress-strain, or stress-stretch, relationship can be calculated using a linear elastic constitutive law.

The concept of stress has existed since the 17th century. In the 19th century, Cauchy was able to establish the first general mathematical model that described stress in a continuous medium. From there sparked mechanical characterisation of industrial materials, however characterisation of biological tissues has only been studied using engineering methods and analyses since the 1950s[8]. Whilst technological advances in the form of new testing machinery have made physical quantities easier to measure, the derivation of the mechanical properties themselves remain fundamentally robust, as they are still based on Cauchy's continuum mechanics theory. In a simplified version of this theory, the engineering stress, σ , and strain, ε , are used to describe a material's mechanical behaviour at small deformations where the cross-sectional area, A , can be assumed to be constant. At large deformations, the cross-sectional area is not constant, therefore, assuming the material is incompressible, this can be accounted for by calculating the true stress, σ_T , and strain, ε_T , which can be expressed as a function of the engineering stress and strain as follows:

$$\sigma_T = \frac{F}{A} = \sigma(1 + \varepsilon) \quad (2.1)$$

$$\varepsilon_T = \ln\left(\frac{L}{L_0}\right) = \ln(1 + \varepsilon) \quad (2.2)$$

Typically, in the engineering field, stress and strain calculations are often used to find the Young's modulus, a material property of linear elastic materials. If a biological sample exhibits a relatively linear stress-strain results curve then an approximate Young's modulus can be calculated. However, most soft biological tissues exhibit a nonlinear stress-strain relationship.

Experimental results for biological tissues usually report the approximated Young's modulus, also known as the effective elastic modulus, E , despite there being increasing evidence that biological tissues are hyperelastic. This is because a modulus is an easily calculated single value that can be readily compared to other materials in order to give an immediate sense of scale. Table 1 summarises the range of elastic modulus values found for a variety of biological tissues reported in literature.

Literature reporting elastic moduli are often aware of the shortcomings of the linear elastic model and will therefore preface their results by warning the reader that this modulus is only valid at small strains, usually below 5% strain. However, when discussing mechanical behaviour

up to larger strains, the accuracy of the linear elastic material model is quickly disregarded, with respect to the more complex and high fidelity hyperelastic models.

Table 1. Elastic modulus, E , of various biological tissues.

Authors	Biological tissue	Testing method	Elastic modulus, E
Taylor and Miller [9]	Brain	Finite element model (FEM)	0.584 kPa
Yeh et al. [10]	Human liver	Compression test, 15% strain	~5 kPa
Collinsworth et al. [11]	Myoblast, differentiated myofibers	Atomic force microscopy (AFM)	Myoblast: 11.5 ± 1.3 kPa Myofiber: 45.3 ± 4.0 kPa
Silver et al. [12]	Human articular cartilage	Tensile test	7.0 GPa
Katsamanis and Raftopoulos [13]	Human femoral cortical bone	Static and dynamic tension-compression test, Hopkinson Bar	Static: 16.2 GPa Dynamic: 19.9 GPa

In the 1940s, Mooney and Rivlin were the first to propose a nonlinear material model that was intended to model rubbers. This type of nonlinear constitutive law became known as the hyperelastic model. The hyperelastic models are derived based on the finite strain theory. The fundamental concept is that the energy put into the material by the applied load, called the strain energy density function, W , is the integral of the Cauchy stress (σ_i) - stretch (λ_i) function, where the subscript i denotes the principal direction. Therefore, the derivative of this work done energy with respect to the stretch is equal to the stress in the material, and is given by the following expression:

$$\sigma_i = \frac{\partial W(\lambda_i)}{\partial \lambda_i} \quad (2.3)$$

This equation shows that the strain energy density function, W , will determine the behaviour of the stress-stretch relationship. Therefore, various strain energy density functions have been proposed over the decades. The main hyperelastic models used to fit biological data are listed in Table 2. Other models include the Polynomial, Saint Venant-Kirchhoff, Yeoh, Marlow, Arruda-Boyce and van der Waals.

Table 2. Summary of the main hyperelastic constitutive laws used to characterise biological tissues, assuming the material is incompressible and isotropic.

Ogden	$W = \sum_{k=1}^N \frac{C_1}{\alpha_k} (\lambda_1^{\alpha_k} + \lambda_2^{\alpha_k} + \lambda_3^{\alpha_k} - 3)$
Neo-Hookean	$W = C_1(I_1 - 3)$
Mooney-Rivlin	$W = C_1 \left(J^{-\frac{2}{3}} I_1 - 3 \right) + C_2 \left(J^{-\frac{4}{3}} I_2 - 3 \right)$
Fung	$W = \frac{1}{2} [C_1(I_1 - 3) + C_2(e^{C_3(I_1-3)} - 1)]$
Gent	$W = -\frac{\mu J_m}{2} \ln \left(1 - \frac{I_1 - 3}{J_m} \right)$

The above constitutive laws are expressed in terms of the deformation tensor invariants and Jacobian. These are a function of the principle stretches given by the following equations:

$$J = \lambda_1 \lambda_2 \lambda_3 \quad (2.4)$$

$$I_1 = \lambda_1^2 + \lambda_2^2 + \lambda_3^2 \quad (2.5)$$

$$I_2 = \lambda_1^2 \lambda_2^2 + \lambda_2^2 \lambda_3^2 + \lambda_3^2 \lambda_1^2 \quad (2.6)$$

In literature, where an elastic modulus is particularly inaccurate but a hyperelastic model is not fitted, the full stress-strain curve is often reported[14,15]. These papers often characterise the material in terms of stress at a given strain to give an easily comparable quantitative sense of stiffness, instead of giving a definitive Young's modulus or hyperelastic model parameters. Nevertheless, the data curves allow other researchers to fit their own model to the experimental data. A typical example of this is the hyperelastic model Mendis et al.[16] suggested that was implemented to fit the experimental data published by Estes and McElhaney[17], mentioned in Table 3. With many available constitutive laws for hyperelasticity, research published in literature typically shows that authors have applied each of the different hyperelastic models to their data in order to evaluate, using a statistical method such as least squares, which model best fits their data.

However, there is no consensus as to which material model is the best to use. This is because biological tissues vary in mechanical behaviour from one tissue to the next. Unfortunately, this makes comparing results across all literature difficult and especially problematic when comparing full model parameters that use different material models, e.g. Mooney Rivlin, van der Waals or Fung, as shown in Table 3. This highlights why researchers may also publish approximate elastic modulus for easier cross-comparison between different soft tissues or only compare the initial shear modulus across different hyperelastic models.

Table 3. Hyperelastic parameters of various biological tissues.

Authors	Biological tissue	Hyperelastic model	Hyperelastic parameters
Mendis et al. [16]	Porcine brain	Uniaxial Mooney-Rivlin	$C_{10}=0.641$ kPa, $C_{01}=0.733$ kPa
Andrikakou et al.[18]	Rabbit lung	Van der Waals	$\mu=3$ kPa, $\lambda=2.4$, $C_2=0.78$, $C_5=0.41$, $a=C_1=C_3=C_4=0$
Gao et al.[19]	Porcine liver	Fung (combined logarithmic-exponential model)	Logathrithmic: $C_1=60$, $C_2=0.63$, $\alpha=-2.54$ Exponential: $C_1=10$, $C_2=1.60$, $\alpha=-3.07$

In the realm where accuracy is imperative, i.e. the computational modelling field, researchers strive to improve their models, and hence require the most accurate material model. Literature in this field therefore focuses on the finding the hyperelastic model parameters that most accurately fit experimental data. New formulations and combinations of existing models are often suggested. The most common computational models are those which model difficult experimental scenarios. An important example of this is traumatic brain injury. There is little experimental data due to the ethical concerns over in vivo testing and the challenging testing conditions. This is where computational modelling is able to establish itself as a key player[20]. Hence, hyperelastic material parameters are often reported by literature in this field. The use of this data in the computational modelling field however means that a material model must be defined. Therefore, the majority of literature focusing on the development of novel material models is centred around applying these models in a computational sense, typically finite element modelling, because their work depends on the accuracy of the constitutive model.

Another important mechanical behaviour is when materials exhibit different responses when loads are applied at different rates. This phenomena is called viscoelasticity. The models that exist to describe this behaviour are based on different arrangements of the spring and dashpot conceptual components[21]. The simplest form is a single spring and dashpot in series, named a Maxwell element with constitutive equation as follows:

$$k \frac{d\varepsilon}{dt} = \frac{d\sigma}{dt} + \frac{1}{\tau} \sigma \quad (2.7)$$

For relaxation, a constant strain is applied, resulting in $\dot{\varepsilon}=0$. Then $\sigma(t)$ may be found through differentiation, shown below, to find the equation describing the stress behaviour over time.

Since the strain is constant, the linear elastic modulus may be found by dividing the equation for stress by the strain to give the following equation:

$$E(t) = \frac{\sigma_i}{\varepsilon_i} e^{\left(\frac{-t}{\tau}\right)} \quad (2.8)$$

The generalised Maxwell model, also known as the Maxwell-Wiechert model, is mainly used to fit experimental data as this allows as many Maxwell elements as necessary to accurately represent the data curve[21–24]. This is shown in Figure 1. However, viscoelastic material properties can be found by fitting the Maxwell model to stress-relaxation test data using the Prony series, where E_∞ , E_i and τ_i are the parameters to be optimised to fit the experimental data:

$$E(t) = E_\infty + \sum_i E_i e^{\left(\frac{-t}{\tau_i}\right)} \quad (2.9)$$

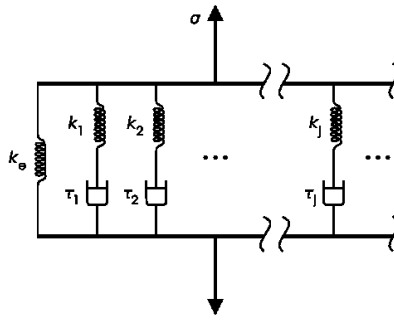


Figure 1: Schematic of generalised Maxwell model [25].

The first characteristic time, τ_1 , represents the immediate rate of decay in the relaxation response and is the parameter that is most relevant for the analysis and evaluation of a material's viscoelasticity. Biological tissues are widely reported to be viscoelastic[26]. Viscoelastic properties may be found by fitting viscoelastic models to experimental data obtained from stress-relaxation tests. The instantaneous, E_i , and equilibrium modulus, E_∞ , also referred to as the relaxation modulus, and relaxation times, τ_1 , are often reported.

Table 4. Viscoelastic parameters of various biological tissues.

Authors	Biological tissue	Viscoelastic parameters
Miller[27]	Porcine brain	$C_{100}=C_{010}=0.263$ kPa, $C_{200}=C_{020}=0.491$ kPa, $t_1=0.5$ s, $g_1=0.450$, $t_2=50$ s, $g_2=0.365$
Mattei et al.[28]	Porcine liver	$E_i= 2.04$ kPa, $E_\infty=0.91$ kPa, $\tau_1=1.10$ s
Marangoni and Glaser[24]	Guinea pig skin	$E_2=34$ Pa, $\eta_1=84$ Ns/m ² , $\eta_2=6.5$ Ns/m ²

Another mechanical property of biological tissues, which is key to the application of this work, is the biphasic solid-fluid phase properties, such as the permeability, which arise due to the porosity of the tissue microstructure. This work will focus on reviewing literature surrounding brain tissue permeability, as the perfusion of therapeutic drugs directly into brain tissue to treat brain tumours is an impactful research area. The majority of brain tumours, gliomas, are derived from the glial cells located in the white matter of the brain. The porous microstructure of the white matter, is anticipated because of its organised microanatomy, which consists of myelinated axonal tracts through which electrical signals, which communicate activity, flow, as illustrated in Figure 2. The mammalian axonal diameter ranges from 1-20 μm and the myelin thickness ranges from 0.5-2.5 μm [29]. At first glance, the axonal microstructure appears to represent a porous medium with pore size (and therefore porosity) related to the axonal diameter. However, the axons are assumed to have impermeable walls due to their myelin sheath, and therefore any perfused fluid flow is assumed to only flow through the interstitial space (ISS) in between the axonal fibres.

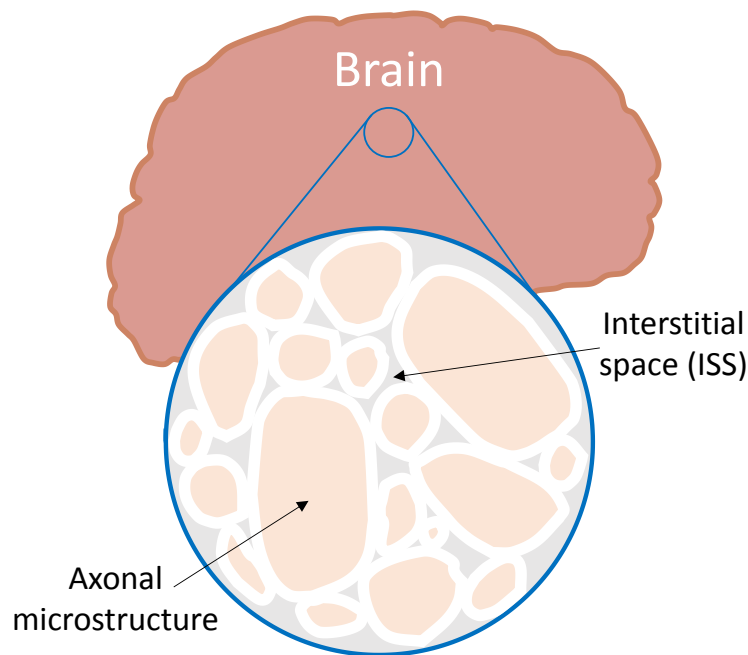


Figure 2: Schematic of the microanatomy of white matter brain tissue.

The composition of the interstitial space is reported to consist of the solid phase extracellular matrix (ECM) materials; collagen, hyaluronic acid (HA) and glycoproteins, and the liquid phase interstitial fluid (ISF) containing; water, ions, gaseous and organic molecules, to name a few[30]. The geometry and arrangement of the ISS is known to change in a tumour microenvironment, therefore cell pathology is one of the main factors that affects the local permeability of the tissue[30]. For example, investigations into the microenvironment surrounding a stage IV brain tumour, glioblastoma, show an increase in the tortuosity and volume fraction of the ECM, resulting in restricted diffusion and a stiffer matrix[30–35]. Additionally, tumour cell invasion

results in the formation of oedema, which raises the intracranial fluid pressure[36]. As such, there is an abundance of literature from the medical field that characterise the changes in brain microenvironment, indicating effects on local permeability and tissue stiffness, which will impact the effectiveness of convection enhanced drug delivery[37–40].

However, actual quantitative measurements of the porosity and permeability of brain tissue are scarcely reported as there is no simple testing method. An alternative method to reverse calculate parameters related to permeability is to develop a poroelastic material model. The first model of this kind applied to brain was developed by Biot[41] in 1956 and the subsequent advancement of this model has led to the use of the hydraulic conductivity, κ , to characterise the solid-fluid interaction. The hydraulic conductivity is the ratio of permeability, k , to pore fluid viscosity, μ , and is quoted as $10^{-11} \text{ m}^2/\text{s}$ [42,43].

In addition, biological tissues vary immensely due to a wide range of environmental testing and physiological factors. One of the main factors is post-mortem time (pm), which is the mechanical response of tissue on the hour time scale following death. There is a large amount of literature focused on this subject, especially around the brain, due to the philosophical speculation over when conscious is lost. Table 5 details the literature surrounding the stiffness of biological tissues with respect to post-mortem time. To compound the complexity of this matter, there is also literature that suggests that the tissue stiffness is greatly affected by the post-mortem storage conditions[44–46].

Table 5. Mechanical dependence of biological tissues on post-mortem time.

Authors	Biological tissue	Testing method	Elastic modulus
Weickenmeier et al. [47]	Human brain, cerebrum	MRE*	In vivo: 1.13 kPa In situ 16 h pm: 2.60 kPa
Vappou et al.[48]	Rat brain	MRE*	In vivo: 7 kPa 0.5 h pm: 13.5 kPa 24 hr pm: 8 kPa
Zhang et al.[44]	Ovine brain	DSC ⁺	0.5 h pm: 1.68 ± 0.31 kPa 1 h pm: 0.99 ± 0.25 kPa 4 h pm: 0.74 ± 0.22 kPa
Rashid et al.[45]	Porcine brain	HRSD [#]	5 h pm: 1.019 ± 295 kPa
Tuttle et al.[49]	Skeletal muscle	Mechanical tension	Unchanged over 7 days: 35 kPa
Yarpuzlu et al.[50]	Bovine liver	Mechanical indentation	5 h pm: 1.57 ± 1.1 kPa 17 h pm: 1.70 ± 1.1 kPa 29 h pm: 7.91 ± 1.8 kPa 53 h pm: 33.60 ± 3.7 kPa

*MRE: Magnetic Resonance Elastography, ⁺DSC: Differential Scanning Calorimetry, [#]HRSD: High Rate Shear Device

Moreover, a wide range of other factors such as disease, age, sex, sample testing temperature and interspecies variation have been reported to affect tissue properties. Summarised here are some key literature that focus on the impact of these factors on the mechanical response of biological tissues. Reported values, and standard deviations where available, for factors affecting tissue stiffness are summarised in Table 6.

Table 6. Other environmental and physiological factors which affect biological tissue stiffness.

Authors	Biological tissue	Testing method	Environmental testing conditions	Physiological factors	Elastic modulus
Murphy et al. [51]	Human brain	Magnetic resonance elastography (MRE)	In vivo	Alzheimer's disease (AD), control normal group (CN+, CN-)	AD: 2.20 kPa CN+: 2.37 kPa CN-: 2.32 kPa
Arani et al. [52]	Human brain, cerebrum	MRE	In vivo	Age	60-69: 2.7 kPa 80-89: 2.3 kPa
Arani et al. [52]	Human brain, occipital and temporal lobes	MRE	In vivo	Sex	Occipital: F>M by 0.23 kPa Temporal: F>M by 0.09 kPa
Lacraz et al. [53]	Rat quadriceps	Atomic force microscopy (AFM)	In vitro	Age	Adult: 3 kPa Aged: 7 kPa
June et al. [54]	Bovine calf patellofemoral cartilage	Unconfined compression, 5% strain, 0.05 mms ⁻¹	Temperature as the independent variable	-	24°C: 182 ± 4 kPa 60°C: 460 ± 26 kPa
Interspecies variation					
Courtland et al. [55]	Mouse, femoral bone	Digital Image Correlation, mechanical tension	In PBS	Intraspecies variation, different mineral composition	High mineral: 34.9 ± 4.4 kPa Low mineral: 27.4 ± 4.1 kPa

2.2. Mechanical Characterisation of Hydrogels

Hydrogels have been used as a biomaterial for a variety of biomechanical purposes[56]. An important use of hydrogels is as a tissue mimicking material. One of the main advantages of developing an artificial tissue material is to perform experiments that would be unethical to test in vivo, such as testing new surgical equipment and understanding the mechanisms behind traumatic injury. This section will review the use of hydrogels as a tissue mimicking material for a wide range of biomechanical applications. The mechanical characterisation of hydrogels is generally less systematic and more application based. This is in part due to the highly flexible and tuneable nature of hydrogels. This means that literature is often structured around mimicking a particular set of mechanical properties that is most relevant for a specific biomedical application.

Although there are many material properties, generally all tissue mimicking materials (TMMs) should mimic the target tissue stiffness to an appropriate degree of accuracy. Therefore, most literature will also report and characterise the mechanical stress-strain behaviour of the hydrogel. As mentioned in section 2.1, they may be characterised using different material models, *e.g.* linear elastic, hyperelastic, viscoelastic. As with all materials, its properties depend on its formulation, constituent polymers, additives and functionalisation, which can all be developed and tuned to increase the accuracy of the soft tissue mimic. An example of this is the development of a hydrogel that is a composite blend of two hydrogels PVA and phytagel (substitute for agar gel). Both hydrogels are well known biomaterials individually; PVA is a cartilage mimic that can achieve a wide range of stiffness and phytagel is a common cell culture substrate with a highly viscoelastic nature[57–59]. Forte et al. combined these hydrogels to find a composition that was able to mimic the stress-relaxation properties of an extremely soft and viscoelastic biological tissue, the brain[60]. This phantom was used to mimic neurosurgical brain shift, where the stiffness and viscoelasticity of the tissue are the main material properties involved in the scenario.

An array of materials have also been developed to mimic the stiffness of soft tissues. Table 7 summarises the stiffness of other synthetic tissue phantoms, which include PVC, silicon-based hydrogels and agar gel. A summary of the stiffness of various soft tissues are displayed in Figure 3 for comparison. The stiffness of soft tissues presented in Figure 3 refers to the linear elastic modulus, which only holds true under the assumption of small strains, usually <5 % [28]. Gelatine, PVC and silicon-based gels exhibit a near linear elastic stress-strain relationship, whilst soft tissues show viscoelastic and non-linear stress-strain behaviour with increasing strain [24]. Therefore, these materials can only be used as accurate tissue phantoms for small

strain needle insertion studies, such as fine needle aspiration (FNA) biopsies, but would not be suitable for traumatic injury studies or laparoscopic surgical procedures. On the other hand, the agar gel and PVA-phytagel phantoms exhibit a greater non-linear stress-strain behaviour, which allows them to match the stress response of real tissue to higher strains[61,62]. In particular, it has been shown that a composition of 5 % wt. PVA 0.59% wt. phytagel composite hydrogel (CH) is able to reproduce the stress-relaxation response of porcine brain up to 95% strain[63].

Table 7. PVC, silicon and agar tissue phantoms and their stiffness found in literature.

Author	Material	Stiffness (kPa)
Li et al. [64]	PVC 50 - 100 %	7 - 43
DiMaio et al. [65]	PVC (% wt. not specified)	27
Okazawa et al. [66]	PVC (% wt. not specified)	53, 100, 157
Wang et al. [67]	Silicon-based 40 % wt.	10.3
Yamaguchi et al. [68]	Agar 3 % wt.	~50
Leibinger et al. [63]	PVA-phytagel 5 % - 0.59 % wt.	2
Datla et al. [69]	PA 8 %, BIS crosslinker 0.35 %	18.15
	PA 11 %, BIS crosslinker 0.22 %	20.15

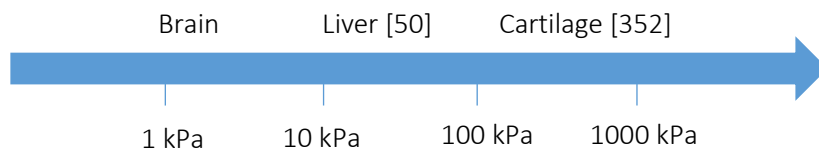


Figure 3: Range of different soft tissue stiffness, *E*.

In addition to hyperelasticity, mimicking the viscoelasticity of soft tissues is especially important when using these phantoms to model impact loads for traumatic injury studies. In the last few decades, traumatic brain injury (TBI) studies have become increasingly more important due to the findings that TBI can increase the risk of developing neurological deficits and diseases decades after the injury, such as Alzheimer's disease and Parkinson's disease[70]. Traumatic and blast injury loads are very complex due to the propagation of pressure waves that impart a combination of highly compressive, tensile and shear loads on the biological tissue[71,72]. Subjecting an accurate tissue mimic to these destructive scenarios allows researchers to understand the multifaceted loading patterns that occur. Cronin et al.[73] investigated blast mine injuries using an artificial leg composed of glass fibre-reinforced epoxy for the bone, a silicone based material for the cartilage and ballistic gelatine to mimic the muscle tissue. Zecheru et al.[74] also used gelatine to model dynamic injury loads, such as stabbing and shooting. Gelatine has also been used to model bird strike, a high impact event that could cause

turbine failure during flight[75]. Researchers have been able to replicate these loads through *in-vitro* testing.

Currently, *in-vitro* testing is limited to the cellular scale and focuses on the signalling response of cells undergoing destructive loads that induce cell alteration and apoptosis, as shown by Chen et al.[76] in Figure 4. Hence, a tissue mimic for this application should be able to mimic the complex mechanical behaviour of soft tissues and also provide an environment for cell culture so that large scale cellular testing becomes feasible[77]. This is important as the load distribution during impact would be affected by the boundary conditions provided by the ECM and architecture of biological tissues, which would undoubtedly vary greatly from that of a collection of cells suspended in fluid. An accurate tissue mimic would also reduce the need for unethical *in-vivo* animal model testing[78,79].

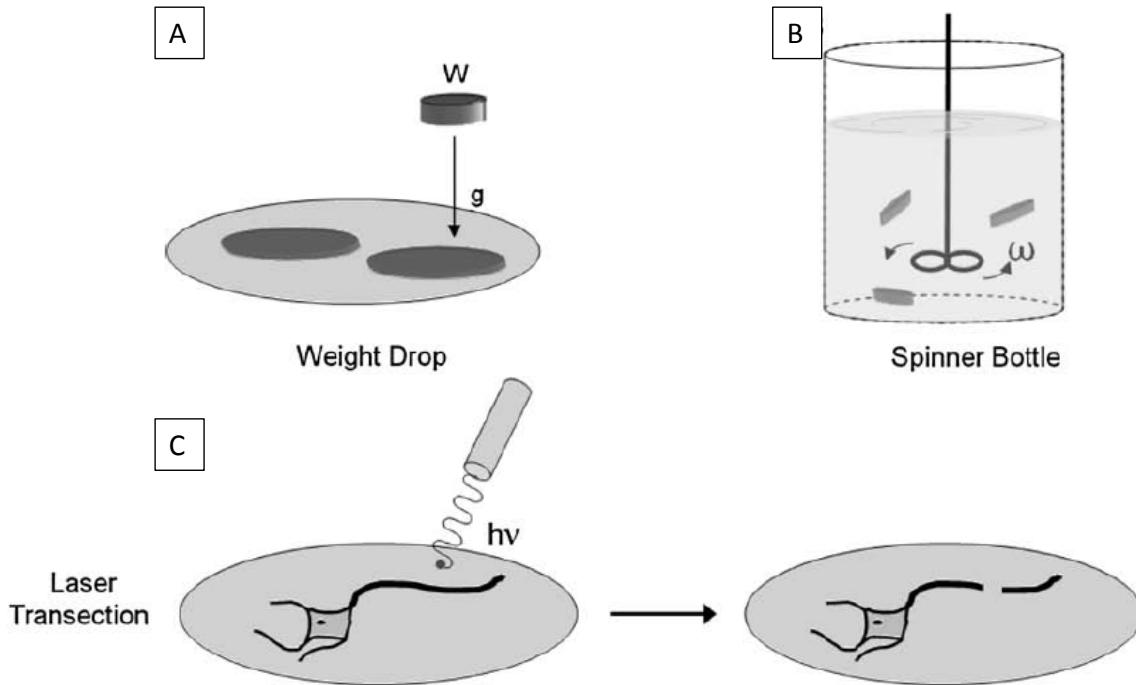


Figure 4: Different methods for inducing cell damage featuring (A) a dropped weight, (B) rotating spinner and (C) focused laser beam to injure specific cell regions[76].

To this end, it is important to develop a mimic that is biocompatible so that live cells may be cultured large scale *in vitro*. Many researchers recognise the potential of this application, not only for traumatic injury studies but also as a mechanically accurate tissue scaffold for tissue engineering applications in general. This is due to the significance of substrate stiffness in stem cell differentiation, which therefore opens up investigations into the role of substrate stiffness in mechanobiology and stem cell therapy[6]. The biocompatibility alongside stiffness tunability of hydrogels is what has propelled this group of materials to become the new standard of cell culture substrates. Thus, there is a large amount of literature discussing the use of hydrogels as *in-vitro* tissue scaffolds.

Ren et al.[80] showed that neural stem cells (NSCs) were able to attach and differentiate into neurons and astrocytes on a polylysine-coated hyaluronic acid (HA) hydrogel. Park et al.[81] reviewed the use of hydrogels to model the complex 3D tumour microenvironments *in-vitro* to better understand how cancer cells proliferate in order to develop new therapies. As mentioned previously in section 2.1, tumour microenvironments are known have a higher stiffness than healthy tissues and Yue et al.[82] were able to show that the presence of cancer cells increased the ECM stiffness around adipocytes seeded on hydrogel substrates of 0.2-3 kPa stiffness, which in turn inhibited adipogenesis, as illustrated in Figure 5. The effect was only significant in a 3D culture, emphasizing the importance of geometrical accuracy for tissue scaffolds[83,84].

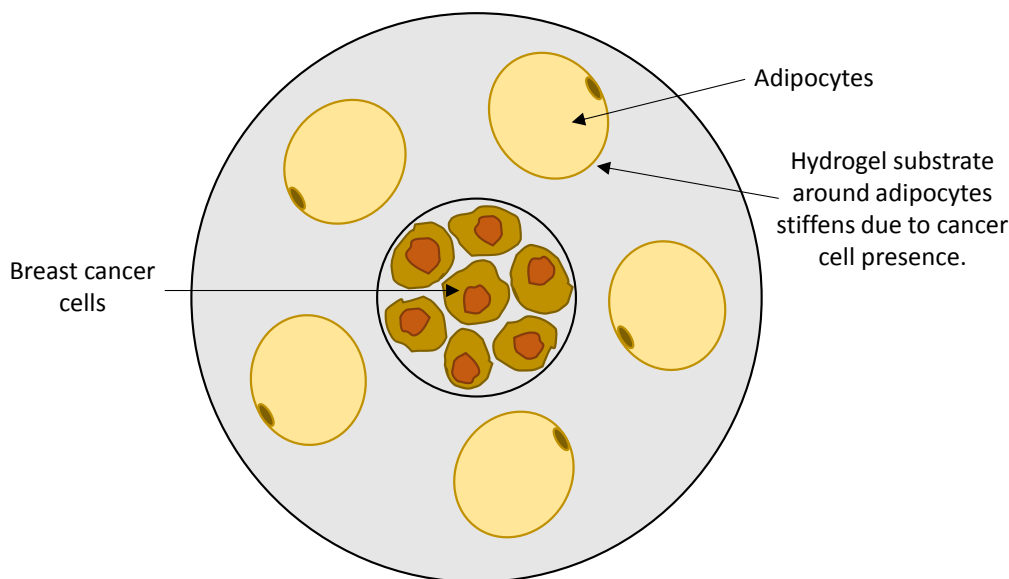


Figure 5: Schematic of the cell culture setup and results from Yue et al. [82] illustrating that nearby breast cancer cells increase adipocyte ECM stiffness.

There is also a large interest in the use of hydrogels for regenerative medicine; as stem cell laden scaffolds, artificial implants or even replica organs[85–91]. Liu et al.[92] reviewed a range of natural and synthetic injectable hydrogels encapsulating stem cells for cartilage repair and found that natural biomaterials, such as chitosan, collagen, alginate and fibrin, performed better in terms of biocompatibility but had less mechanical strength and stability than synthetic hydrogels, such as PEG and PVA. Alexandre et al.[93] discussed the feasibility of using PVA discs for vascular grafting and showed that mesenchymal stem cells (MSCs) exhibited the correct surface marker expressions, hence determining sufficient biocompatibility and hemocompatibility, however as expected, an inflammatory response occurred due to the organism's foreign body reaction (FBR)[93,94]. The FBR remains a large challenge in the realm of using biomaterials to create synthetic organs for transplants but the development of hydrogels has at least opened up the question.

Another biomedical application of hydrogels is their ability to swell in water. This is due to their hydrophilic microstructure, which is very adept at capturing and storing free water[4]. This has

led to the development of mechanisms that are able to control and utilise hydrogel swelling. Chirani et al.[3] summarised that hydrogels may respond by swelling under certain stimuli, such as temperature, pH, ionic concentration, light, magnetic and electric fields. Ma et al.[95] utilised the pH controlled swelling behaviour of polymethylacrylic acid hydrogels to regulate the frictional properties of a porous composite material, as shown in Figure 6. Overall, the bulk of the literature surrounding smart hydrogels show their use for controlled drug releasing applications, stents, and stem cell therapies[96–100]. Therefore the work in this field focuses on characterising the chemical and biological properties, and less so the mechanical properties.

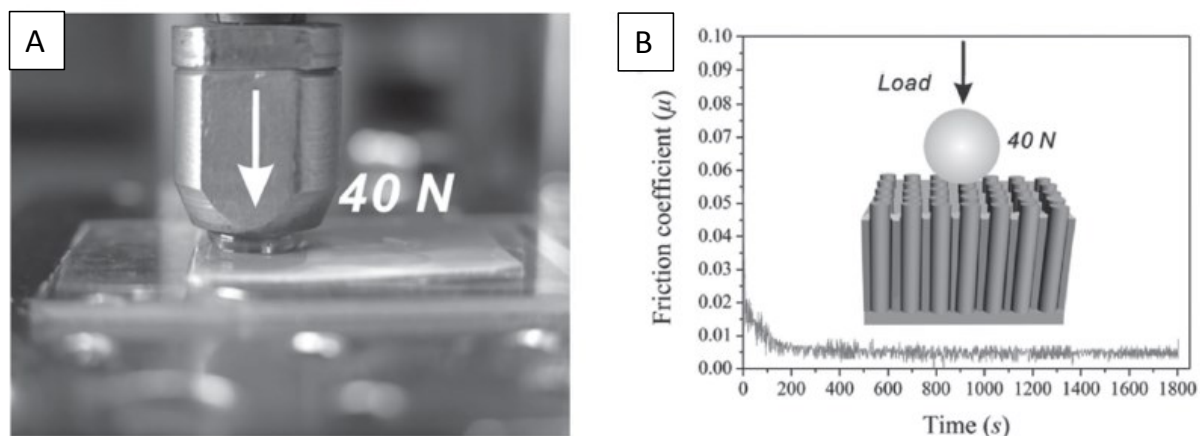


Figure 6: Porous nanocomposite material constituting of anodic aluminium oxide (AAO) substrate and hydrogel fibres showing (A) sliding test set up at a load of 40 N and sliding velocity of 0.01 m/s (B) typical coefficient of friction results graph where hydrogel fibres swell in basic media (pH 10)[95].

Other popular biomedical applications where physical properties are important include using hydrogels as a tissue mimic for elastography. This involves developing a material with an accurate acoustic characterisation to biological tissue, which eludes to properties such as the ultrasound velocity, attenuation coefficient and acoustic impedance[101,102]. A particular subset is called optical elastography for which optical properties such as the scattering coefficient, scattering anisotropy and refractive index should be mimicked[101]. Furthermore, thermodynamic properties are important when mimicking a tissue's response to wound cauterizing, thermal ablation and targeted laser therapies[103].

Finally, the fracture properties coupled with the stiffness are important factors for surgical tissue mimics. Many surgical procedures involves needle insertion processes, which lead to the characterisation of complex insertion force profiles that are greatly influenced by the tissue's architecture. Parameters that are often identified and aimed to mimic in this field are the cutting energy, average insertion force, number of puncture peaks. The next section expands on literature reporting fracture properties since they are usually used to characterise a tissue mimicking hydrogels for surgical training applications.

2.3. Soft Tissue Phantoms for Surgical Applications

In experimental research, tissue mimicking materials (TMMs) are an attractive alternative to real tissues due to their synthetic nature, allowing for better accessibility and more ethically viable procurement. Therefore, the development of a mechanically accurate and reproducible TMM is a valuable field to explore. Thus, a wide variety of TMMs have been developed for a range of applications, both medical and non-medical. TMMs detailed in previous literature are reviewed here, focusing on soft tissue phantoms used in surgical needle insertion experiments to study tissue-needle interactions.

The selection of a soft tissue phantom material is typically chosen based on the material properties associated to specific applications. For example, material properties required for phantoms in ultrasound biopsy are acoustical properties, such as the speed of sound and attenuation coefficient[104,105]. Whereas, mechanical properties, such as the fracture toughness and friction coefficient are required to be matched in phantoms used in surgical intervention experiments[67]. In all cases, the stiffness of the phantom should be in good accordance with that of the real tissue being studied[63].

For the application of surgical needle insertion, there is often a specific and delicate target area that must be reached, which may be for example a target tumour or the target site for drug delivery. The insertion of a needle towards the target will cause tissue deformation resulting in the target being displaced[106]. This displacement is called target motion. The insertion force is related to the tissue deformation by the material stiffness, which is therefore a key material parameter that must be matched by a phantom material in order to accurately study target motion.

Table 8. Gelatine concentrations and their stiffness found in literature.

Author	Gelatine (wt%)	Stiffness (kPa)
Leibinger et al. [63]	3.4	-
Oldfield et al. [106]	6	-
Swaney et al. [109]	10	-
van de Berg [111]	10	O(10)
Podder et al. [112]	5, 10, 20	-
van Veen et al. [110]	8, 14.9, 20	8.7, 35.5, 58.1

As such, gelatine is often chosen as a soft tissue phantom due to its non-toxicity, accessibility and tunability of its stiffness, which is primarily controlled by varying its concentration[107].

Additionally, gelatine is brittle and results in needle insertion forces close to that of brain[63]. Therefore, gelatine has been used as a phantom in surgical needle insertion studies as the stiffness may be tuned to approximate that of various soft[108–110]. Table 8 lists a summary of the gelatine concentrations found in literature that have been used as phantoms for various soft tissue studies.

In addition to the stiffness, the fracture mechanics of the phantom material when subjected to needle insertion should also be matched with real tissue studies. For soft delicate tissues, performing tensile tests to determine the fracture toughness is challenging due to the difficulties encountered when fixing the specimen to the testing machine platen. Moreover, for tissue phantoms such as gelatine, stress concentrations due to the shape of the tensile samples and imperfections on the surfaces cause premature fractures that are difficult to avoid and time consuming to correct.

Therefore, the fracture toughness is often characterised by measuring the insertion force in the axial needle direction and obtaining the fracture toughness from fracture mechanics or energy methods[113–115]. For the work presented in this report, fracture is characterised by crack growth, which is more adequate for use in a model of needle insertion. There is also literature reporting the use of an experimental wire cutting technique to obtain the fracture toughness [107,116–118]. The findings from these two methods on gelatine are found summarised in Table 9 along with literature values of real soft tissues for comparison. There are fewer literature describing the fracture toughness of other soft tissue phantoms compared to gelatine and what has been found is also shown in Table 9.

Table 9. Energy release rate, G_c , of gelatine phantoms.

Author	Method	Material	G_c (J/m ²)
Forte et al. [107]	Wire cutting	gelatine 10 % wt.	1.1
Czerner et al. [116]	Wire cutting	gelatine 10 – 33 % wt.	5 - 34
Oldfield et al. [114]	Needle insertion	gelatine 6 % wt.	17.43
Datla et al. [69]	Needle insertion	PA 8 %, BIS crosslinker 0.35 %	136
		PA 11 %, BIS crosslinker 0.22 %	38
Dumitriu et al.	Wedge penetration	Agar	4 - 32
Azar et al. [115]	Needle insertion	Porcine Liver	76 - 185

From the findings for gelatine phantoms in literature, it is apparent that these two methods lead to different values for the energy release rate, which is related to the fracture toughness of the material. It is difficult to conclude which method leads to more accurate results, however the needle insertion method is suitable for the interests of this work and has been shown to be used more widely for the study of real soft tissues in literature as well[115,119,120]. The strain energy release rate, G_c , can be obtained from needle insertion test results by the following equation derived from an energy balance:

$$W_{G_c} = W_{ext} - W_f - W_\varepsilon \quad 2.7$$

Where W_{ext} is the external work done by the needle and W_f is the frictional work, which are obtained experimentally, and W_ε is the material deformation strain energy estimated computationally, leading to the resolution of W_{G_c} : the energy used to create the crack. W_{G_c} is defined by the energy release rate, G_c , given in Eq. 2.8. The crack width, w , is a function of the penetration depth, x , and X_{max} is the full needle penetration depth. In an article by Oldfield et al.[121] the crack width is assumed to be constant through the depth of the sample, $w(x) = 15$ mm. Eq. 2.8 can therefore be solved to find G_c .

$$W_{G_c} = \int_0^{X_{max}} G_c w(x) dx \quad 2.8$$

The PA gels show that the fracture toughness may be controlled by varying the concentrations of the constituting monomer and crosslinker concentrations so that the stiffness remains near constant, referring to Table 8, but the fracture toughness is greatly different. However, the energy release rates obtained at by the PA phantoms at the compositions described in the literature are too high to mimic that of delicate soft tissues such as brain. In addition, in real tissues, the range of energy release rate found can vary widely, which occurs due to the inhomogeneity of real soft tissue[115]. Few literature exists characterizing the fracture toughness of brain due to its scarcity and difficulties encountered when measuring the crack width, a key parameter when calculating the energy release rate.

Overall, it may be concluded that gelatine has been used as a tissue phantom for many needle insertion into soft tissue studies due mainly to its low cost, accessibility and ease of use above other phantom materials. However, a novel composite hydrogel material, PVA-phytagel, has been introduced, which can be tuned to more accurately mimic the viscoelastic behaviour of soft tissues[63]. There is therefore scope to appreciate the capabilities of this new phantom material by systematically testing and understanding the effect of PVA and phytagel concentration independently on the viscoelastic and fracture properties of the phantom material. One of the aims of this work is to construct a full mapping of the compositions of CH required to produce a phantom with the mechanical properties of a wide range of soft tissues.

2.4. 3D Printing of Hydrogels for Tissue Engineering

Since the introduction of additive manufacturing 3D printing technology, there has been scope to develop these machines to print with a wide range of different materials, ranging from concrete to food[122,123]. Any flowing substance that can be turned into a stable matrix upon extrusion may be used as an ink. Therefore, with the constant development of new soft tissue phantom materials that can be used as printing inks, the field of 3D printing biological structures has been growing for the past three decades. It is even possible to extrude living cells suspended in the printing ink, which gives it the name bioprinting[124]. Munaz et al.[125] completed a recent review of a wide range of bioinks used for both hard and soft tissue scaffolds, which is shown in **Table 10**. Along with many different bioinks, there are various nozzle head configurations that can be used to deposit the bioinks. The main three categories for these are 1) ink-jet, 2) extrusion and 3) laser jet and examples of schematics of these print heads are shown in Figure 7.

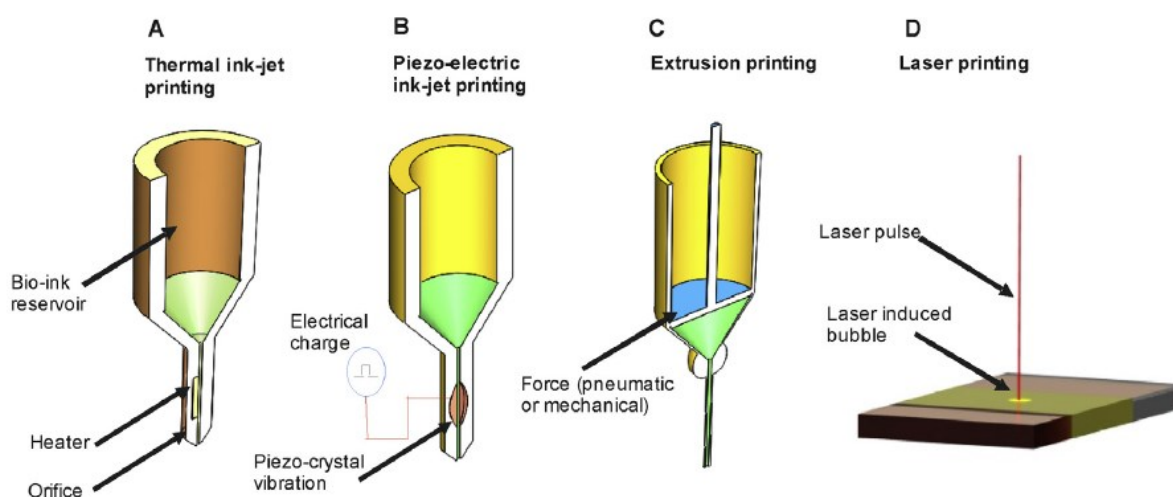


Figure 7: Schematic diagrams of a) thermal ink-jet, b) piezo-electric ink-jet, c) extrusion and d) laser-based printing systems [125].

The advantages of an extrusion-based system is that it does not rely on generating high temperatures to allow the bioink to flow, therefore cells suspended in the ink are more likely to survive the printing process. However, the ink-jet and laser-based systems have the advantage of being able to print bioinks with high viscosity, which are difficult to print with extrusion-based systems, by creating tiny bubbles of high pressure and temperature to force the ink out of the fine needle. A recent development in the field is the use of laser-based stereolithography, which utilises UV radiation to cross-link UV curable hydrogels. Due to the focused laser beam technology, the stereolithographic technique allows intricate 3D constructs to be created. This has allowed researchers to investigate the impact of how the geometry of a cell seeding substrates affects cell behaviour, including adhesion, proliferation and migration[126–129].

Table 10. Summary of bioinks found in literature [125].

Printed objects	Printing technique	Bio-ink formation				Media	Implants	Ref.
		Scaffold	Encapsulator	Cells/Protein	Cross linker			
Hard Tissues	Thermal	CaP:CaSO ₄ , HA:CaSO ₄ and, β -TCP:CaSO ₄	-	-	Water based binders	-	In-vitro	[130]
	-	β -TCP, bio-active glass (45S5 Hench glass)	-	-	H ₃ PO ₄ , H ₇ P ₂ O ₇	-	In-vitro	[131]
	Thermal	CaP solutions with α -TCP and HA	-	C3H/10T1/2	Collagen, Acidic binder (phosphoric acid)	-	In-vitro/ In-vivo	[132]
	-	PLA coated with PDA	-	hADSCs	-	DMEM, FBS, penicillin, streptomycin	In-vitro	[133]
	Extrusion	PCL, PLGA	Collagen, gelatine	hTSMCs, rhBMP-2	-	DMEM, FBS	In-vitro/ In-vivo	[134]
	Thermal	Chondrogenic progenitor plugs (bio-paper)	PEGDMA	Human articular chondrocytes	Photo initiator	DMEM, Human serum, penicillin, streptomycin, glutamine	In-vitro	[135]
Soft Tissues	Laser	Titanium powder	-	Human osteogenic sarcoma (MG63)	Silica sol	-	In-vitro	[136]
	Extrusion	-	Agarose rods	CHO, HUVMSCs, HSFs, PASCs	-	DMEM, FBS, antibiotics (penicillin, streptomycin, gentamicin), Geneticin, Hams F12, glutamine, gelatine	In-vitro	[137]
	Extrusion	gelatine, alginate, chitosan, fibrinogen	gelatine, alginate, chitosan, fibrinogen	Hepatocytes, ADSCs	Thrombin, CaCl ₂ , Na ₃ P ₃ O ₁₀ and glutaraldehyde	DMEM, FBS, penicillin, streptomycin, aprotinin	In-vitro	[138]
	Extrusion	PCL	DAT	hASCs	-	DMEM, FBS, penicillin, streptomycin	In-vitro	[139]
	Extrusion	PCL	adECM, cdECM, hdECM	hASCa, hTSMCs	-	DMEM, α MEM, FBS, antibiotics (penicillin, streptomycin)	In-vitro	[140]
	Extrusion	-	RGD-GG	Primary cortical neural cell	DMEM or CaCl ₂	Collegenase, FBS, neurobasal media, glutamine, penicillin/streptomycin	In-vitro	[141]
	Extrusion	-	Sodium alginate, gelatine	Primary myoblast (BL6)s	CaCl ₂	DMEM, FBS, penicillin, streptomycin, Hams F10, glutamine	In-vitro	[142]
	Ink jet	-	Gellan gum	ECs and SMCs	CaCl ₂ , gelatine	EGM-2 (endothelial growth medium)	In-vitro	[143]
	Piezo-Ink-jet	Collagen bio-paper	gelatine, fibrinogen, 4 arm PEG amine	C2C12, PC12 and L929	Poloxamer 188 (P188) and/or fluorinate	DMEM, FBS, HS (horse serum)	In-vitro	[144]
	Extrusion	-	-	HDFs, HUVECs	PEGX-gelatine, PEGX-fibrinogen, EDC {N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide}, NHA (N-Hydroxysuccinimide), thrombin	PBS (phosphate-buffered saline) or DMEM, FBS, antibiotics (penicillin, streptomycin)	In-vitro	[145]
	Thermal	Sodium alginate-collagen composite	Hydrogel solutions (Bovine type B gelatine)	hAFSCs, dSMCs, bECs	CaCl ₂	MEM, DMEM, EBM-2, clonetics, FBS, glutamine, penicillin/streptomycin	In-vitro/ In-vivo	[88]
	Extrusion	gelatine methacrylamide	-	HepG2	1-[4-(2-Hydroxyethoxy)-phenyl]-2-methyl-1-propane-1-one, and 2,2'-Azobis[2-methyl-N-(2-hydroxyethyl)propionamide]	DMEM, FBS, penicillin and streptomycin	In-vitro	[146]
	-	-	Sodium alginate	HAFs and HUVECs	-	DMEM, penicillin/streptomycin, EGM-2	In-vitro	[147]
	Extrusion	PCL, alginate solution	-	Chondrocytes, osteoblast	CaCl ₂ , NaCl solutions	DMEM/FBS/penicillin and streptomycin	In-vitro	[148]

The majority of studies utilising stereolithographic printing methods focus on the use of polyethylene glycol (PEG) hydrogel as a printing ink, which is widely used in the tissue engineering field as a result of its biocompatibility, biodegradability and, most importantly in this context, cross-linked by UV radiation. An example of this is the work by Fozdar et al.[128] who have investigated the effect of the cell seeding substrate geometry on cell migration by 3D printing auxetic PEG constructs, which are structures that exhibit a negative Poisson's ratio (NPR). The structure is able to demonstrate this material property thanks to its re-entrant feature, as shown in Figure 8 (A) and (B). Soman et al.[129] showed the feasibility of seeding fibroblast cells onto these structures to set the stage for investigations into mechanical strain mediated cell differentiation, as shown in Figure 8 (C).

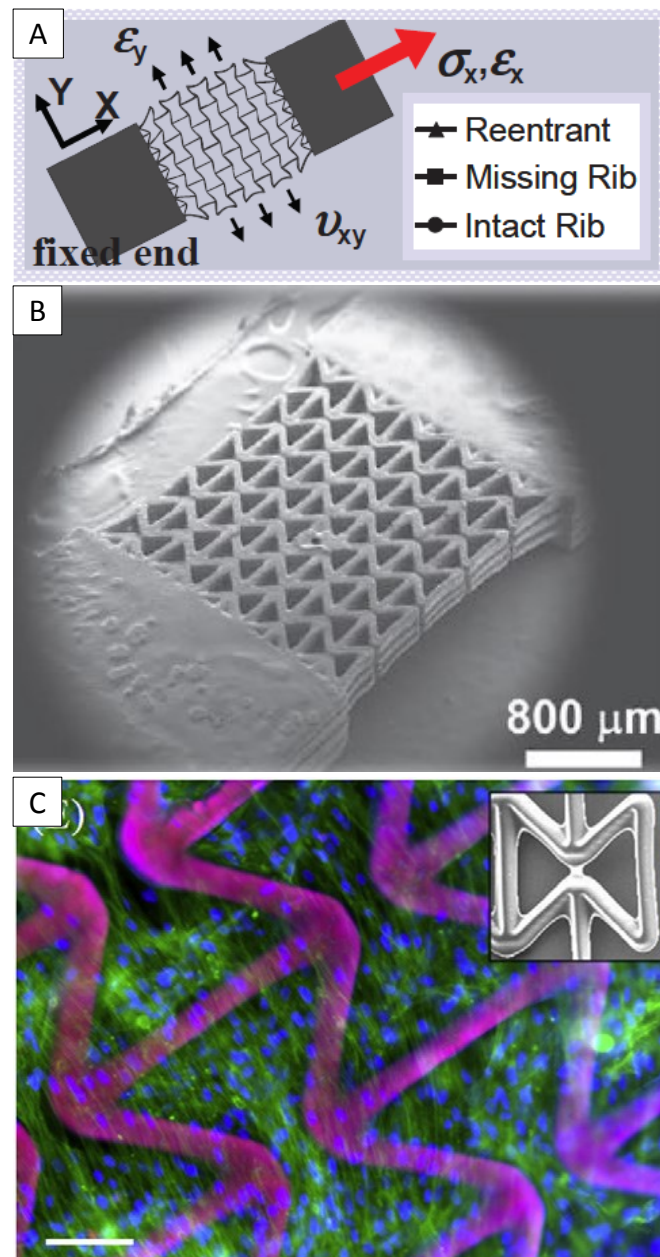


Figure 8: Use of stereolithographic 3D method to achieve complex 3D structures showing (A) a schematic explaining the behaviour of an auxetic structure[128], (B) 3D printed auxetic structure[128] and (C) application of these scaffolds in tissue engineering studies of fibroblasts, scale bar 125 μm [129].

However, traditional stereolithographic 3D printing of hydrogels does not allow *in situ* cells to be printed successfully. This is because the high level of laser energy used by the stereolithographic technique is too harmful for the cells suspended in printing media to survive. Due to this, cells are usually seeded after the construct has finished printing and as such, this method is not true 3D bioprinting. Just recently, Grigoryan et al.[149] pioneered the use of food colouring dye and as a cross-linking agent, which requires much lower levels of radiation, enabling actual cell-laden bioprinted constructs to be created, as demonstrated in Figure 9.

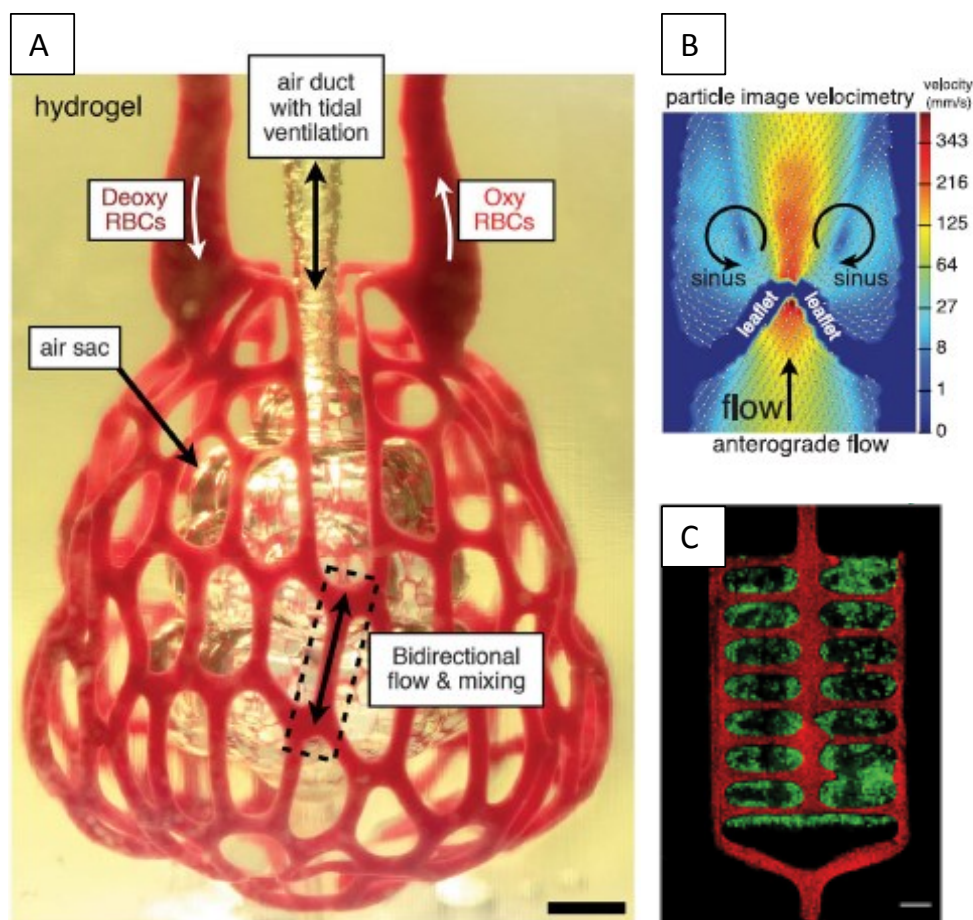


Figure 9: 3D printed, cell laden constructs using stereolithography of (A) lung aveoli structure, (B) bicuspid valve and (C) hepatic hydrogel carriers for liver disease therapy[149].

The application of this method to help the shortage of organ donors by printing full human organs has been popularised by surgeon Anthony Atala[87]. Hence, bioprinting is a rapidly developing field despite the technological and biological challenges. Hinton and co-workers have demonstrated the extrusion-based printing of biological structures, such as the arterial branches shown in Figure 10 using alginate, collagen and fibrin gels with < 500 kPa stiffness, using a gelatine slurry support bath[124]. Hinton and co-workers also demonstrate the precision of their system by printing a scaled down human brain using an alginate bioink, shown in Figure 11. However, the stiffness of this ink is $O(10)$ kPa and therefore does not match that of real human brain, which is $O(1)$ kPa[124,150].

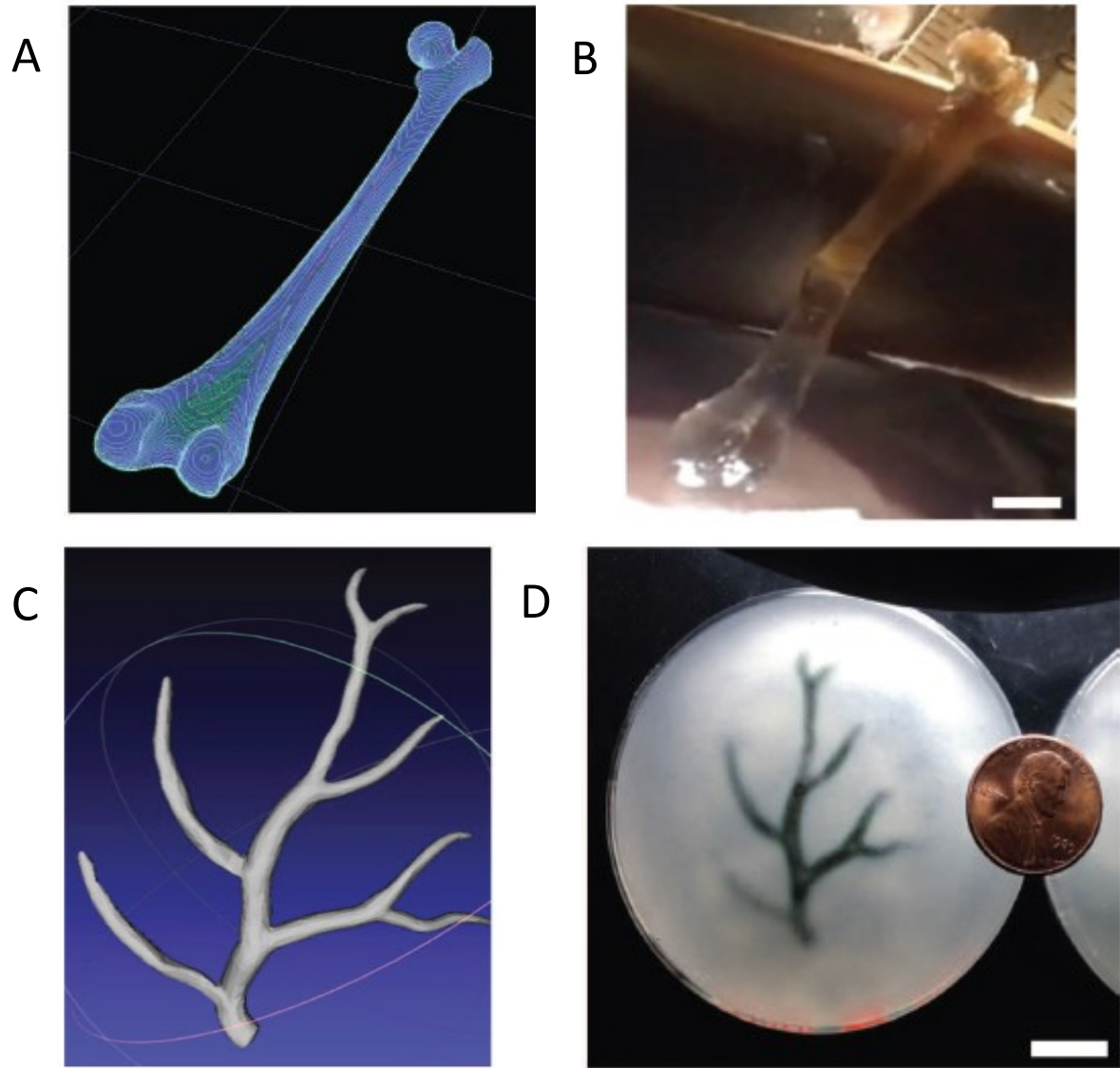


Figure 10: Biological structures; (A) model of femur, (B) printed alginate femur, (C) model of human coronary arterial tree and (D) printed arterial branch [124].

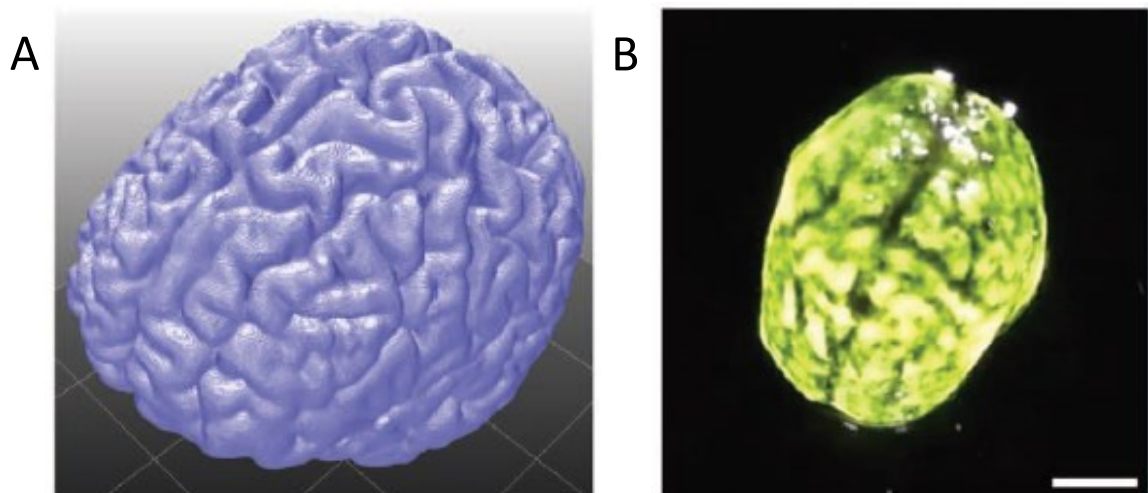


Figure 11: (A) model of human brain from MRI data and (B) printed alginate brain with scale bar 10 mm.

In another study, Lozano et al.[141] used a RGD modified gellan gum 1 % wt. hydrogel bioink with encapsulated cortical neuron cells to demonstrate the ability to print soft 3D cell-laden constructs, although the material stiffness was not characterised. The neurons exhibited good attachment and proliferation inside the gellan gum scaffold as shown in Figure 12. However, the structure was printed using a hand-held extrusion-based syringe, which lacks the precision required for printing complex structures in 3D.

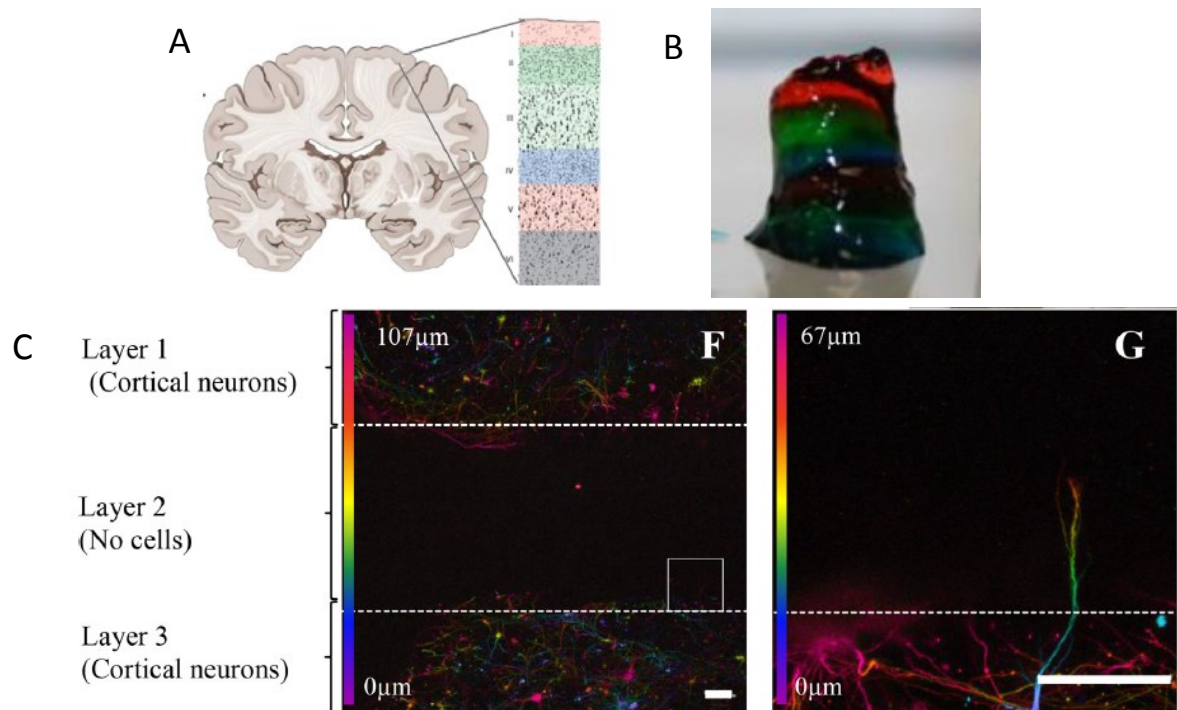


Figure 12: (A) Different layers in brain grey matter, (B) printed structure where the colours represent different layers and (C) confocal microscope of neurons in the layers after 5 days culture [141].

The challenge remains to print very soft structures with stiffness close to brain. This is because the printed structure is so soft it is unable to hold its shape or allow further layers to be built on top of it. Aside from utilising a supporting slurry bath as mentioned previously, the hydrogel solution can be solidified through an almost instantaneous freezing method. In their PhD thesis, Barnett[151] demonstrated the 3D printing ability of intricate ice sculptures by utilising the phase change of water. Since super soft hydrogel solutions are around 95 wt% water, the freezing point and rate is very close to water. Adamkiewicz et al.[152] have gone on to show that a cryogenic method can be used to 3D print hydrogel by means of a liquid nitrogen bath. As such, the following chapters will focus on finding the tissue mimicking material formulations of the composite hydrogel and concluding with the final chapter, which will be dedicated to the development of a new 3D printing technique that is able to create super soft tissue-mimicking constructs for tissue engineering.

3. Mechanical Characterisation of Soft Biological Tissues

This chapter has been partly published in Materials and Design[153].

3.1. Introduction

The mechanical characterisation of real soft tissues, such as brain, lung and liver, is vital as it allows us to understand how they deform during a range of important scenarios. Through this enlightenment, ways to reduce damage to living tissues during real life situations can be identified and developed. For most applications, it is necessary to characterise the tissues physical properties as this describes how the material reacts to an applied load[154]. The majority of literature in this field focuses on characterising the most relevant mechanical properties of a biological tissue for a particular medical scenario.

Diagnostic imaging is an essential medical tool by which the properties of the tissue can be well characterised to allow the physicians to understand the images. For ultrasound, the speed of sound, c_1 , and density, ρ , varies in different biological tissues, which allows users to distinguish between different tissues and evaluate its elasticity. In magnetic resonance imaging (MRI), it is the energy given off by the realignment of the tissues' protons in the magnetic field that can be quantified and characterised for different tissues[155]. Optical, nuclear and radiation based imaging tools have also been developed to exploit the many variances in physical properties of different tissues[156–158]. This knowledge can be used to diagnose abnormalities in the scanned image when compared to healthy scans.

Another important field is blast injury studies. An example of this scenario is the injury caused by a blast mine explosion, where the damage is focused in the area below the knee. To understand how dynamic loading rates result in blast injury mechanisms, the mechanical properties of bone and muscle tissue have been reported at initial strain rates in the order of magnitude of 1000 s^{-1} [159,160]. Andrikakou and co-workers evaluated the hyper-viscoelastic material parameters of rabbit and rat lungs at high speed loading rates under tension and compression using a van der Waals material model[18]. These model parameters will allow computational and phantom materials models to be built to more ethically test a wider range of blast injury scenarios.

In many literature that report the material properties of biological tissues, the consideration of anisotropy due to the effect of the fundamental internal structures of the tissue is often disregarded as its inclusion would bring another degree of complication to the experimental setup and, in the case of computational modelling, require excessive computational power.

Whilst there is undisputable evidence that the architecture of the biological tissues exists, there has been little effort to systematically characterise the mechanical directionality relative to the substructures. In literature, tested samples are often taken without respect to the direction of the internal structures as it is often quite difficult to do so because of the size and orientation of the internal structures of interest[14]. For example, de Jong et al.[161] performed needle insertion tests on human liver, in an arbitrary direction and discussed that a number of large penetration peak forces, reported as outliers, may actually be interactions with the internal liver vasculature.

All biological tissues are organised with a very clever architecture that constitutes a complex yet organized internal structure. In the brain, the white tracts exist to connect and carry signals across different regions of the brain. These tracts are made up of myelinated axons that have a diameter ranging from 1-10 μm , making them impossible to characterise at the macroscopic level. As a result, diffusion tensor imaging (DTI) coupled with diffusion weighted imaging (DWI), based on MRI signals, has been developed to measure the microstructural properties of brain. Budde and co-workers were able to analyse the anisotropic nature of axonal fibres using histological staining to confirm the directionality of the axons implied by DTI results[162]. Komlosi and co-workers used diffusion MRI techniques to study the regional anisotropy of lung[163]. Using a different technique, Mitzner and co-workers found that a rat lung is anisotropic and therefore the lung air volume/surface ratio cannot be estimated using the mean airspace chord length, as it depends on the orientation of the lung section[164]. However, the literature mentioned here only briefly reports that anisotropy exists but has not quantified how this affects the mechanical properties of the tissues.

Muscle fibres are among one of the most well studied biological tissues involved in extensive mechanical testing. Kuthe and co-workers reported elastic moduli for skeletal muscle of 1.59 MPa in a loading direction parallel to the fibres and a significantly lower 0.43 MPa in the perpendicular direction[165]. As such, it is becoming increasingly important to accurately determine the mechanical characteristics of biological tissues with respect to the orientation of their internal and inherent structures. There are very few studies that test, evaluate and compare the directional mechanical properties of a range of different biological tissues. The aim of this work is to systematically evaluate the mechanical properties of a range of different biological tissues. By applying the same testing methods and conditions to each biological tissue, the results are made more relevant and valid for cross-tissue comparison. Particular attention is paid to the effect of the biological substructures that introduces anisotropy to the material and may also impart significant directionality.

In order to study this, the mechanical properties that will be measured and compared must be selected. Since the focal application of this work is surgery, there are a number of crucial mechanical properties that define the behaviour of the regime. Surgery is a highly complex procedure, which involves many different surgical tools imparting a variety of different mechanical loads on the biological tissues. Among the many tools involved in surgery, the grasper is a device commonly used to hold and manoeuvre tissue. Li and co-workers measured the frictional behaviour between a medical grade steel surgical grasper and porcine liver tissue[166]. The applied load of the grasping motion was mimicked by a clamp-drag experimental setup and the authors found that trauma at the tool-tissue interface was observed in the form of haemorrhage at a clamping force or greater than 3.5 N[166]. The results of this study were able to find force thresholds that ensure no tissue damage or tool slippage, leading to effective tool usage.

In this chapter, the surgical scenario has been simplified to focusing on a single needle insertion procedure and defining the mechanical properties related to this setting. The key mechanical properties that have been identified to be crucial parameters during this procedure are the compressive stress-strain relationship, the average insertion force, and the average friction force between the needle and the tissue[167]. Experimental ex-vivo and in-vitro needle-insertion tests have been carried out on a range of biological tissues. Tonke and co-workers found the average peak insertion force that occurred during needle insertion into ex-vivo human livers was 0.18 N, with outliers of up to 1.7 N, which were due to interactions with the hepatic vasculature[161]. Azar and Hayward went on to characterise the fracture toughness of liver from needle insertion using an energy method and reported values for J_{IC} of 75.8- 185.6 Jm⁻², where the range of values was attributed to the inhomogeneity of the tissue[115]. Separately, it has also been shown that the frictional interaction at the needle-tissue interface depends on the material of the needle and any applied coatings and the needle surface roughness[168]. Not only do the the results from these tests elucidate the surgical needle-tissue interaction, but they can be used to define computational models.

The development of computational models has risen in popularity as an alternative to the experimentally destructive, and hence unethical, nature of *in-vivo* testing. Although the animal models are anaesthetised before being subjected to *in-vivo* mechanical testing procedures, they are often euthanised after the end of the test due to the terminal damage caused, which raises ethical issues regarding sacrifice of life[169-171]. A more ethical approach would be to test biological tissue after the natural death of an animal. However, *ex-vivo* testing has shown that the mechanical properties of biological tissues fluctuate with respect to hourly post mortem time, which increases the difficulty of obtaining accurate soft tissue mechanical

characterisation[44,48–50,172]. Therefore, since most soft tissue material properties found in literature are based on post-mortem tissues, their accuracy is limited by these ethical factors.

Furthermore, understanding these mechanical behaviours could help researchers, clinicians and surgeons understand how the different mechanical behaviours of each tissue impacts its biology. The discovery that not only chemical but also mechanical stimuli affects biological processes was highly impactful and gave rise to the field of mechanobiology[6,83,173–177], which studies the influence of mechanical stimuli on biological processes, such as stem cell differentiation. It is even well known that mechanical stimuli of these cells increase the risk of developing fibrosis[178].

Additionally, the mechanical characterisation of biological tissues will also provide the ground truth to which artificial tissue mimicking materials should aim to match. This is a major incentive as there are many applications that require an accurate tissue mimicking phantom. The most common is the use of tissue mimicking materials during the prototype testing of novel surgical tools. The development of a tissue mimicking material that is able to match the mechanical properties related to surgical procedures would therefore be highly suitable for use as a surgical phantom, clinical path planning and surgical training.

There are a number of inherent physiological and environmental factors. The main factors that have been addressed in this study are post-mortem time and directionality. Post-mortem time until testing is known to affect the stiffness of the tissue drastically. However, it was infeasible to acquire *in-vivo* mechanical properties in this project due to the destructive nature of these tests raising ethical limitations for both human and animal tissues. This further highlights the importance of developing an artificial tissue mimicking material for destructive testing scenarios.

3.2. Materials and Methods

3.2.1. Biological Tissue Sample Preparation

Porcine brain, lung and liver were procured from a supplier within 24 hrs post-mortem. The porcine brain was kept hydrated until tested. A biopsy punch and surgical scalpel were used to prepare the samples for testing. For compression tests, samples of 9.70 ± 0.90 mm diameter and 6.85 ± 1.61 mm height were cut using a biopsy punch. The biological samples obtained using a biopsy punch were often conical in shape due to the dragging of the tissue surface in contact with the punch, before cutting occurred. Therefore, the average diameter was reported and used for calculation of true stress in this work, as is also performed by Miller et al.[179].

All biological tissue samples (n=6 per organ) were tested 1 hr after procurement. White and grey matter samples from all the regions (posterior, superior and anterior) of the cerebrum were tested. White matter samples from the corona radiata were taken with respect to the anatomical planes (coronal, sagittal and transverse) to assess directionality, as depicted in Figure 13. Samples from the cerebellum were also mechanically tested and compared to cerebrum results as the cerebellum represents a mixed white and grey matter composition. For lung and liver, samples were taken in the transverse direction relative to the internal structures of the tissues, which are respectively, the bronchi and major hepatic veins (n=6).

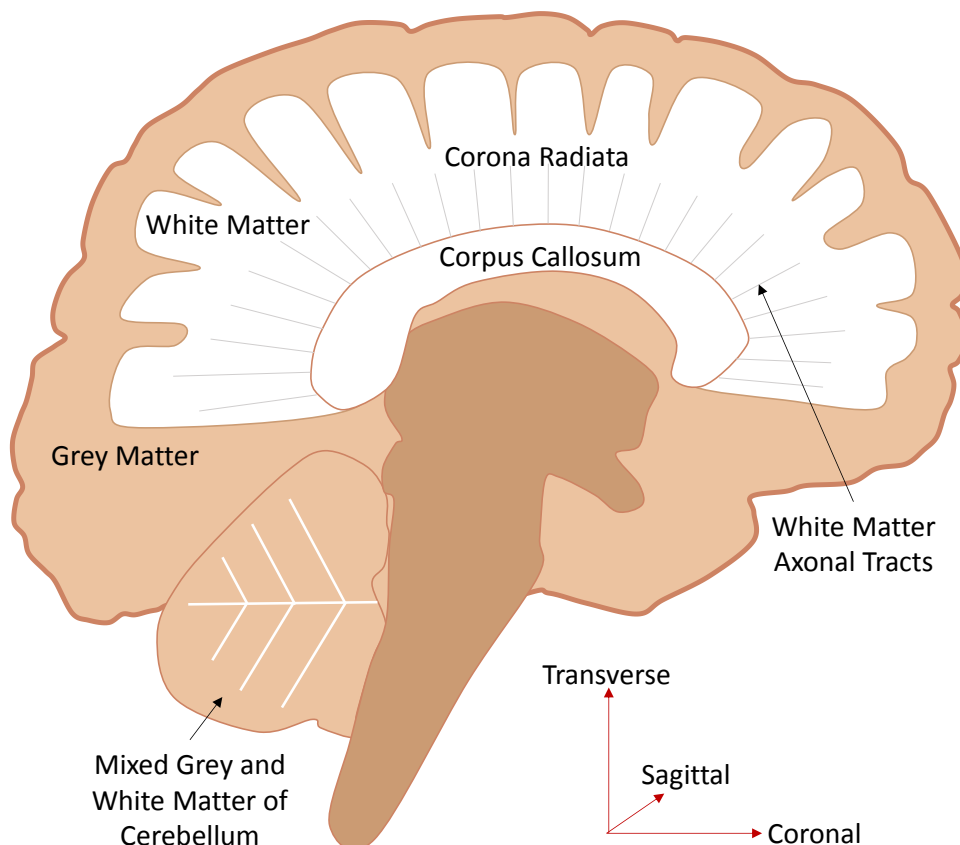


Figure 13: Schematic of brain showing areas and directions in which samples were taken.

The architecture of the lung consists of a deeply branching bronchial tree that maximises the capacity of air intake and hence respiratory function of the organ, as depicted in Figure 14. The bronchial walls are composed of many cartilaginous platelets, which allow them to expand and contract to match the expansion of the soft parenchymal tissue[180,181]. This cartilaginous material has a greater stiffness than the functional parenchyma[182]. It follows that this stiffer internal structure would impose anisotropy and mechanical directionality on the tissue. It is therefore important to study the mechanical properties of the lung with respect to the direction of the local bronchial branches. The individual stiffness of the bronchi was also measured in the axial direction ($n=3$) and compared to the parenchymal tissue samples taken in the axial and transverse directions.

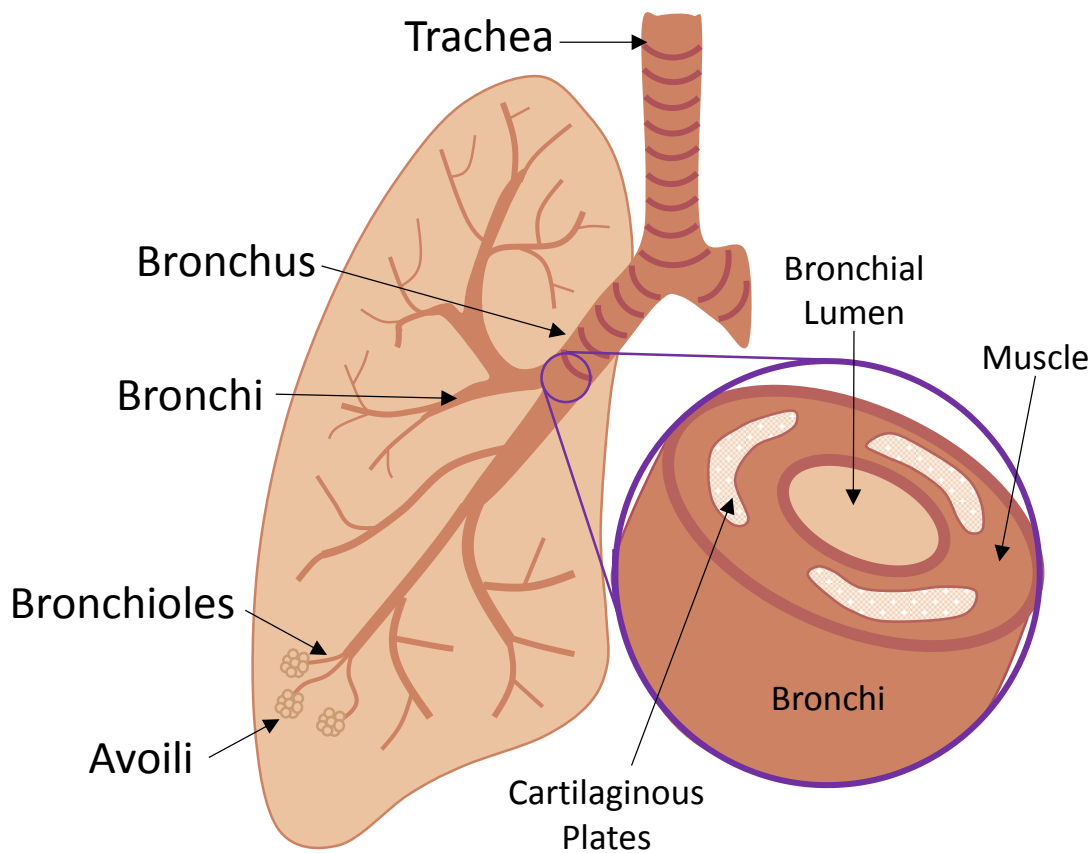


Figure 14: Schematic of lung showing the internal branching bronchial tree structure.

The characterisation of liver tissue involved taking into account the main internal vasculature feature, the hepatic veins, as shown in Figure 15. The effect of the hepatic vein orientation on the anisotropy of mechanical properties in liver was therefore investigated. However, it was not possible to isolate and test the compressive stiffness of the venous membrane itself as it is not cartilaginous but has the standard venous membrane structure comprising of a collagenous outer wall, a muscle and elastin inner wall and is lined with endothelium cells[183]. However, the venous architecture involves a vena cava of larger diameter and thicker membrane which

was related to the local thickness of the liver. Therefore, the effect of liver thickness on the compressive stiffness was also studied by comparing samples taken near the vena cava to those at the edges of the liver where the veins are very small. In addition, to investigate the effect of post-mortem time on tissue degradation over longer periods, porcine liver samples were kept at 4°C and tested after 7 days.

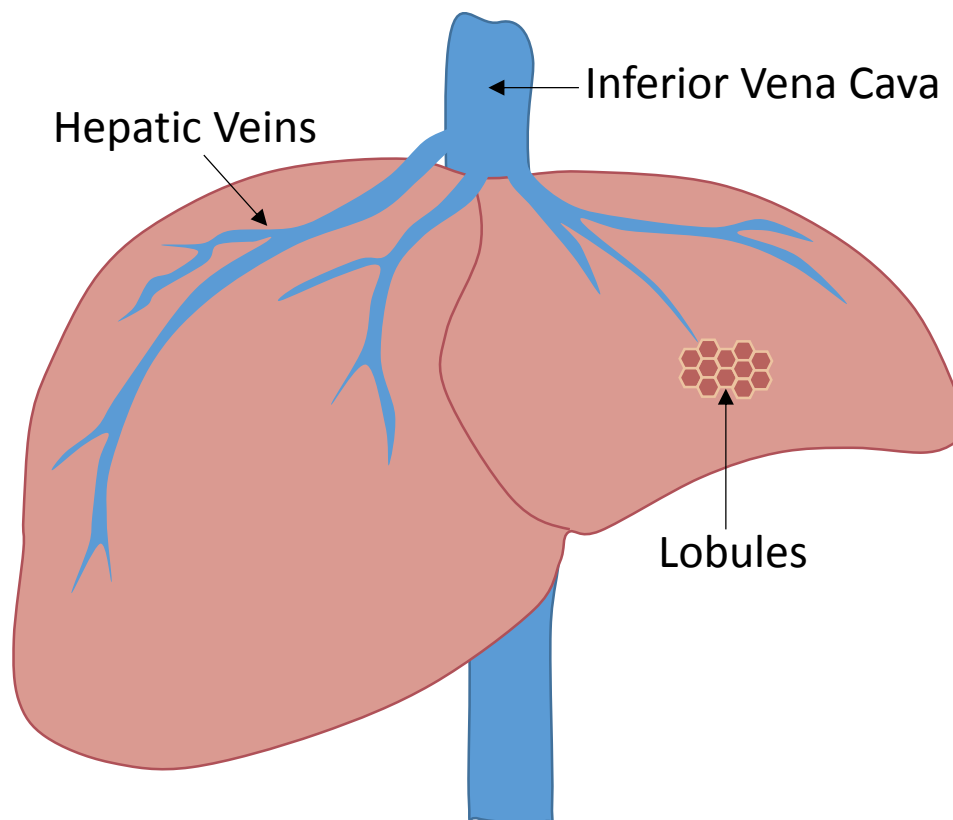


Figure 15: Schematic of liver showing the hepatic veins and the hexagonal lobular tissue structure.

The sample direction is illustrated in **Figure 16**. The lung samples therefore contained minor bronchi running transversely through the diameter of the sample. For porcine liver, samples were taken in the central region of the liver. Since the major hepatic veins in porcine liver are larger than the sample diameter, it was possible to avoid taking samples with significantly sized veins running through them.

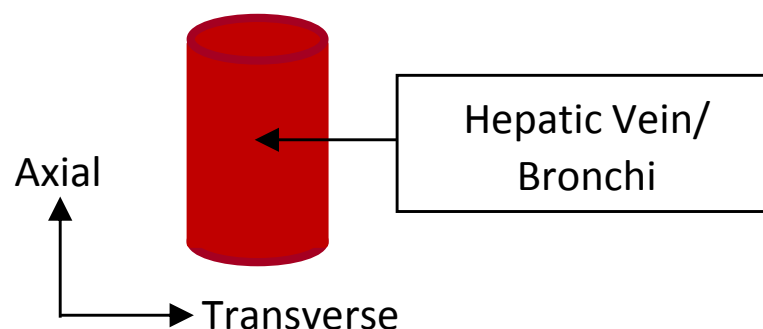


Figure 16: Schematic showing the definition of axial and transverse directionality.

3.2.2. Compression-Relaxation Tests

Three mechanical characteristics were used as criteria to find the best CH compositional match: true stress at 30% strain, average insertion force and average friction force. The tests were performed on the biological porcine brain, lung and liver samples. Directionality with respect to the internal biological macrostructures in lung and liver was assessed by testing samples taken in the axial and transverse directions relative to the structure ($n=6$), as depicted in Figure 16. Unconfined uniaxial compression tests to 30% strain at 0.01 s^{-1} initial strain rate were performed using a Mach-1TM mechanical testing system (Biomomentum, Canada), the same parameters as used in other literature [14,18,60]. Initial strain rate is calculated by dividing the crosshead speed by the sample height, therefore the crosshead speed was varied according to sample height to maintain a constant initial strain rate. All strain rates referred to in this thesis and literature are obtained using this initial strain rate calculation. The unconfined compression rig is equipped with two stainless steel plates, a fixed bottom platen and a controlled top platen connected to the load cell directly. The testing set-up is shown in Figure 17 (A). Two load cells were used to measure the forces on the testing samples over a wide range of magnitude, which were a 1.5 N load cell (Honeywell, USA) and 70 N load cell (Biomomentum, Canada). The compression test took under 30 seconds to complete, which is not long enough to induce any dehydration effects in the biological samples according to Forte et al.[15], therefore the samples were not kept in a hydration bath. True stresses and strains, along with error bars depicting standard deviation, were calculated and displayed in the results graphs of all unconfined compression tests.

3.2.3. Needle Insertion Tests

Needles with a diameter of a few millimetres are commonly used in neurosurgery, for example in minimally invasive biopsy procedures [167,184]. The needle insertion tests were performed using a rigid 3D printed needle with 4 mm diameter and 40° conical tip connected directly to the load cell in a similar setup to Leibinger *et al.*[63]. The needle displacement rate was 0.01 mm/s . The biological samples were placed in a $60 \times 60 \text{ mm}$ perspex box, which was large enough to avoid any edge effects, as is shown in Figure 17 (B). Each hemisphere of the porcine brain was tested separately. Additionally, the tests were focused on the cerebrum of the porcine brain and not the midbrain or cerebellum as the internal structure is significantly different, as depicted in Figure 17 (C). The porcine lung and liver test specimens were 25 mm sample depth. The needle was inserted all the way through the sample and out the bottom surface of the box, through a 10 mm diameter laser cut hole, designed to minimise alignment issues and excessive resistance of the tissue as the needle completed its travel through the bottom end of the box. The mechanical behaviour of the biological and CH samples was then analysed.

From the experimental data obtained during the unconfined compression tests, the true stress was calculated at 30% engineering strain under incompressibility assumption, which is commonly used to model soft tissues[15,17]. The average insertion force was calculated as the average force of the entire insertion stage, including repeated rupture, and the average friction force was calculated as the average force after the needle passed through the entire sample and through the bottom of the box. For the analysis of the insertion testing results, engineering strains were used to describe the position of significant features, namely the first penetration peak, and are obtained by calculating the displacement at which the feature occurs over the sample thickness. Therefore, a penetration peak taking place at an engineering strain of greater than 100% should be understood as having occurred after the needle has passed through the bottom hole of the box.

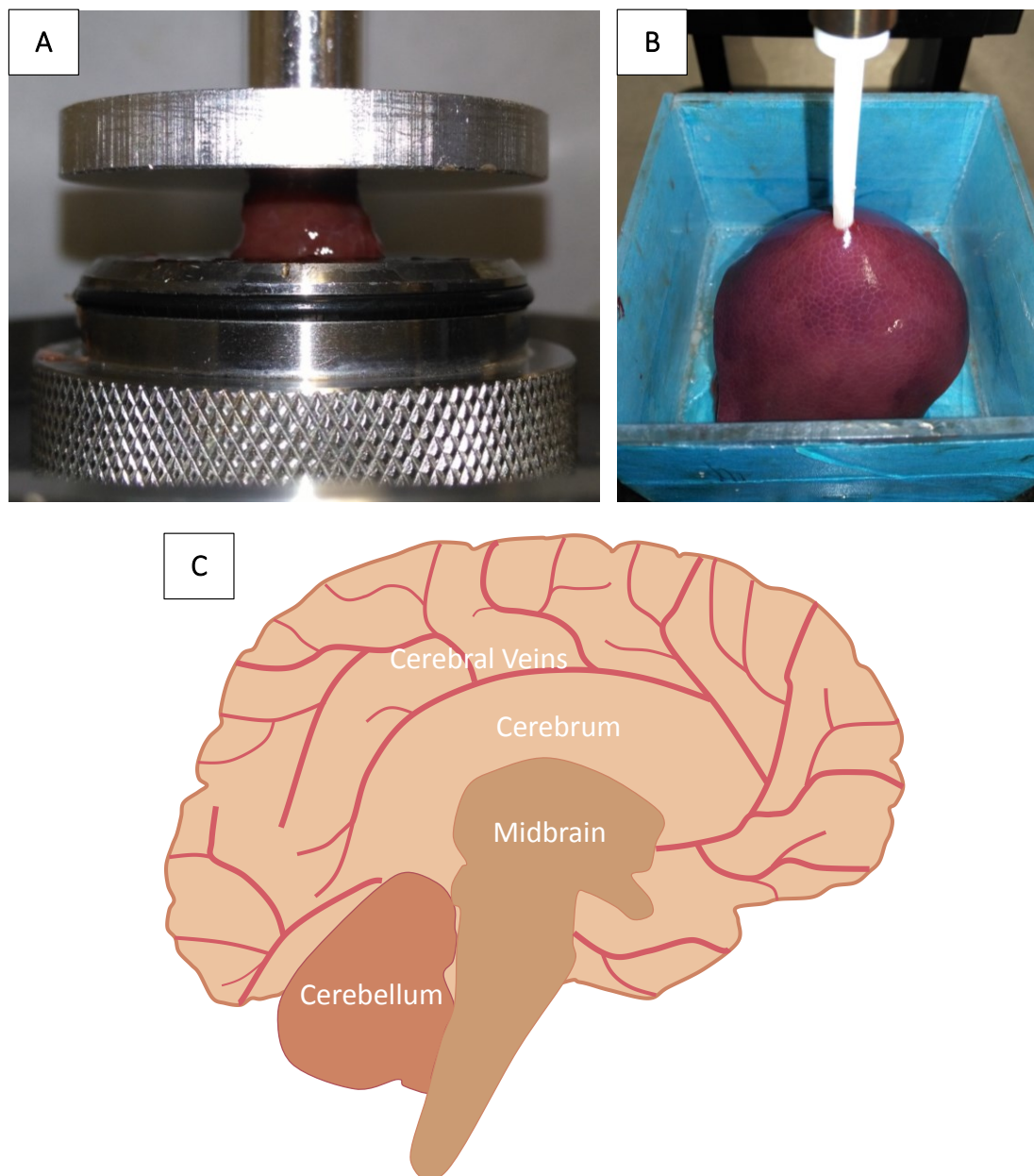


Figure 17: (A) Shows the mechanical testing setup for uniaxial unconfined compression, (B) needle insertion testing setup and (C) illustration of the outer surface of the brain showing the cerebral veins.

3.2.4. Hyperelastic Constitutive Material Model

As established in Chapter 2, section 2.1, a hyperelastic material model is more accurate at large strains than the linear elastic modulus when describing the relationship between material deformation and force experienced by biological tissues. Large strains are critically experienced during surgical intervention; it is therefore important to accurately predict the behaviour of tissues at high strains. The Mooney-Rivlin hyperelastic model was fitted to the experimental results as it has been shown in literature to be a good constitutive model for soft biological tissues[16,185–188]. Since the work focuses on compression only, a first order Mooney-Rivlin model was deemed sufficient to match the experimental results. However, it should be noted that in the literature it has been shown that a ninth order Mooney-Rivlin model has been able to represent the behaviour of brain under a wide range of tensile and compressive strains[189].

The general formula for principal Cauchy stress, σ_i , in compressible Mooney-Rivlin model is given by the following expression:

$$\sigma_i = 2C_1 J^{-\frac{5}{3}} \left(\lambda_i^2 - \frac{1}{3} I_1 \right) + 2C_2 J^{-\frac{7}{3}} \left(\lambda_i^2 (I_1 - \lambda_i^2) - \frac{2}{3} I_2 \right) + 2D_1 (J - 1) \quad (3.1)$$

Where λ_i is the principal stretch. For unidirectional loading, i.e. uniaxial compression, $\lambda_2 = \lambda_3$. Since $\lambda_1 \lambda_2 \lambda_3 = J$, this leads to $\lambda_2 = \lambda_3 = \sqrt{J/\lambda_1}$. Therefore the expression for the invariants becomes:

$$I_1 = \lambda_1^2 + \lambda_2^2 + \lambda_3^2 = \lambda_1^2 + \frac{2J}{\lambda_1} \quad (3.2)$$

$$I_2 = \lambda_1^2 \lambda_2^2 + \lambda_1^2 \lambda_3^2 + \lambda_2^2 \lambda_3^2 = 2\lambda_1 J + \left(\frac{J}{\lambda_1} \right)^2 \quad (3.3)$$

As $\sigma_2 = 0$ for the unidirectional loading case,

$$\sigma_1 = 2 \left(C_1 J^{-\frac{5}{3}} + C_2 \frac{J^{-\frac{4}{3}}}{\lambda_1} \right) \left(\lambda_1^2 - \frac{J}{\lambda_1} \right) \quad (3.4)$$

Biological tissues are often assumed to be incompressible as their measured Poisson's ratio is close to 0.5[185,190,191]. The incompressibility assumption results in $J = 1$, leading to the simplified expression for Cauchy stress in terms of axial stretch:

$$\sigma_1 = 2 \left(C_1 + C_2 \frac{1}{\lambda_1} \right) \left(\lambda_1^2 - \frac{1}{\lambda_1} \right) \quad (3.5)$$

Where the constants C_1 and C_2 are to be tuned to match the experimental results. The material constants were determined using the MATLAB curve fitting tool which utilises a least squares corrected fit to output the coefficients which result in the best fitted model.

3.2.5. Viscoelastic Material Model

Biological tissues are well known to be viscoelastic. Viscoelasticity is the dependency of mechanical properties on the time. Viscoelasticity can be studied through creep or relaxation tests. In compression-relaxation tests, the strain applied in the compression step is kept constant. If the testing material is viscoelastic, the force will change although the applied strain is kept constant. The material properties that are used to describe this behaviour can be defined using the Maxwell generalised model as introduced in Chapter 2, section 2.1.

A third order Maxwell element equation was used to model the stress-relaxation test data for the biological tissues. The Prony series expression, where E_i and τ_i are the parameters to be optimised to fit the experimental data, $E(t)$, is as follows:

$$E(t) = E_{\infty} + \sum_i E_i e^{\left(\frac{-t}{\tau_i}\right)} \quad (3.6)$$

Where E_{∞} is the equilibrium modulus that can be found by calculating the ratio between the equilibrium true stress, $\sigma_{T\infty}$, and equilibrium true strain, $\varepsilon_{T\infty}$, which is equal to 0.35 at 30% engineering strain:

$$E_{\infty} = \frac{\sigma_{T\infty}}{\varepsilon_{T\infty}} = \frac{\sigma_{T\infty}}{0.35} \quad (3.7)$$

3.3. Results and Discussion

3.3.1. Porcine Brain

Firstly, the comparison between white and grey matter samples show that white matter is stiffer by 28.7% than grey matter, with stress values at 30% engineering strain of 475 ± 132 Pa and 369 ± 91 Pa respectively. This finding confirms results presented in the literature[15,192–194]. For example, white matter was reported to be 36% stiffer than grey matter, with values for elastic modulus of 1.895 ± 0.592 and 1.389 ± 0.289 kPa, respectively, by Budday et al.[193]. An average shear modulus of white and grey matter of 1.027 ± 0.422 and 0.741 ± 0.118 kPa, respectively, which corresponds to a 39% difference between the tissues, was reported by van Dommelen et al.[192]. Forte and co-workers investigated the compression at three compression rates (500, 5 and 0.05 mm/min) and found that white matter was on average 80% stiffer than grey matter[15].

The reason for the difference in mechanical properties between the white and grey matter is down to the different materials that the individual areas are made of. The organisation of the white and grey matter microstructure are also likely to affect their mechanical properties. Grey matter, forming the outer layer of the cerebral cortex, consists of the neural cell bodies. Different regions of grey matter are connected to each other via axons (nerve fibres that conduct brain signals), which make up the white matter. Axons are encapsulated in oligodendrocytes which wrap around the axonal core, forming long cylindrical highways for electrical pulses. The oligodendrocytes produce myelin which acts as an insulator to improve the connection between neurons. Myelin is the component that gives this material its opaque white colour, and so giving rise to the name white matter. Literature has shown that myelin content is one of the factors that gives rise to a stiffer material response[195,196].

Figure 18 also shows the results obtained from the porcine cerebellum. The cerebellar cortex consists of a tightly folded layer of grey matter. Under this is the white matter that has the same properties and functions as in the cerebrum; connecting the different parts of the cerebellar cortex. It is called the arbor vitae due to its branching geometry, and then within this lay the deep cerebellar nuclei, composed again of grey matter. This complex architecture of grey and white matter gives rise to a composite material structure where the overall stiffness should theoretically be an average of the individual counterparts. Indeed, Figure 18 shows that the cerebellum stress-strain results lie in the middle of the white and grey matter results with an average stress at 30% strain of 432 ± 172 Pa. The large standard deviation attached to this set of

results covers the entire range of white and grey matter results. This highlights the fact that the cerebellum is indeed an intricate mix of both white and grey matter.

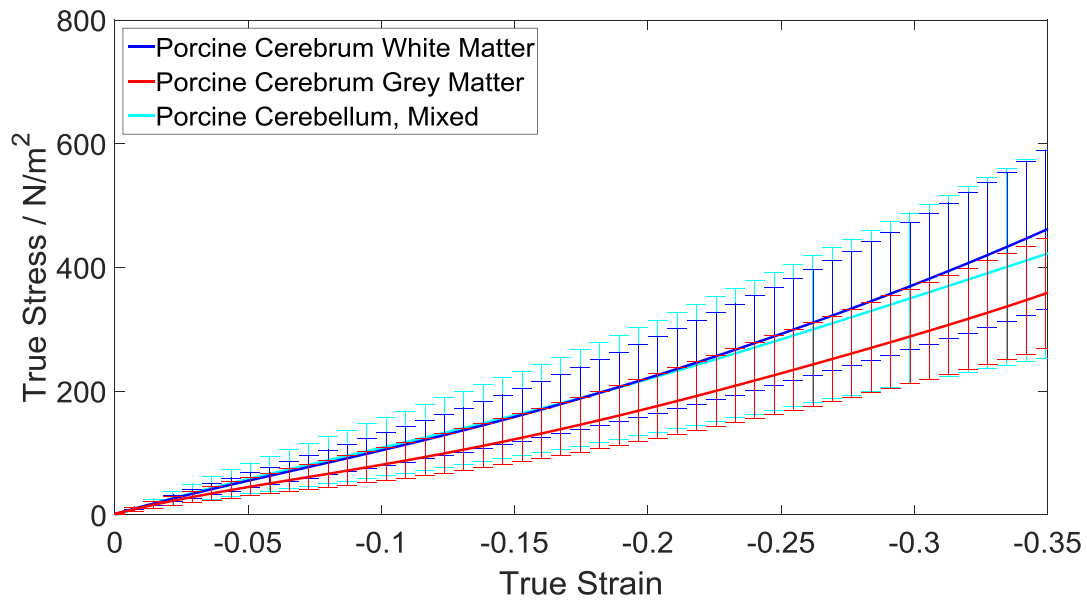


Figure 18: Comparison between porcine cerebrum white and grey matter.

The architecture of the axons in the brain is fascinating and much of its structure can be analysed thanks to DTI technology, where the movement of water molecules is related to the directionality of the white matter tracts[197–199]. The images from DTI studies have shown that the white matter tracts exhibit a clear organised structure, designed to efficiently connect important areas of our brain for functioning[200,201]. Since this architecture clearly affects the diffusion of water molecules in the microstructure, it is important to understand if it also has an effect on the mechanical properties. The most intuitive method to analyse the effect of the cylindrical axonal bundle geometry on directionality is to take samples transversely and axially with respect to the axonal direction.

Given the fact that our investigation focuses on the macroscopic response of the tissue, during the sample preparation of porcine brain (also in the absence of detailed imaging of the brain) it was not possible to take samples relative to the axons as the sample diameter (around 10 mm) was many orders of magnitude greater than an axons diameter (1-10 μm). This in turn hinders the possibility to have a coherent orientation of the fibres throughout the sample volume[202–204]. Furthermore, it is also discussed in literature that anisotropy due to the axonal microstructure is unlikely be found from tests carried out on the millimeter scale but may be elucidated through microindentation and imaging methods[205–207]. It is for this reason that we chose to study the directionality of brain in the three anatomical planes to assess whether there was any macroscopic directionality effects. The study of directionality in grey matter was also of interest as anatomically speaking, there shouldn't be any significant directionality since there are no axonal tracts. However, taking samples of grey matter with respect to the

anatomical planes was not feasible at the testing scales of this work as the ratio of white to grey matter decreases the greater the size of the brain[208,209]. For porcine brain, it is easier to take consistent cylindrical samples out of the white matter than the relatively thin grey matter area, hence, this work will focus on the characterisation of white matter.

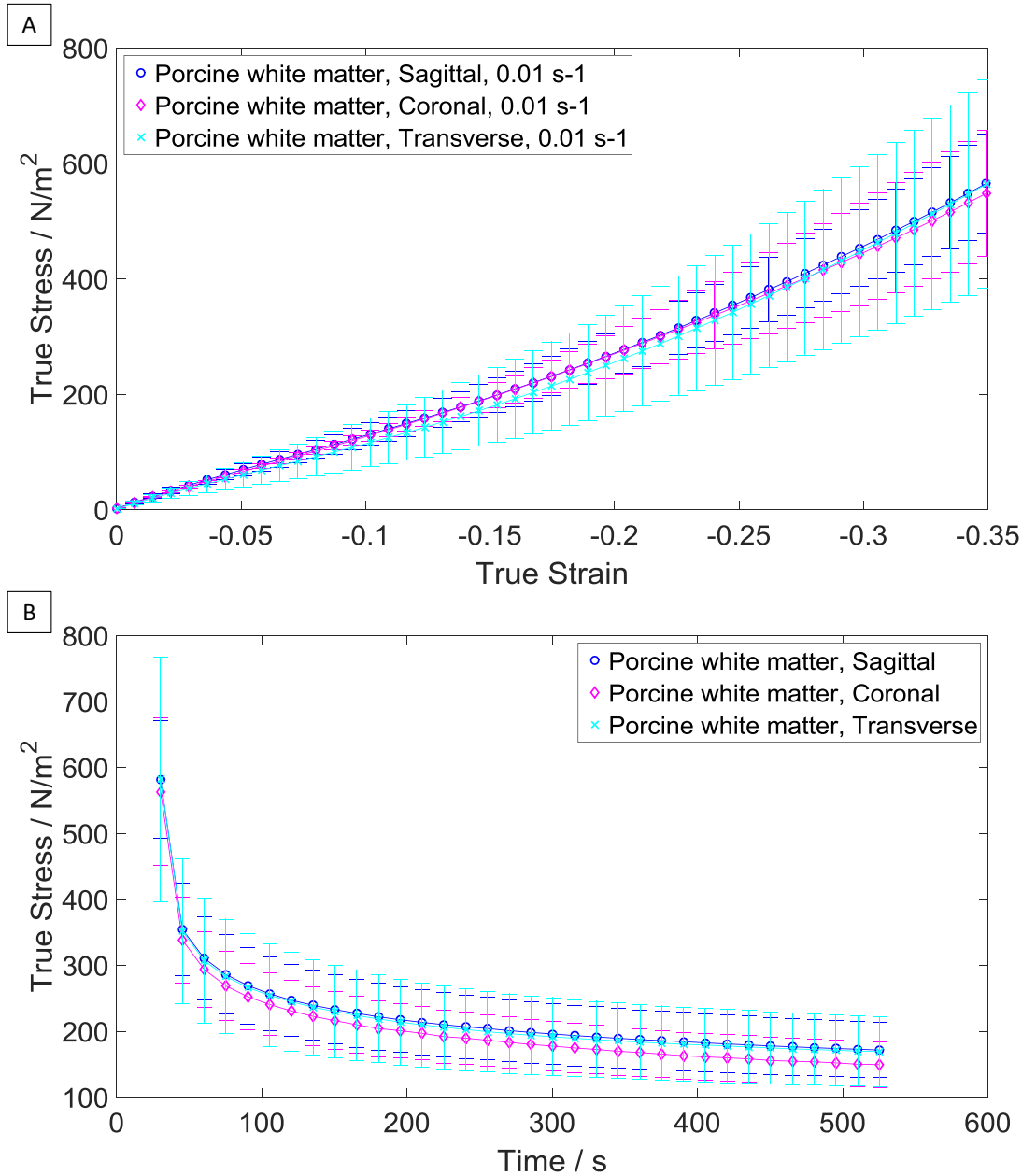


Figure 19: Directionality results for porcine brain in terms of the anatomical planes showing (A) the stress-strain response during loading and (B) the relaxation response at constant 30% strain.

The porcine brain results from the unconfined compression-relaxation tests are shown in Figure 19. Comparison between the three anatomical planes (sagittal, coronal and transverse) showed little variation and were all well within one standard deviation of each other. From this we can conclude that there are no significant directionality effects at the macroscale tested here, though there are thousands of axons positioned in numerous directions within each sample. It would be of interest to investigate the directionality effects at smaller scales using nano-

indentation to understand the difference in mechanical properties between the axon and the surrounding extracellular matrix for scenarios such as diffusion and tumour cell growth, which are sensitive to the local behaviour of the tissue. The coupling of this work with diffusion tensor imaging (DTI) results is being investigated in parallel as part of the Horizon2020 EU funded EDEN2020 project, which aims to model the diffusion of therapeutic agents during drug delivery.

Due to the limited directionality effects, the average was taken and compared to literature results. Figure 20 shows that the porcine brain results are within the range reported by literature[8]. The standard deviation is due to the sensitivity of the biological tissue and matches the spread reported by Forte *et al.*[60]. The porcine brain results are particularly well matched to those reported by Forte *et al.*[60]. This may be because the biological tissue was procured from the same provider, and the sample preparation and unconfined compression tests followed the same procedure and used the same testing setup and environmental conditions. The results also fall within the results range reported by Prevost *et al.*[210] and Vappou *et al.*[211], whose results show the variability and sensitivity of the biological material when performing these tests, which are mainly due to the biological and environmental testing conditions; post-mortem time, age of animal, testing temperature and humidity.

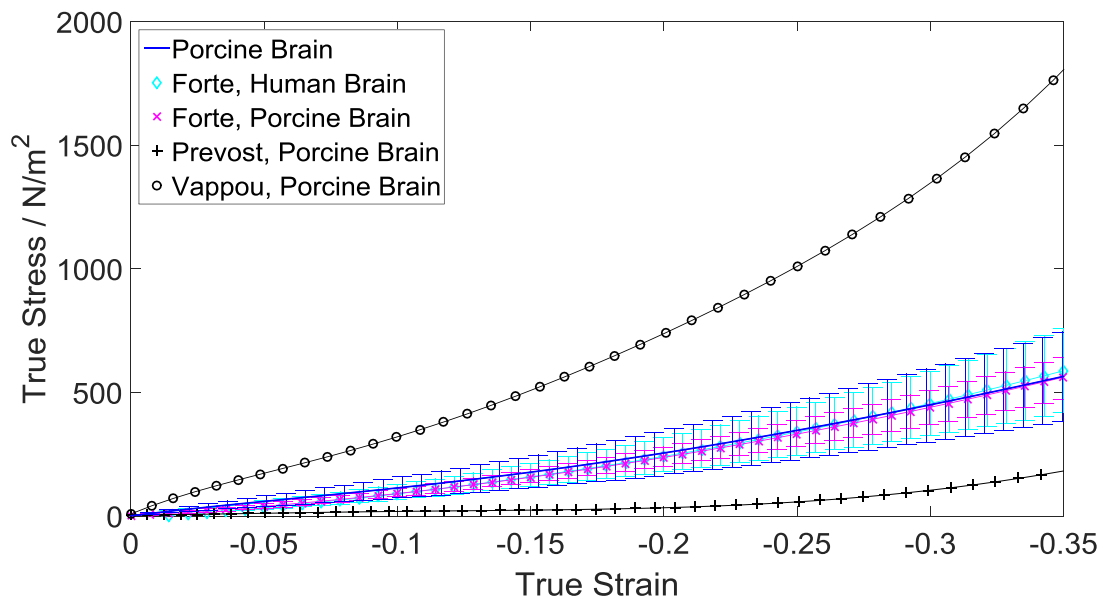


Figure 20: Porcine tissue stress-strain results at 0.01 s^{-1} strain rate compared to literature results for brain. Results obtained for human brain tested using the same procedure used for the porcine sample are also reported to show that porcine and human samples produce very similar results.

To characterise the mechanical behaviour of the experimental results for porcine white matter, the Mooney-Rivlin hyperelastic model and the generalised Maxwell viscoelastic model were

implemented. The model parameters found to give the best fit to the experimental data are summarised in section 3.3.4 Table B.

The best fitting Mooney-Rivlin parameter for C2 was computed as a negative constant. Negative coefficients have been reported in the literature and should be checked for stability over a wide range of strains before being implemented as material constitutive parameters in computational models. Therefore, Figure 21 shows the stress-strain response for brain compared to the computed Mooney-Rivlin model to assess the stability of the hyperelastic parameters at strains outside of those to which the model is fitted; the model was extended from 50% compression to 50% tension.

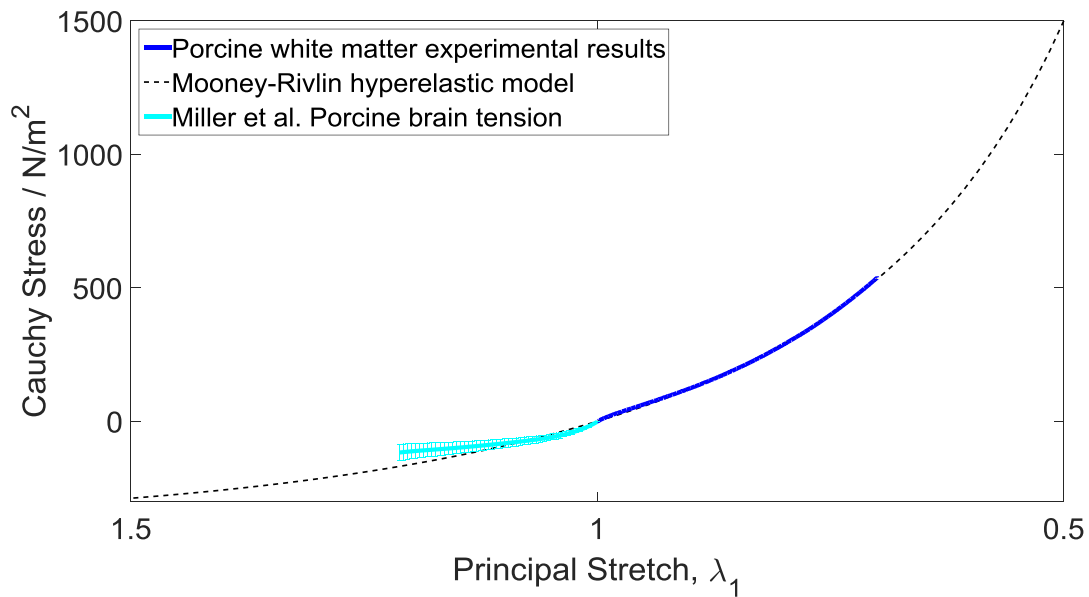


Figure 21: Mooney-Rivlin hyperelastic model fitting compared to experimental results for average porcine white matter brain and tensile results by Miller and Chinzei[179].

The graph shows that the Mooney-Rivlin parameters are very well matched to the experimental results range of 0-30% compression. Whilst experimental data for tension was not obtained in this work, literature results for porcine brain in tension up to 20% strain in tension have been supplemented to the graph. Figure 21 shows that the model is even able to reasonably match the tensile experimental data obtained from Miller and Chinzei[179]. A possible explanation for the lower stress results reported by Miller and Chinzei is that their applied strain rate was 0.0064 s^{-1} which is slightly lower than the strain rate applied to the samples tested in this work (0.01 s^{-1}), for which the model was originally tuned to.

Miller and Chinzei also fitted a hyperelastic model to their own experimental results of brain in compression and tension, where the stretches were raised to a fractional power of -4.7[179], as opposed to 2 as in this work. Their model was closer to the experimental results in tension but less accurate in compression. Separately, the model suggested in this work is stable in tension

and there is no stationary point unlike the results reported by Bergström[212]. In addition, the tensile properties of brain were also investigated as part of a Master's project in which the author played a supervisory role, which reported a significant difference between the stress at 30% strain in tension and compression[213].

The average insertion force for porcine brain was 0.083 ± 0.026 N and this was found to be in line with those reported by Leibinger *et al.*[63]. The needle insertion force profiles captured multiple sharp peaks throughout the needle insertion regime, which dropped off sharply once the needle had passed entirely through the brain hemisphere, as shown in Figure 22. Due to the softness of the brain tissue, the organic material seemed to be slightly dragged by the needle into the laser-cut hole at the bottom of the perspex box, which resulted in a transient drift of the measured force. However, once the needle passed an initial critical distance, the force became steady and the friction force was calculated by computing its average over this region of the plot.

For the brain, the area of insertion was very important because of the sulci and gyri folds that create the complex geometrical structure of the cerebral cortex. These folds have tiny cerebral veins spread across them, which increase the resistance to fracture, as illustrated in Figure 17 (C). This is depicted in the results graph in Figure 22 as the numerous sharp penetration forces. These peaks are thought to correspond to the fracture of the cerebral veins as the needle passes through the tissue. The average insertion force and friction force for the porcine brain was very low compared to lung and liver, confirming that the brain is not only very soft but also very delicate.

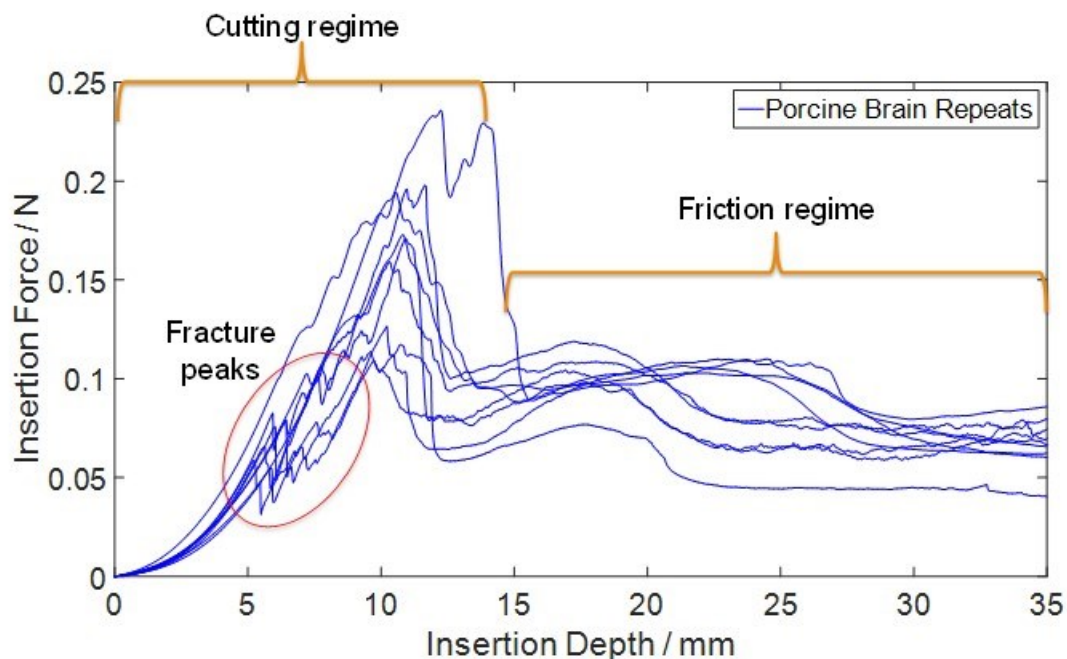


Figure 22: Porcine brain needle insertion results with individual repeat tests, not averaged in order to show unique fracture characteristics.

3.3.2. Porcine Lung

Firstly, the results shown in Figure 23 of the stress-strain response of the different components of the lung show striking difference between the cartilaginous bronchi and the parenchymal lung tissue. The average stress at 30% strain of the bronchi was 4.501 kPa compared to 0.527 kPa and 0.119 kPa of the transverse and axial lung parenchymal tissue, respectively. The unusual shape of the curve is due to the non-linear deformation of the bronchial plates coupled with the membranous muscle that holds the plates together, providing additional support in the circumferential direction. The average diameter of the isolated axially tested bronchi samples was 9.35 ± 0.23 mm and therefore larger than the bronchi found in the transverse samples, which were around 3 mm in diameter. Larger bronchi have thicker walls, hence explaining the significantly stiffer response. In the transverse loading direction, the bronchial plates, which are not rigidly bound together, would be subjected to circumferential hoop stresses which would cause a much more compliant deformation.

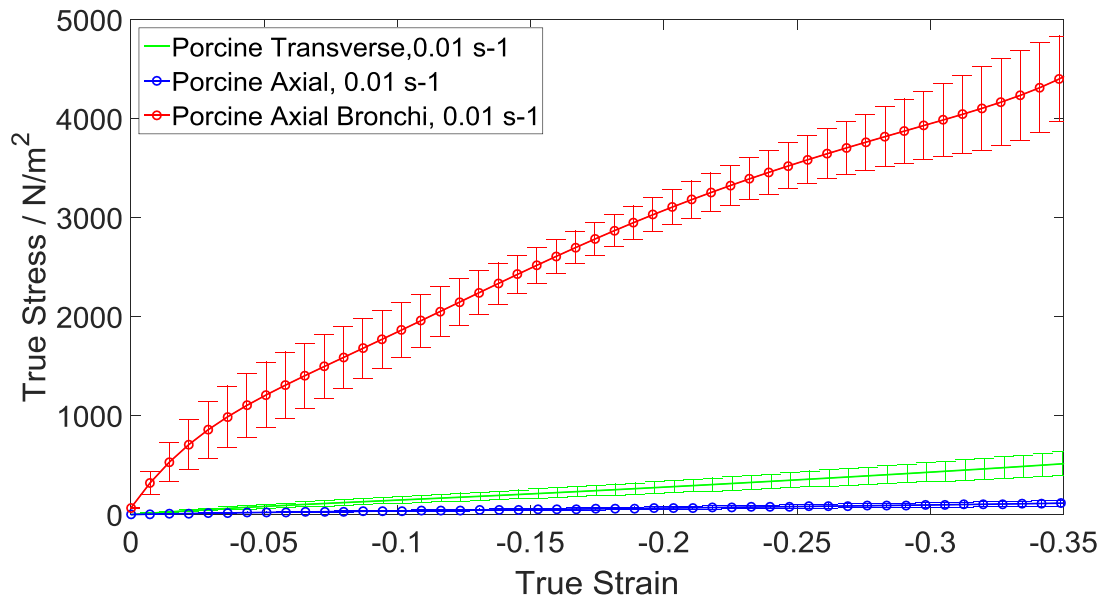


Figure 23: Stress-strain response of isolated bronchi in axial direction compared to lung tissue in transverse and axial directions.

However, comparing the stiffness of the sample with the transversely included bronchi with the sample obtained in the axial direction but without bronchi inclusion, it is obvious that the inclusion of the bronchi increases the stiffness of the material regardless of the direction, which can be seen more clearly in Figure 24. The samples taken in the transverse direction show stresses around 4.5 times higher than the ones measured in the axial direction. With a clear indication of the effect of bronchi on the stiffness of the lung, more effort should be considered in modelling the lung as an anisotropic organ with a bronchial tree structure that is an order of magnitude greater than the surrounding, extremely soft parenchymal tissue.

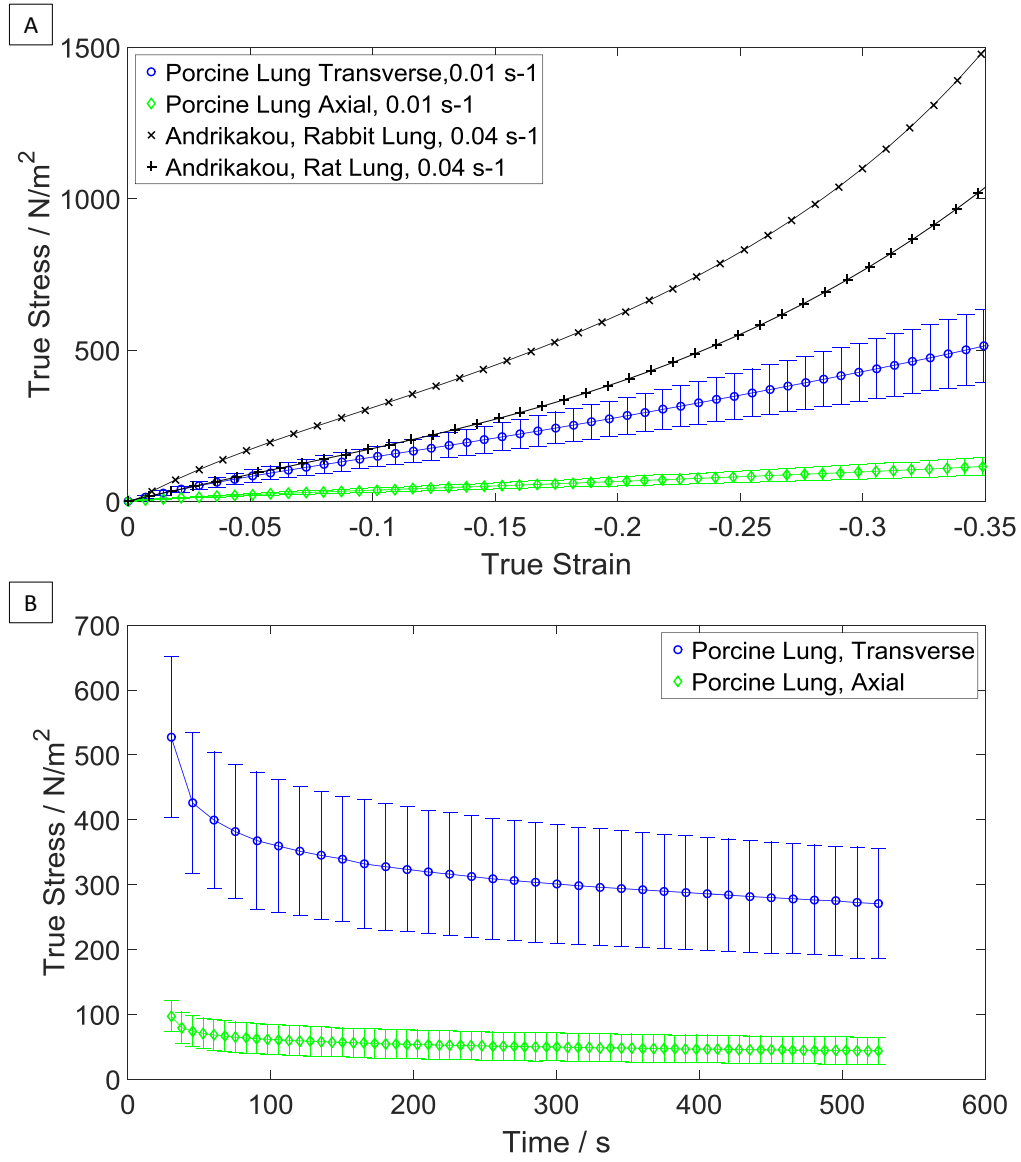


Figure 24: Stress-relaxation response of porcine lung samples taken in the transverse and axial directions relative to the bronchi compared to literature where (A) shows the strain-strain response under compression and (B) the relaxation response over time as strain is kept at 30%.

Existing literature on the mechanical testing of lung obtained under comparable testing conditions is sparse and therefore direct comparison to the results found in this work is limited. Weed et al.[214] performed unconfined compression experiments on mouse lung but applied a higher load of 98 N at and faster strain rate of 0.1 s⁻¹ resulting in a much more dramatic stress-strain response than the results reported here. Other literature involved in characterising the mechanical behaviour of lung has previously focused on air inflation methods to obtain pressure-volume curves[215,216] or the use of imaging elastography techniques to characterise lung stiffness and therefore stress-strain curves cannot be obtained for comparison[217].

The most comparable study was carried out by Adrikakous *et al.*[18], whose results, depicted in Figure 24 (A), show that rabbit and rat lungs are significantly stiffer than the porcine lung results found in this study, which may be due to the discrepancy in strain rate[18]. The strain

rate applied by Andrikakou *et al.* is 0.04 s^{-1} , 4 times higher than in this study, which must be taken into consideration as it has been shown that most of organic tissues are strain rate dependant[8,26,218]. Another issue is the effect of intraspecies variation. Andrikakou and co-workers reported a difference of $\sim 500 \text{ Pa}$ at 30% engineering strain between the rabbit and rat lung, therefore, there may also be a difference in stiffness between these species and porcine tissues[18]. Another important factor is the variation of species, which affects the size of the native organ. In rat, a 10 mm diameter cylindrical sample would contain almost the entire bronchial branch as the native tissue is 15-20 mm diameter[219]. Therefore, the bronchial density is different, leading to a discrepancy in stiffness.

Furthermore, the stress-strain behaviour of the porcine lung depicted in Figure 24 (A), in both directions, are relatively linear compared to the curved response reported by Andrikakou *et al.*[18] Analysing the relaxation response shown in Figure 24 (B) leads to the observation that the tissue have comparable relaxation ratios of 1.95 and 2.21 in the tranverse and axial directions, respectively. The difference in relaxation ratio here is not large enough to draw conclusions on whether the inclusion of the transverse bronchi has an effect on the relaxation behaviour.

A Mooney-Rivlin hyperelastic model was also fitted to the experimental data for both transverse and axial directions. The model was extended to $\pm 50\%$ engineering strain and literature results for rabbit and rat lung in tension from Andrikakou *et al.*[18] were superimposed onto the graph for comparison. Figure 25 shows that the tuned model parameters reported in Table 12, section 3.3.4, whilst negative, produced a model which is stable in tension but does not match the tensile experimental results.

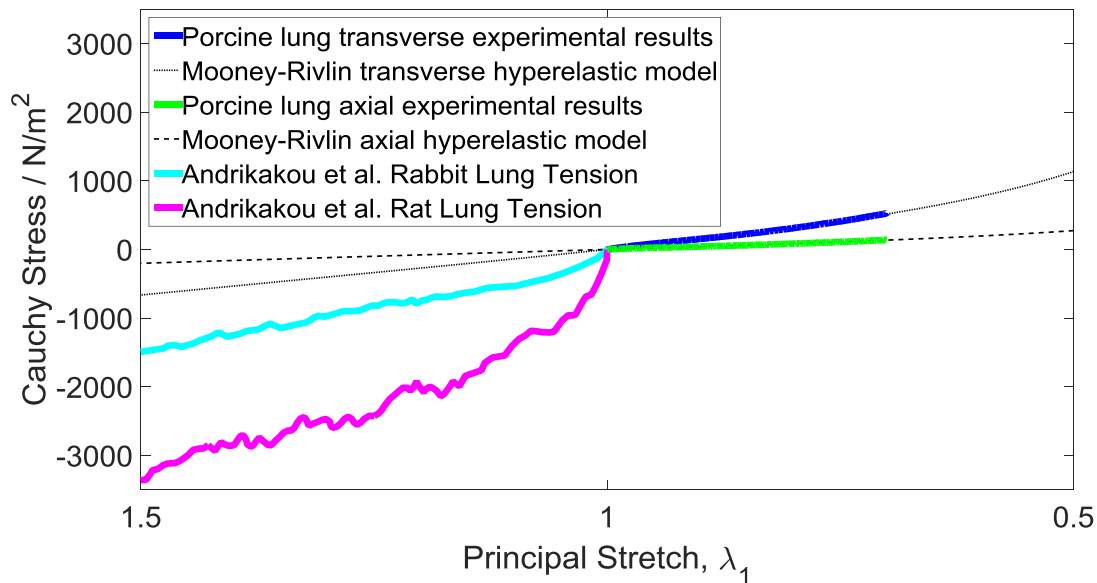


Figure 25: Mooney-Rivlin hyperelastic model compared to experimental results for porcine lung samples tested transversely and axially to the bronchi compared with tensile lung literature[18].

The rabbit and rat results reported by Andrikakou et al.[18] consistently exhibit a stiffer response in both tension and compression than the porcine results obtained in this work. Although the strain rate applied by Andrikakou and co-workers was 0.004 s^{-1} , and therefore slightly lower than the 0.01 s^{-1} that the model was fitted to, this discrepancy is unlikely to be great enough to account for the difference in behaviour that the tensile results exhibited in Figure 25 suggests. Instead, the large difference is more likely due to the fact that the bronchial density is greater in smaller volumes which correlates to the smallest lung volume of rat exhibiting the greatest stiffness, followed by rabbit and then porcine. Additionally, the greater the bronchial density, the greater the likelihood of samples containing bronchi branching out in perpendicular directions relative to the testing direction, which again would result in a greater stiffness.

Furthermore, the tensile stress-stretch behaviour shown in Figure 25 is not smooth, suggesting that there was some breakdown of the rabbit and rat samples throughout the test. The results for rat lung in tension showed an even greater irregular and discontinuous mechanical response. This is further evidence that the intraspecies effect on material behaviour is partly due to the testing scale size and hence the volume and directionality of the bronchi in the samples.

The insertion force profiles shown in Figure 26 for lung feature sharp peaks with significant drops in force after the needle has fractured the material. Some insertion force profiles demonstrate particularly large penetration peaks, which indicate where the needle has encountered larger bronchi. The bronchial stiffness and toughness is proportional to its diameter, resulting in higher insertion forces and a greater resistance to fracture[220].

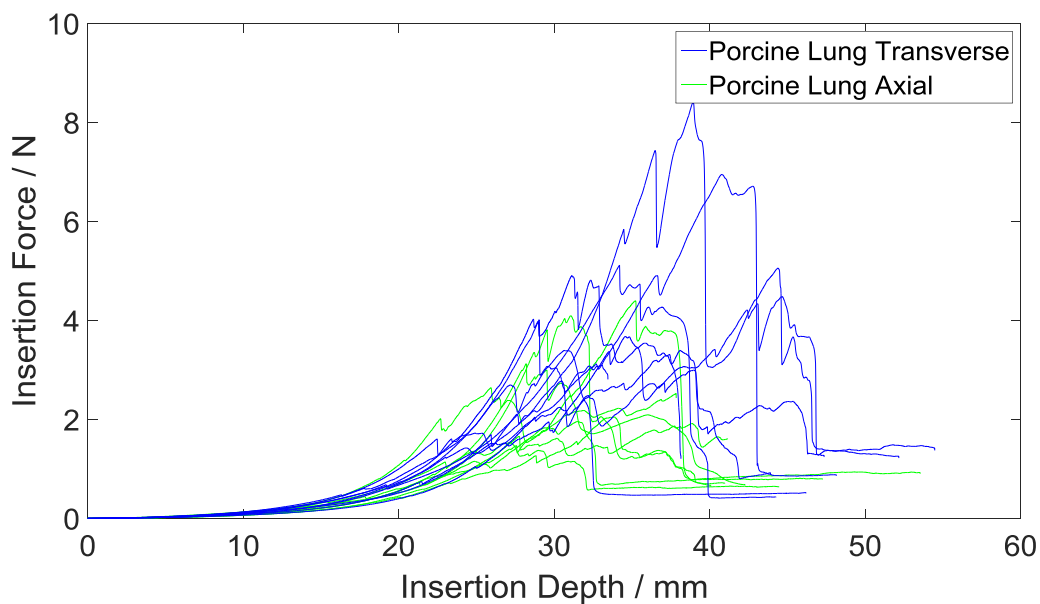


Figure 26: Insertion force profiles of porcine lung samples taken in the transverse and axial directions relative to the bronchi.

Analysing for directionality, the average insertion force of porcine lung samples punctured in the transverse direction was 0.47 N greater than in the axial direction which is not a significant difference given that the standard deviation was ± 0.38 N. This is because although samples were taken in the axial direction relative to the main bronchi, many smaller bronchi branch out perpendicularly from this main stem. Therefore, when the needle is inserted in an “axial” direction, it will still encounter many smaller bronchial branches that have a higher resistance to fracture than the parenchymal aveoli tissue, which causes many sharp insertion force peaks.

3.3.3. Porcine Liver

The major architecture in liver is the branching hepatic venous system, which exists to maximise the efficiency of the liver function, which is to cleanse the blood of toxins, a schematic of which can be referred to in Figure 15. On a smaller scale, the parenchymal tissue is organised in lobules which are roughly hexagonal, consisting of a central artery and vein surrounded by hepatocytes. The size of these lobules are on the millimetre scale and are found to vary between 1-5 mm[221]. Much like the bronchial tree in lung, the branching nature of the hepatic veins results in larger, thicker veins being present near the entrance of the liver, where the main hepatic vein, the inferior vena cava, connects the liver to the rest of the body. The veins become smaller as they branch out to the liver extremities.

As such, a notable observation from the results of porcine liver is that the stiffness of the transverse samples depend on the thickness of the liver and the area from which they were extracted, as shown in Figure 27. Samples of the same height taken from the thickest area of the liver are a factor of about 10 times greater in compressive stress at 30% strain than samples taken from the thinnest areas of the liver which are at the liver edge. The thickness of the liver is correlated to the size of the local veins since the liver is thickest around the inferior vena cava and thinnest at the outer edge of the liver.

The thickness dependency of liver is even more interesting as the sample diameter, height and testing strain rate were kept constant, suggesting that the lobular microstructure in the thicker regions of the liver were inherently stiffer and more elastic than lobules at the liver edge. Although there were no observed differences between the lobular microstructure in the centre of the liver compared to the lobules at the liver surface, it is advised that more microstructural imaging and analysis should be conducted to investigate the effect of the lobular microstructure on the stiffness in future. Due to this discrepancy, the transverse and axial samples were taken

in the same regional thickness of liver to ensure that the effect of liver thickness would not affect the assessment of directionality.

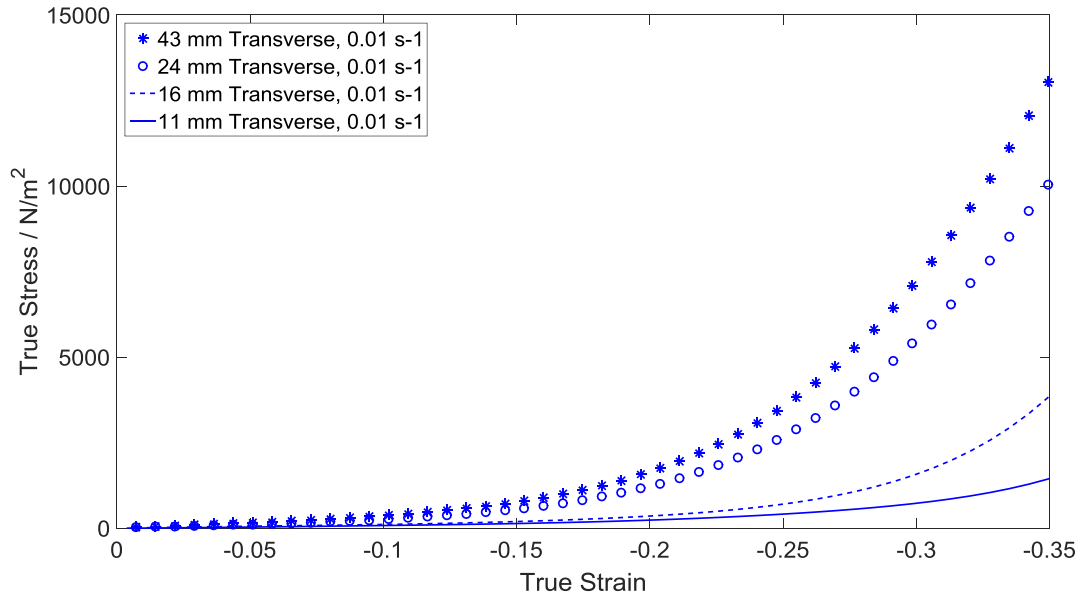


Figure 27: Thickness dependency of liver on stiffness.

The results for porcine liver show a relationship between the direction the samples were taken in and their stiffness, where the transverse samples are stiffer than the axial samples. In 3D, the liver lobules have a hexagonal prism geometry that means they are anisotropic by nature. They are orientated so that the hexagonal faces are in plane with the circular cross-section of the veins, the axial direction. However, the problem is not only one that can be solved by analysing the geometry as there are complex boundary conditions and mechanical interactions at the interface between each lobules. The lobules are firmly connected to each other by a tough membrane. Additionally, there is a portal triad, consisting of a portal vein, hepatic artery and bile duct, running axially along the edges at every vertex of the hexagonal face, as well as a central vein in the middle of the lobule[222]. As future work, a computational simulation should be applied to study the influence of geometrical alignment, membrane toughness and localised boundary conditions.

The zoomed in insert in Figure 28 (A) shows the a comparison between these results and literature results by Mattei *et al.*[28] and Roan *et al.*[14]. The results are very well matched to literature up to 10% strain. Since Roan *et al.*[14] tested transversely obtained samples, their results should be compared to the transverse liver results reported in this work. At 30% engineering strain, we see that the results from Roan and co-workers are lower than the results of this study. Investigating this further, it was found that Roan *et al.*[14] obtained their samples from the half of the liver furthest away from the inferior vena cava. Given the evidence of thickness dependency of the liver previously discussed, it follows that the results from Roan *et al.*[14] would be softer as they were taken from the thinner region of the liver.

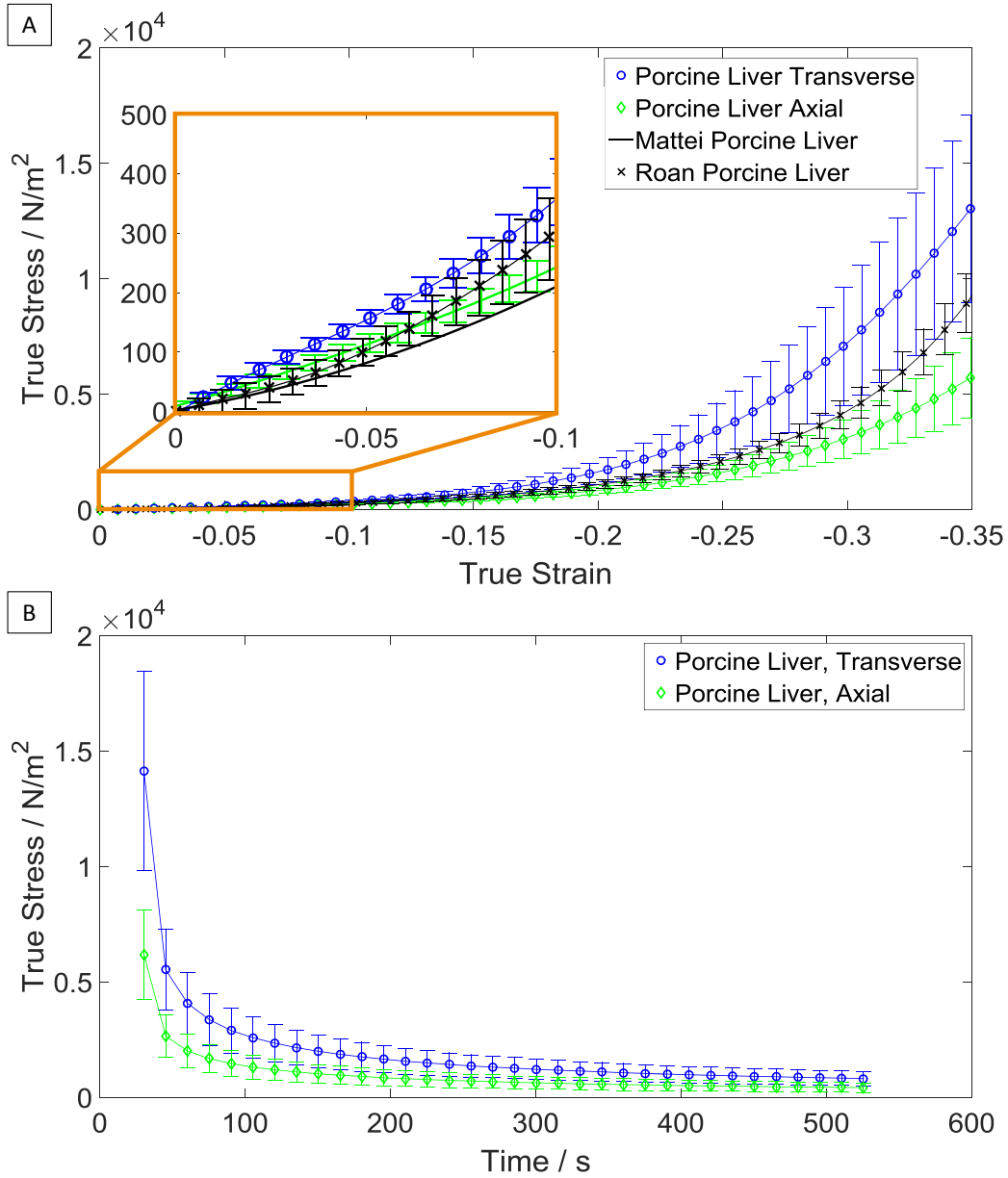


Figure 28: Mechanical behaviour of porcine liver samples taken in the transverse and axial directions relative to the hepatic vein for (A) compressive stress-strain response at 0.01 s⁻¹ strain rate and (B) relaxation response over 500s.

The characterisation of the viscoelastic behaviour of liver has shown that the relaxation response is the most severe out of the biological tissues tested in this study. Although the transverse and axial samples relax to a very similar equilibrium modulus, E_{∞} , at 500s, with a very small standard deviation, as is shown in Figure 28 (B), the relaxation ratio of the transverse samples are significantly higher, which is mainly due to the fact that the stress at the start of the relaxation is much higher than the axial results. As previously discussed, this is because the evidence shows that samples taken transversely to the hepatic vein exhibit a greater degree of strain stiffening and reach a stress at 30% strain that is over twice as high as axial samples. But since the equilibrium modulus is of a similar value, this would suggest that the transverse samples are much more viscoelastic, although the reason for this is not clear.

The Mooney-Rivlin hyperelastic model fitting for porcine liver was the least successful. Figure 29 shows that at a stretch of 0.7, the experimental results begin to deviate significantly from the model curve. This means that at larger strains, the MR model will underestimate the stresses that the biological liver experiences. Again, the model was extended to $\pm 50\%$ strain, as shown in Figure 29, and literature values were added to complete the tensile side of the mechanical behaviour. It is clear that the MR model computing to be best fitting for the compressive experimental results for liver, is not stable in the tensile direction and cannot be used to define material parameters for computational model analyses.

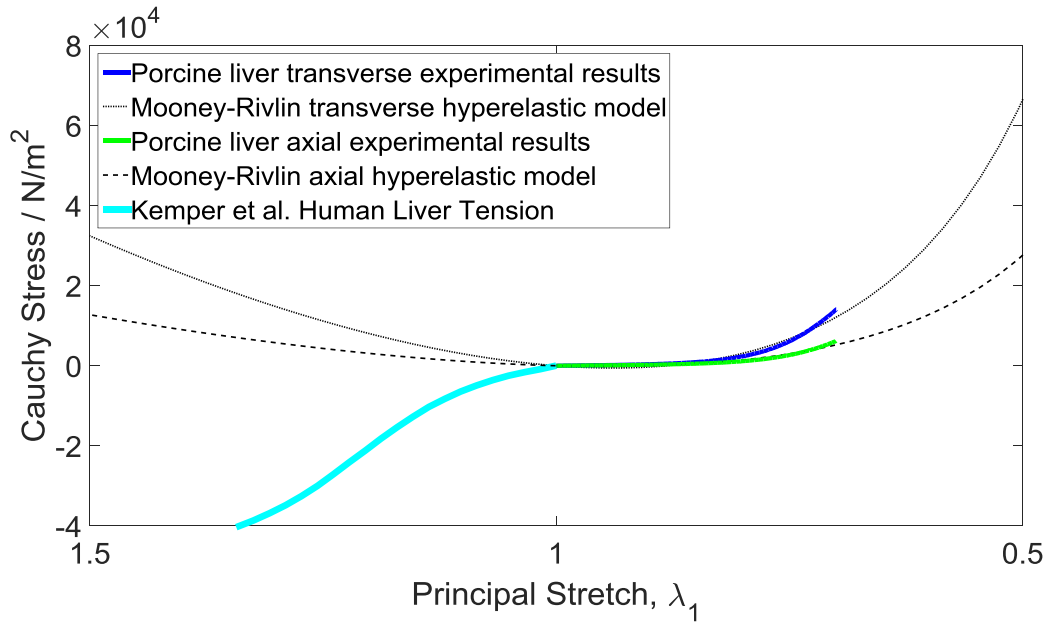


Figure 29: Mooney-Rivlin hyperelastic model fitting compared to experimental results for (A) porcine liver samples tested transversely to the hepatic veins and (B) axial porcine liver test results.

A possible solution would be to use higher order models, as Chui et al.[189] demonstrated with their ninth order Mooney-Rivlin model, and also the logarithmic version of the Fung-Demiray model[223]. Moreover, the difference in behaviour could also be due to the intricate lobular structure whose hexagonal geometrical microstructure may also impact the stress-strain curve shape. It is also interesting to note that the experimental liver result from Kemper et al.[224] appears to be much stiffer, 36 kPa at 30% tensile strain, compared to the value obtained in this study, 14 ± 4 kPa at 30% compressive strain. This may be due to intraspecies variation, as Kemper *et al.* tested human liver and the results of this study are from porcine liver, but it may also be highlighting a fundamental difference in mechanical behaviour between the tensile and compressive loading scenarios. Hence, further work with the aim of investigating the mechanisms that could elucidate the non-linear and discontinuous properties of soft tissues across all strains should be carried out.

Conversely to the lung, the insertion force results for liver show a significant difference between the transverse and axial directions, where the average insertion force in the transverse direction is 1.1 N greater than in the axial direction with a standard deviation of ± 0.5 N. This may be because the liver is closer to a disc shape which makes the definition of transverse and axial relative to the primary hepatic vein simpler. In addition, the results for axial samples of liver show less dramatic declines in insertion forces directly after puncture and are instead characterised by many small and steady tissue ruptures as the needle travels through the tissue.

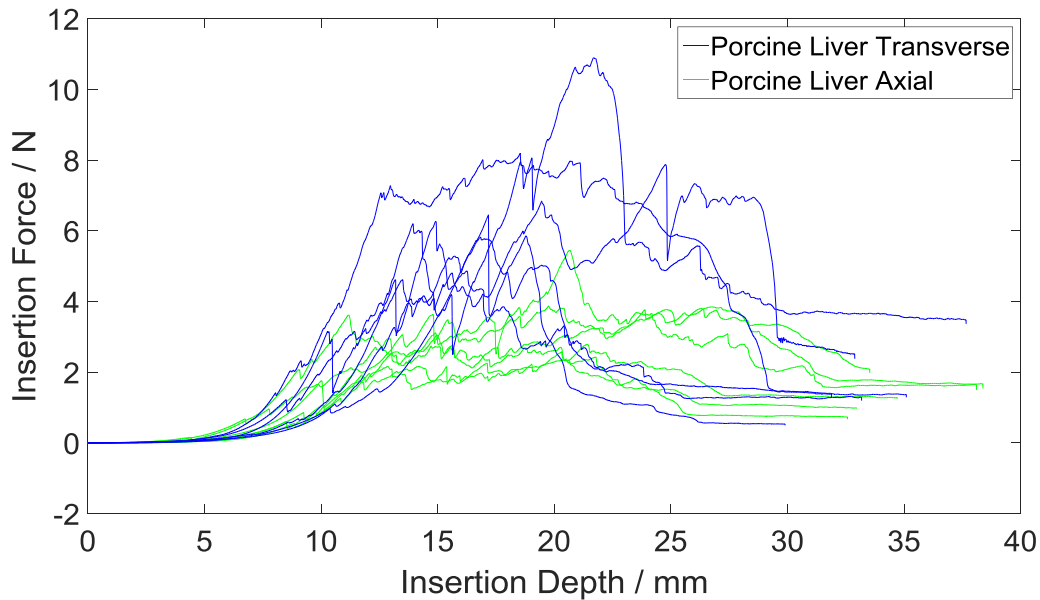


Figure 30: Insertion force profiles of porcine liver samples taken in the transverse and axial directions relative to the hepatic vein.

The insertion force results of liver, shown in Figure 30, are the largest obtained for the soft tissues tested in this study. The large peaks correspond to the rupture of the large hepatic veins, which show that the vein membrane is much tougher than the surrounding tissue. This behaviour was also reported by de Jong et al.[161] but was discussed as an outlier from the results obtained. On the contrary, the results produced in this work indicate that the penetration peaks are a key characteristic of the needle insertion force profile of the liver and should not be discounted as the branching venous structure makes it difficult to distinguish a threshold at which the fracture peaks should be counted as outliers. Figure 30 shows that there are also a multitude of smaller peaks throughout the insertion procedure that occur at every millimeter of the insertion depth. This may be evidence of the fracture of the lobular microstructure since the lobules are around 0.5-2 mm in diameter and are therefore on the same scale as the diameter of the needle, which is 4 mm[221].

3.3.4. Cross-tissue Mechanical Behaviour Comparison

One of the main goals of this work is to mimic a variety of biological tissues using different formulations of the same composite hydrogel material to show its versatility. Consequently, the biological tissues studied in this chapter were chosen as they represent a wide range of mechanical properties. In fact, the results in this chapter have gone further to show that the behaviour of these mechanical properties; stiffness, fracture toughness and frictional interaction, in biological tissues are independent of each other. In this section, the parameters for material characterisation are summarised and compared to each other in more detail to quantify their differences and obtain values that the composite hydrogel will aim to mimic, as revealed in Chapters 4 and 5.

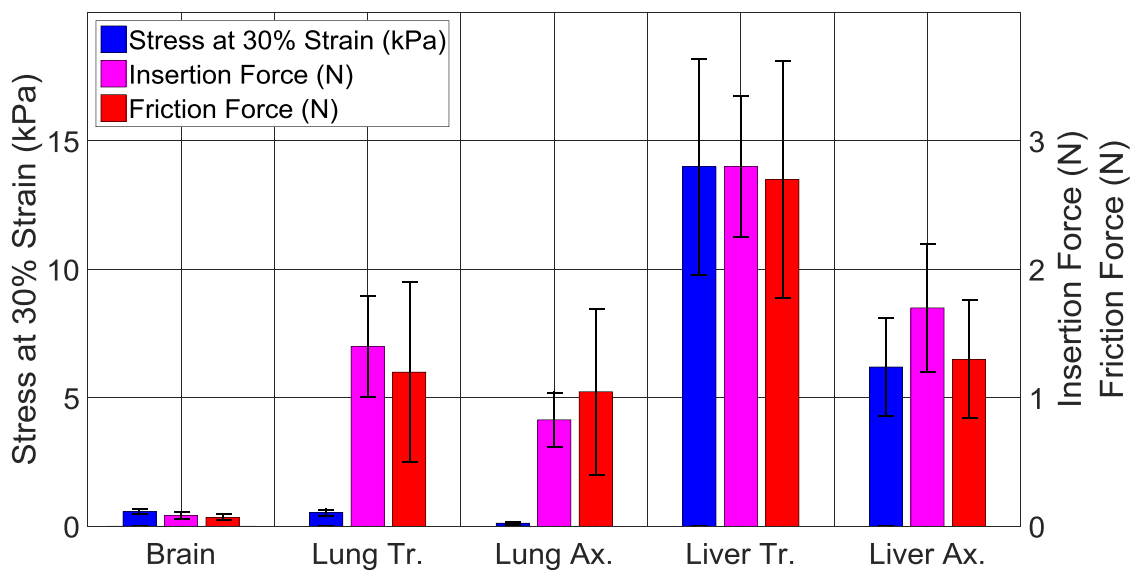


Figure 31: Testing results of porcine brain, lung and liver in terms of their mechanical properties of stress at 30% strain, insertion force and friction force for comparative analysis.

Firstly, the results for true stress at 30% engineering strain across the soft tissues tested, porcine brain, lung and liver, are compared. Although this characterisation does not take into account the development of stress-strain curve, it is a simplistic method to easily compare the approximate material characteristics of different biological tissues and is akin to comparing the linear stiffness of a material, without stating the assumption of linear elasticity. The results for stress at 30% engineering strain displayed on the left y-axis in Figure 31 show that brain and transverse lung samples reach a very similar stress at 30% strain, which is very soft compared to liver that is two orders of magnitude higher. The average values are reported in Table II.

Table II. Biological soft tissue mechanical testing results.

	Stress at 30% Strain $\pm$ SD (kPa)	Insertion Force $\pm$ SD (N)	Friction Force $\pm$ SD (N)
Porcine Brain, white matter	0.57 ± 0.11	0.083 ± 0.026	0.069 ± 0.023
Porcine Lung, transverse	0.53 ± 0.12	1.4 ± 0.39	1.2 ± 0.70
Porcine Lung, axial	0.12 ± 0.029	0.83 ± 0.21	1.0 ± 0.65
Porcine Liver, transverse	14 ± 4.2	2.8 ± 0.55	2.7 ± 0.92
Porcine Liver, axial	6.2 ± 1.9	1.7 ± 0.5	1.3 ± 0.46

Interestingly, the stiffness of the lung samples taken in the axial direction, which is comprised of only parenchymal tissue and no bronchi, is 4.8 times lower than that of white matter. This shows that the parenchymal lung tissue is much softer than both white and grey matter in the brain, the latter of which is often regarded as one of the softest biological tissues. The softness of lung can be understood by realising that the interstitial spaces in the lung aveoli are designed to be filled with air and not fluid. This result is poorly studied as often, the entire lung is tested due to the species and therefore size of the organ. In this case, porcine lung is much larger than smaller animal species, which allowed for the testing of the bronchi and parenchymal tissue individually. The results indicate that the inclusion of the bronchi in the lung has a large impact on the tissue mechanical properties and is able to raise the average stress-strain response of the overall organ by an order of magnitude.

A different narrative is apparent when analysing the insertion and friction forces. For brain and liver, the stiffness is positively correlated with measured insertion and friction forces. However, the same cannot be concluded for the lung. The lung is the only material in this study where its stiffness does not accurately correlate to the measured insertion forces, as shown in Figure 31. In comparison to brain, where a low stiffness leads to a low insertion force, the average insertion forces for lung are an order of magnitude higher than brain, despite their stiffness being very similar. Hence, this suggests that tissue stiffness is independent to the puncturing and cutting forces experienced by the tissue during needle insertion. The correlations made in this work refer to the average insertion force as a simplistic representation of the insertion force profile so that the scale of forces can be compared across different tissues. Future work should focus on advancing the characterisation of the insertion force profile with respect to tissue

penetration depth by introducing and analysing parameters such as the number of penetration peaks, the maximum peak stress value and strain at which the first penetration peak occurs.

For lung, for example, the first penetration peak occurs at a much higher strain than in brain and liver; at over 100% strain on average, demonstrating the compressibility and toughness of lung compared to brain and liver. Whilst remarkable at first, this is quite easily understood when considering the biological role that the lung plays, which is one that requires constant cyclic expansion and contraction to high strains that few other organs are required to do. This necessitates a material with low stiffness and high toughness, which would lead one to consider that this biological tissue evolved in such a way to serve its purpose. A full and detailed analysis of each experimental insertion force profile result was beyond the scope of this work but future work incorporating more insertion profile characteristics should elucidate interesting results that could help explain physiological phenomena.

The Mooney-Rivlin hyperelastic model parameters best fitted to each biological tissue, and accounting for directionality, are listed in Table 12. The model parameters with the lowest R^2 values are those for porcine lung in both directions. This may be because the stress-strain curve for lung is the most linear compared to that of brain and liver. Although the best fitted model parameters for brain have a larger R^2 value than lung, the extension of the model to 50% strain in the tensile and compressive loading directions and subsequent comparison with tensile literature results show the better stability and fit, especially in the tensile direction, compared to lung. The model parameters for liver show the greatest deviation from the experimental results. This is particularly apparent when extrapolating the model in the tensile direction leads to an unstable and physically inaccurate prediction.

Table 12. Mooney Rivlin hyperelastic parameters.

	C1 (Pa)	C2 (Pa)	R^2
Porcine brain, white matter	77.8	-253.2	0.9725
Porcine lung, transverse	-152.8	-85.58	0.9981
Porcine lung, axial	-56.79	-10.74	0.9967
Porcine liver, transverse	2.485e+04	-2.191e+04	0.9659
Porcine liver, axial	9975	-8928	0.9634

It is apparent that the Mooney-Rivlin material model does not adequately describe the stress-stretch relationship of liver, which stiffens with increasing strain at a greater rate than the first order MR model can account for. A possible explanation for this extreme strain stiffening

behaviour is that the microanatomy of the liver governs its mechanical behaviour, more so than in brain and lung. In the liver, each lobule is surrounded by a layer of impermeable connective tissue consisting of venous membranes and fibrous material that are much tougher than the parenchymal hepatic tissue. However, the connective tissue is flexible and as such, at low strains, the liver appears to be soft as the soft parenchymal tissue deforms. Because the connective tissue is almost impermeable, as the strain increases, the fluid pressure in each liver lobule, even at low surgical speeds, builds up. Since the connective tissue is also very tough, it is able to sustain massive fluid pressures before it fractures. This behaviour can be likened to the deformation of an inflated balloon; the initial compression does not require a lot of force, however, it becomes increasingly more difficult to achieve greater compression due to the pressurisation from the air that cannot escape and the balloon membrane that does not burst. Furthermore, the entire liver consists of a densely packed organisation of these individual lobules, which only compounds the effect. Comparatively, whilst the axons in brain are also considered impermeable, the brain is 2 orders of magnitude softer so the axons would likely not be able to withstand the same level of pressure resulting in a material collapse. The lung shows the smallest relaxation ratio because it is highly porous and should be filled with air. The little relaxation it does show is likely due to the interstitial fluid of mucin, which serves to lubricate the expansion of the lung.

Future work should focus on implementing higher order MR terms to model as it has been previously shown that a 9th order MR model can successfully model biological tissues across a wide range of strains, from tensile to compressive[189]. Alternatively, a more accurate material model derived from a physiological perspective should be explored, ideally from understanding the effect of the microstructure on the deformation behaviour. This could involve the development of a model that takes into account the hexagonal lobular microstructure of the liver, for example the transversely isotropic constitutive model proposed by Chui et al.[223].

The viscoelastic models parameters were fitted to the average relaxation curve of the porcine tissue results. The 3rd order Prony series fitted parameters are listed in Table 13. The first set of parameters describes the initial progression of the relaxation curve. The third set of parameters describes the relaxation curve as it settles to E_{∞} . The E values reveals the weight of the time constant on the fitting of the curve. The dominant E value signifies the overriding time constant. For example, if $E_1 \gg E_3$, then τ_1 has a more significant role in the relaxation response of the tissue compared to τ_3 . Since τ_1 is the shortest time constant, a large E_1 value signifies that the relaxation response is much greater. This is confirmed when comparing the trend between E_1 and the relaxation ratios calculated for each tissue and listed in Table 14.

Across the three tissues tested, the liver shows the greatest relaxation response, E_1 , and consequently, the highest relaxation ratio. The equilibrium stiffness, E_∞ , is also the greatest, which is easily understood as the liver has already been shown to be the stiffest biological tissue out of those tested in this study. The relaxation ratio of brain is the next highest value, followed closely by lung. The brain E_i values are relatively consistent across the three orders compared to lung and liver. This is interesting because the relaxation ratio for brain and lung are of comparable magnitude, yet the fitted parameters indicate that the progression of the relaxation curve for lung is dominated by E_3 , but E_1 in brain. This suggests that lung has a softer relaxation response and that the longer time constant has a greater influence on the curve. This quantification is confirmed by observing the relaxation curves in Figure 19 (B) and Figure 24 (B), where it is evident that the relaxation curve for brain is steeper in the first 100 s than lung.

Table 13. 3rd order Prony series viscoelastic parameters.

		Prony series order term		
		1	2	3
Porcine brain, white matter	τ_i (s)	2.25	16.5	150
	E_i (Pa)	366	404	409
Average Porcine Lung, Transverse	τ_i (s)	2.16	15.9	171
	E_i (Pa)	124	213	397
Average Porcine Lung, Axial	τ_i (s)	1.39	14.1	168
	E_i (Pa)	15.5	44.8	74.1
Average Porcine Liver, Transverse	τ_i (s)	2.56	17.6	130
	E_i (Pa)	15691	13742	8660
Average Porcine Liver, Axial	τ_i (s)	2.41	17.5	132
	E_i (Pa)	6039	6099	4334

Table 14. Relaxation Ratio.

	Relaxation Ratio
Porcine Brain,white matter	3.89 ± 0.54
Porcine Lung, transverse	1.95 ± 1.09
Porcine Lung, axial	2.21 ± 1.04
Porcine Liver, transverse	17.5 ± 6.3
Porcine Liver, axial	14.7 ± 7.0

The effect of directionality on the viscoelastic properties in lung and liver are not as significant as the variability among different tissues. In lung, the average relaxation ratio is insignificantly

greater in the axial samples compared to transverse results, as the standard deviation is very wide greater than the difference. The Prony series parameters reveal greater detail about the evolution of the relaxation curve. Although the relaxation ratio seems to be greater in the axial samples, E_3 is 4.8 times greater than E_1 , compared to the 3.2 times in the transverse samples. Hence, whilst the axial samples relax more overall, their relaxation is over a slightly longer time scale. Generally, the trend of the values across the terms are consistent across transverse and axial lung results, with $E_3 > E_2 > E_1$. The opposite trend is true and consistent for liver samples, where $E_1 > E_2 > E_3$, and E_1/E_3 is 1.81 and 1.39 for transverse and axial samples, respectively. This shows that relaxation response is more severe in the transverse samples, compared to axial, as the stress at 30% strain is much greater than the equilibrium stress. This result is also confirmed when recognising that the relaxation ratio is greater in the transverse direction by 19% than axial results.

The organs tested in this work were chosen to be studied because they were deemed to have a wide range of mechanical properties and the results have clearly shown this. From a biological stand point, the parenchyma; axons, aveoli and hepatic lobules, of each biological tissue serves an extremely different function. In addition, the interstitial fluid; CSF, air and blood, in brain, lung and liver, respectively, have different rheological properties. Also, the mechanical properties of the internal structures; myelinated axons, cartilaginous bronchi and membranous hepatic veins, are also vastly different. The organisational variety of each tissue's internal structure only serves to confirm the amazingly complex and efficient methods to achieve their desired function.

Once this is understood, it is obvious that the application of a single hyperelastic material model is inadequate. Although the Mooney-Rivlin model is a constitutive law derived from finite strain theory and is fundamentally defined by the strain energy density function, this work has shown that is not possible to model all biological tissues using a single type of model. There are a vast array of different constitutive models suggested in literature that can be extended to include many ordered terms in order to find a closely fitting model. However, this study has not found evidence of a model that is derived with respect to the significance of the internal structures or the effect of the intricate microstructural geometry that could help explain the results found in this work. Consequently, an ambitious future work would be the derivation of a constitutive law that couples geometrical influences with the limitations of the strain energy density function in order to more accurately define the anisotropic response of complex materials. Applications for such a scalable material model would not only be limited to biological materials, but could also include any complex material that is inherently anisotropic

and has a defined geometrical structure, for example the increasingly popular study of three dimensional auxetic and origami structures[225,226].

When analysing the viscoelastic material properties, the relaxation ratio and fitted Prony series parameters are often used in literature to cross compare different data set results. Since the trends are consistent between the results of the two methods, it is advised to quote the relaxation ratio for simple comparison between different analyses. If further detail about the evolution of the relaxation response is of interest, or for use as a material definition for computational modelling, the Prony series parameters can be studied and used.

The work produced in this chapter was aimed to explore and describe the effect of some main factors on the mechanical properties. The key material characteristic that was identified to be of interest for this study was the effect of directionality with respect to the internal biological structures, and how this influences the compression-relaxation response. Additionally, a central application when dealing with biological organs is surgery, and so the biological tissue fracture properties, using a needle insertion methodology, were also of high interest. Considering the scope of the task, unfortunately, not all equally intriguing parameters were able to be studied, including many physiological and environmental factors, such as disease or testing temperature. The next chapter will describe the process of attaining detailed maps that will be used to determine the tissue mimicking hydrogel compositions that match the results found in this chapter.

4. Mechanical Characterisation of Composite Hydrogel

This chapter has been partly published in Materials and Design[153].

4.1. Introduction

In the previous chapter, some of the crucial mechanical properties of real biological tissues were characterised. However, the measurement of those results are influenced by the effect of environmental factors on the tissue's mechanical properties, which results in high standard deviations and great variability amongst literature reported results[8]. In addition, ethical limitations that hinder procurement and the range of tests that can be carried out *in-vivo* constrain the advancement in study of a number of important topics; the most notable of which are the study of traumatic brain injury, disease progression on a macroscale and the testing of new surgical equipment.

In traditional engineering, where the testing of the real system is not possible, a model system is created to try to predict the outcome of certain events as accurately as possible. These models involve both physical and computational elements and both fields of research symbiotically increase the other's accuracy. To that end, there is a great interest in developing a synthetic material that is able to be used as a tissue phantom model to be subjected to any test without raising ethical issues.

Consequently, a variety of materials have been studied and developed to mimic specifically the mechanical properties of different biological tissues, which include PVA, PVC, natural and silicone rubber, gelatine and hydrogels[60,63,64,161,227–230]. Among these materials, hydrogels have gained popularity due to the wide range of applications that have arisen from their development[4,95,231,232]. The biocompatibility and stiffness tunability of hydrogels has also allowed them to be used for tissue engineering scaffolds[60,63,84–86,93,228,233–235]. This is because, in mechanobiology, the mechanical fidelity of hydrogels is vital as mechanical traction forces are generated at the cell-substrate interface due to cell adhesion, migration and proliferation, and it is also known that substrate stiffness affects cell biology[6,236–242].

Out of the variety of materials reviewed, a promising solution is the composite hydrogel (CH) that has been developed by Forte and co-workers to match the complex viscoelastic behaviour of brain[60]. Its constituents are poly(vinyl alcohol) (PVA) and phytagel (PHY). The CH can be tuned to achieve different mechanical properties by varying the concentrations of each hydrogel component. By observing the change in these properties as a function of PVA and PHY concentration, one can map the mechanical response of the CH at different PVA/PHY

concentrations and subsequently find the mixtures that best match the mechanical responses of different soft tissues. It is for this reason that the CH was selected as the tissue mimicking material in this work. The homogeneous response of the synthetic material is compared to the real biological tissues. Since structural anisotropy is not present in the CH and heterogeneity is confined to scales much smaller than those of interest for the characterisation performed here, attention is also focused on potential differences emerging in the comparison with real tissues when these show a more heterogeneous response due to their internal structures being prominent at the scales tested here. Three maps are hereby presented for the mechanical properties: stress at 30% strain, insertion force, friction force. Through the combination of these maps, an evaluation of a single hydrogel composition that best matches all three mechanical characteristic responses for each of the soft tissues can be made.

The work in this chapter will describe the characterisation of the composite hydrogel to a high level of detail. The mechanical behaviours that are considered and studied in this work include the compressive stress-strain response over a range of strain rates (from equilibrium to dynamic), stress relaxation over time at a fixed strain, fracture properties and frictional interactions between the hydrogel and other materials relevant to specific applications. The first item mentioned above is the compressive stress-strain response, from which the mechanical stiffness of a material can be implied. This is one of the crucial mechanical behaviours that is relevant to any application in which load bearing applications, such as tool/construct interactions, are of interest. As introduced in chapter 3, the stress at 30% strain is often used to characterise and infer the stiffness of a material because it gives an immediate sense of scale. However, the composite hydrogel also displays a non-linear stress-strain curve and so, in order to properly describe the shape of the stress-strain curve, the Mooney-Rivlin material model for hyperelasticity is also evaluated for every hydrogel composition. The viscoelastic response of the material is also mapped from the relaxation tests performed on each hydrogel composition.

A number of methods exist for measuring a material's ability to resist fracture. Traditionally for metals, this material property, called fracture toughness, may be measured using the Charpy impact test, where a notched sample is impacted and the energy required to cause complete failure is recorded. However, this testing method is not sensitive enough for extremely soft materials, such as biological tissues. When these types of loads are experienced by the human body, it is usually a case of broken bones. For soft tissues, damage is usually encountered during physical trauma and surgical procedures. The loads applied in these scenarios cause penetration and cutting of the soft tissue. Therefore, this mechanical behaviour is often characterised as a resistance to penetration and cutting, and most literature found characterising this behaviour derives from needle insertion experiments[108,114,243,244]. Additionally, the friction force

experienced between the needle and the hydrogel can be isolated and friction coefficients can be determined.

The frictional interaction between two materials is a system property, which means that it depends on the materials in contact and the lubrication mechanism or agent. A range of novel state of the art materials and biomaterials are now being developed for surgical use, including intricately designed 3D printed needles made from polymer resins[245–247]. The frictional interaction between these advanced catheter materials, real biological tissues and tissue phantoms should be investigated and compared to traditional surgical tool materials, such as stainless steel. Separate frictional sliding tests can be used to compute the friction coefficient between a hydrogel substrate and a stainless steel material for comparison to other material systems.

The structure of this chapter is organised by reporting the result of each testing method in the form of 3D maps that show how the mechanical behaviour is affected by the hydrogel composition. In the first section, the stress at 30% strain, strain rate dependency and Mooney-Rivlin parameters are mapped. Then, the relaxation ratio and Prony series relaxation parameters are reported. This is followed by the characterisation of fracture by means of average insertion force and complete sample penetration depth. Finally, the frictional interaction is evaluated by studying the average CH/needle friction force and the friction coefficient between the CH and stainless steel. Following this, Chapter 5 will detail the exact utilisation of the results found here to propose and evaluate certain hydrogel compositions that focus on mimicking biological tissues for certain applications.

4.2. Materials and Methods

4.2.1. Composite Hydrogel Sample Preparation

PVA (molecular weight 146,000-186,000 Da), PHY and deionised water were supplied by Sigma Aldrich, USA. The CH was prepared by dissolving various amounts of the PVA and PHY powders (exact compositions can be found in Appendix A, separately in deionised water for 1h at 90 °C. The separate solutions were combined in a 1:1 weight ratio and stirred at 70 °C for an additional 30 min. The mixed solution was then poured into a petri dish and allowed to cool to room temperature. The range of hydrogel compositions chosen to be tested was limited to combinations within the ranges of 2.5-15 wt% PVA and 0-5 wt% phytagel. The lowest formulation tested, deemed structurally stable and hence included in the results was 2.5 wt% PVA and 0.5 wt% phytagel. In the other extreme, when increasing the concentration of each constituent hydrogel, there are two factors that hinder sample production; the difficulty in dissolving the polymer powder increases, and the hydrogel solution viscosity increases, making it difficult to cast-mould. This limits the feasibility of producing high concentration formulations and hence, the highest achievable composition tested was 15 wt% PVA and 5 wt% phytagel. Although results from higher concentrations are reported in literature, these are typically obtained using more advanced fabrication methods involving multiple autoclave cycles to achieve high enough pressure to dissolve the powder[248–250].

The CH is physically cross-linked by hydrogen bonds that form during the thawing process when the hydrogel undergoes a freeze-thaw cycle. Therefore, the solution is frozen and stored at -25 °C for 15 hrs. Before testing, the samples are thawed to room temperature in a controlled environment (~19 °C). For needle insertion testing, the tissue mimicking compositions corresponding to brain, lung and liver, were cast moulded in a 60 x 60 mm perspex box with a thickness of 17.60 ± 1.25 , 18.32 ± 1.55 and 22.02 ± 0.37 mm, respectively. The hydrogel samples were kept hydrated until tested.

4.2.2. Compression-Relaxation Test

Unconfined uniaxial compression tests to 30% strain at 1, 0.01 and 0.0001 s⁻¹ strain rates and 500 second relaxation time were performed using a Mach-1™ mechanical testing system (Biomomentum, Canada), the same parameters as used in other studies reported in the literature[14,18,60] to test the biological tissues. Two loads cells were used to measure the forces on the testing samples over a wide range of magnitude, which are the 1.5 N load cell (Honeywell, USA) and 70 N load cell (Biomomentum, Canada). The unconfined compression rig is equipped

with two stainless steel plates, a fixed bottom platen and a controlled top platen, connected to the load cell directly. The interfaces between the sample and the machine platen were lubricated using silicon oil to reduce the effect of the lateral friction force during compression. The compression test took under 30 seconds to complete, which is not long enough to induce any dehydration effects in the hydrogel samples according to Forte et al.[15] For compression testing, cylindrical samples of 8.81 ± 1.87 mm diameter and 8.07 ± 0.78 mm height were cut from the petri dish bulk material using the same biopsy punch as the biological samples. The diameter was measured using callipers placed around the middle of the sample height. The height was measured after securing the sample on the testing apparatus by recording the distance between the two testing platen before the start of the test.

From the experimental data obtained during the unconfined compression tests, the true stress was calculated at 30% engineering strain under the assumption of incompressibility, which is commonly used to model soft materials[15,17]. A more accurate and descriptive evaluation of the deformation behaviour was obtained by fitting a first order Mooney-Rivlin material model to every tested hydrogel composition at 0.01 s^{-1} strain rate. The model parameters for C1 and C2 were plotted in a similar map to analyse the effect of PVA and phytigel concentration on the hyperelastic parameters. The relaxation response was fitted with a 3rd, 2nd and 1st order Prony series viscoelastic model for the 1, 0.01 and 0.0001 s^{-1} strain rate results, respectively. The model parameters involved include the stiffness E_i , and time constant τ_i . Maps for each of the terms with respect to the hydrogel composition were also obtained and discussed.

4.2.3. High Strain Rate Tests

The composite hydrogel samples were prepared according to the method described in section 4.2.1. The samples were cast-moulded to achieve cylindrical samples of 29.4 ± 0.4 mm diameter and 19.3 ± 0.2 mm height. Unconfined uniaxial compression tests were performed at 60 s^{-1} strain rate, which is in the order of magnitude of speed required to initiate traumatic brain injury damage[187]. The impact tests were conducted using a high speed testing rig with a fixed bottom platen and a moving top platen, as shown by the schematic in Figure 32. The impact testing machine utilises magnetic repulsion forces from magnetic rings surrounding the top and bottom platen to decelerate the top compression platen, which is travelling at a speed of around 0.55 m/s. To ensure the safety of the rig, the magnetic plates require a certain amount of time to decelerate the impacting top plate. Although the samples were curated to be compressed by 30% strain before the plates touched to match the low rate compression tests, due to the deceleration of the plates, only results up to 20% strain were meaningful for analysis.

The impact rig used to test the hydrogels is typically used to test metals and polymers. As such, the only available and feasible load cell that could be used given the safety constraints of the rig was a 25 kN load cell. This load cell was not sensitive enough to obtain valuable data for the lowest hydrogel concentrations and as such, only a preliminary map showing how hydrogel composition may affect the compressive stress at dynamic loads was obtained from these results. The raw data was extracted from the testing machine and post-processed using a Savitzky-Golay filter in MATLAB. The true stress at 20% strain was calculated for each hydrogel sample and a map representing the stiffness dependency on hydrogel composition at a dynamic strain rate of 60 s^{-1} was plotted.

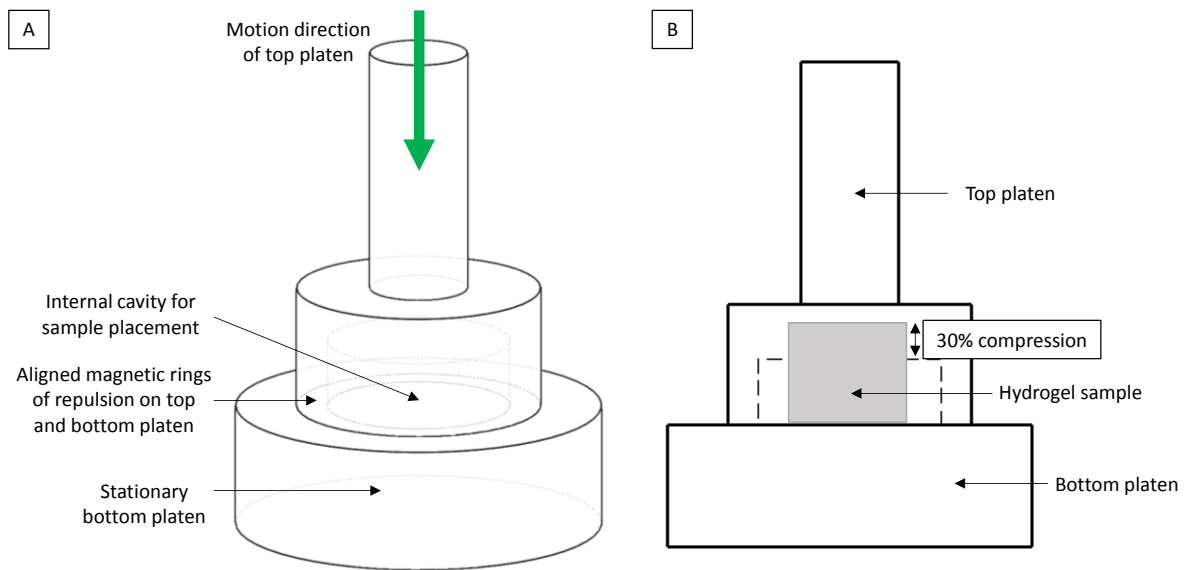


Figure 32: Schematic showing the impact testing rig setup in (A) isometric view and (B) determination of impactor depth based on sample height to achieve 30% compression.

4.2.4. Fracture Behaviour Tests

Needles with a diameter of a few millimetres are commonly used in neurosurgery, for example in minimally invasive biopsy procedures[167,184]. The needle insertion tests were performed using a rigid 3D printed needle with 4 mm diameter and 40° conical tip connected directly to the load cell in a similar setup to Leibinger et al.[63]. The insertion speed was based on the same medium strain rate (0.01 s^{-1}) as in the compression tests so that the results can be combined to evaluate a surgical phantom, as we shall see in the next chapter. The CH samples were placed in a $60 \times 60 \text{ mm}$ perspex box, which was large enough to avoid any edge effects. As with the methodology of the biological samples, the needle was inserted all the way through the hydrogel sample and out the bottom surface of the box, through a 10 mm diameter laser cut hole, designed to minimise alignment issues and excessive resistance of the tissue as the needle completed its travel through the bottom end of the box. The insertion force profile of the CH samples was then analysed.

The average insertion force was calculated as the average force of the entire insertion stage, including repeated rupture. The influence of ductility on the fracture behaviour was also characterised by analysing the depth at which the sample had fully fractured, i.e. when cutting had finished. The average friction force between the needle and the hydrogel was calculated as the average force after the needle passed through the entire sample and the bottom of the box, which is a protocol carried out similarly by Oldfield et al.[114]. The coefficient of friction was also evaluated to give a deeper understanding of the frictional behaviour between the needle and the composite hydrogel. The coefficient of friction is the ratio of friction force over normal force. The normal force on the needle is governed by the stiffness of the material. Therefore, it was interesting to study the coefficient of friction separately from the friction force as it gives valuable insight on how these properties relate to one another, with respect to the hydrogel composition.

For the calculation of friction coefficient, the normal force was determined from a FEA model of a 2 mm diameter needle deforming a 60x60 mm hydrogel block, as shown in Figure 33. A 2D quarter symmetry model consisting of 8 noded tetrahedral elements was used. A rigid body constraint was applied to the needle as the needle was assumed to be much stiffer and hence undeformable compared to the hydrogel. A linear elastic material model was used to define the hydrogel block. The linear stiffness (Young's modulus) was calculated as the gradient of the stress-strain curve up to 6.67% strain as this is the strain that the 2 mm diameter needle would impart on one half of the entire 60x60 mm block. At this strain, the compressive behaviour was deemed linear enough to allow for this assumption. The Poisson's ratio was set to 0.475 as the hydrogel was assumed to be incompressible[114].

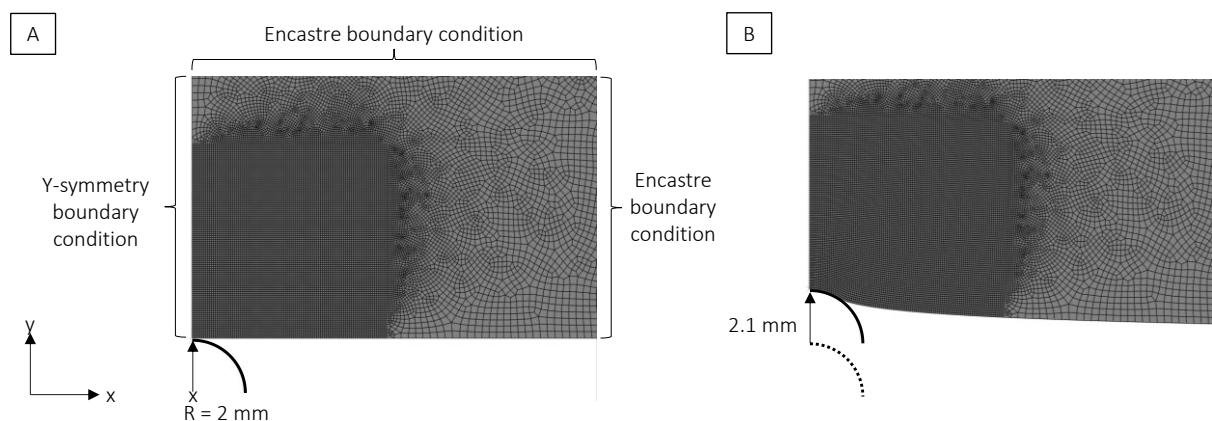


Figure 33: ABAQUS finite element model used to find the normal force of the surrounding tissue on the needle showing (A) undeformed with boundary conditions labelled and (B) an example of the deformed results.

Figure 33 shows the setup of the finite element model with boundary conditions labelled. The needle was initially placed 0.1 mm away from the surface of the hydrogel block so the displacement step was 2.1 mm to result in the needle reaching a 2mm depth into the block. Since this model is a quarter of the hydrogel block, the total normal force was calculated as 4 times the y reaction force produced by the model. The friction coefficient was then calculated as the ratio between the measured friction force from the needle insertion experiments and the normal force calculated from the FEA model. 3D maps outlining the effect of CH composition on the average insertion force, entire sample penetration depth, average friction force and coefficient of friction were created from the results calculated for every hydrogel composition tested.

4.2.5. Frictional Sliding Tests

Since friction is a system property, the friction force between the composite hydrogel and stainless steel was investigated and compared to that of CH/3D printed needle. Using the Mach-1™ mechanical testing system (Biomomentum, Canada) and a six axes 70 N load cell (Biomomentum, Canada), a spherical 6 mm diameter stainless steel indenter was displaced 2 mm into the height of the sample. A sinusoidal reciprocating motion was used to conduct the frictional sliding tests. Three compositions that were determined to have the same stiffness from the results of the CH stiffness mapping were tested to analyse the effect of hydrogel composition on frictional behaviour, independent of stiffness. The determination of friction coefficient is a first step towards analysing frictional behaviour at the surgical tool-tissue interface.

4.3. Results and Discussion

4.3.1. Linear Elastic Parameters

The effect of the constituent PVA and PHY on the stiffness of the overall composite hydrogel is shown in *Figure 34*. The values for stress at 30% strain for each CH composition tested and the standard deviation can be found in the Appendix B. Increasing the concentration for both PVA and PHY increases the maximum stress at 30% compressive strain. Interestingly however, the absolute percentage weight contribution of PVA and phytigel have different effects on the stress at 30% strain. A 5wt% increase in phytigel with any blend of PVA concentration results in a much stiffer composite hydrogel than a 5wt% increase in PVA. Also, a 5 wt% increase in phytigel in combination with 10 wt% PVA results in a greater rate of stiffness increase, compared to a 2.5 wt% PVA blend. The reverse is also true if phytigel concentration is kept constant and the relationship between PVA and stiffness is analysed.

Additionally, at increasing strain rates, increasing the concentration for both PVA and phytigel results in a non-linear increase in the maximum stress at 30% strain. This confirms findings in previous literature that a strain rate dependent model must be adopted to characterise soft tissue[187]. Figure 35(A) shows that the strain rate dependency significantly increases as the concentration of phytigel increases, keeping PVA concentration constant. Subsequently, Figure 35(B) highlights the effect of changing PVA concentration when phytigel concentration is kept constant. The logarithmic curves show less difference in gradient, especially between the 5 and 10 wt% PVA compositions. This suggests that the phytigel has a greater effect than PVA on the strain rate dependency of CH. The logarithmic equations best fitted to the data can be found with their respective R^2 values in the Appendix B.

The strain rate dependency of soft tissue is important as this allows surgeons to understand that the stresses soft tissues are subjected to during surgery are affected by the speed at which they interact with the tissue. It also allows for more sensitive surgical planning and handling procedures to be developed with the intention of reducing stresses in the tissue, which may lead to tissue injury. At higher compression speeds, the strain rates are those that may be experienced during impact loading, for example in car crashes and explosions, resulting in traumatic injury[251], which is therefore also extremely important to understand. The results in Figure 35 show that the stress at 30% strain increases logarithmically with increasing strain rate, which allows the stresses experienced at strain rates within the range reported (0.0001 to 1 s^{-1}) to be interpolated.

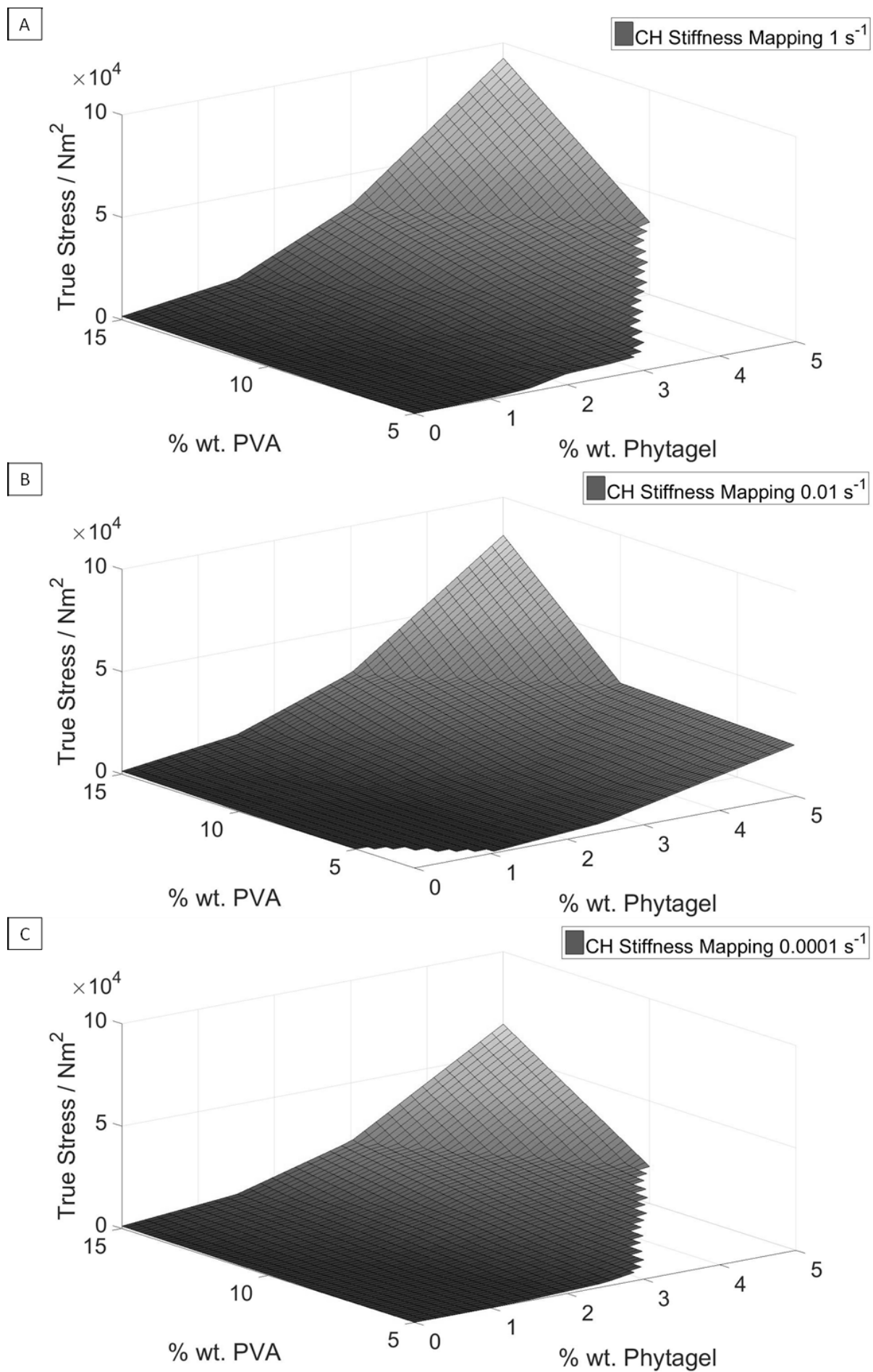


Figure 34: Stress at 30% strain mapping with respect to hydrogel composition at (A) 1 s^{-1} , (B) 0.01 s^{-1} and (C) 0.0001 s^{-1} strain rates.

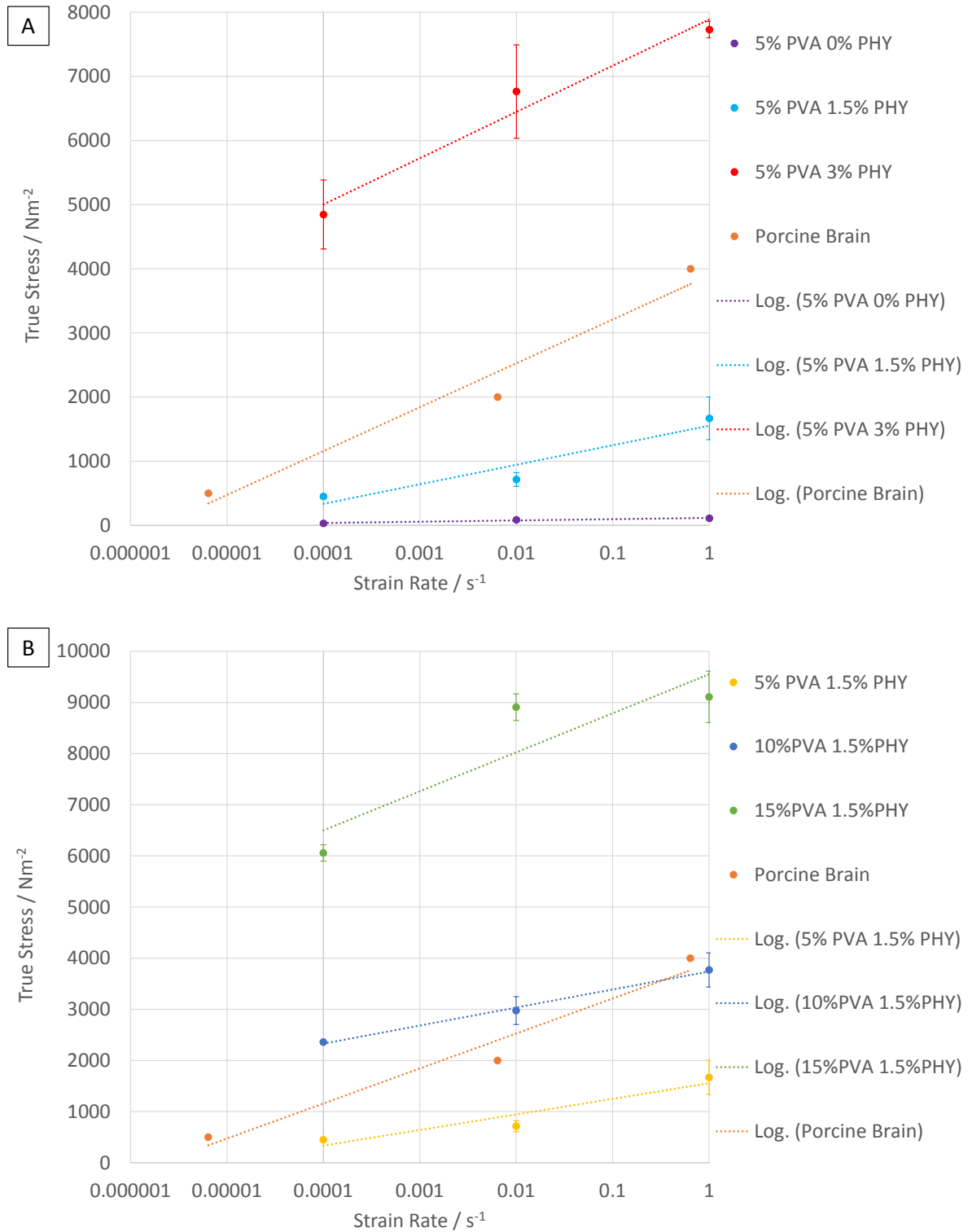


Figure 35: Strain rate dependency of composite hydrogels where wt% PVA is constant and wt% phytigel is changed for (A) 5 wt% PVA and (B) 10 wt% PVA compared to the literature values for brain reported by Miller and Chinzei[252].

The experiments performed by Miller and Chinzei[252] use the most similar strain rate testing range to this work, hence allowing the results to be compared, as shown in Figure 35. A logarithmic trend line, $R^2 = 0.96$, can also be fitted to the results to model the strain rate dependence of brain. Comparing the strain rate trend lines of brain to hydrogel, brain exhibits a steeper rate, most equivalent to high concentrations of phytigel, for example 5 wt% PVA and

3 wt% phytigel. Unfortunately, those concentrations result in a material much stiffer than brain. Therefore, the appropriateness of mimicking stiffness or strain rate dependency must be weighed against one another when selecting a single tissue mimicking composition, as there is no obvious formulation of PVA-phytagel composite hydrogel that is able to mimic the highly strain rate dependant behaviour of real brain across 5 orders of magnitude.

4.3.2. Hyperelastic Parameters

The fitted hyperelastic Mooney-Rivlin model parameters show a similar story to the map for stress at 30% strain, where the general trend is that increasing the concentration of PVA and phytigel results in an increase in stiffness.

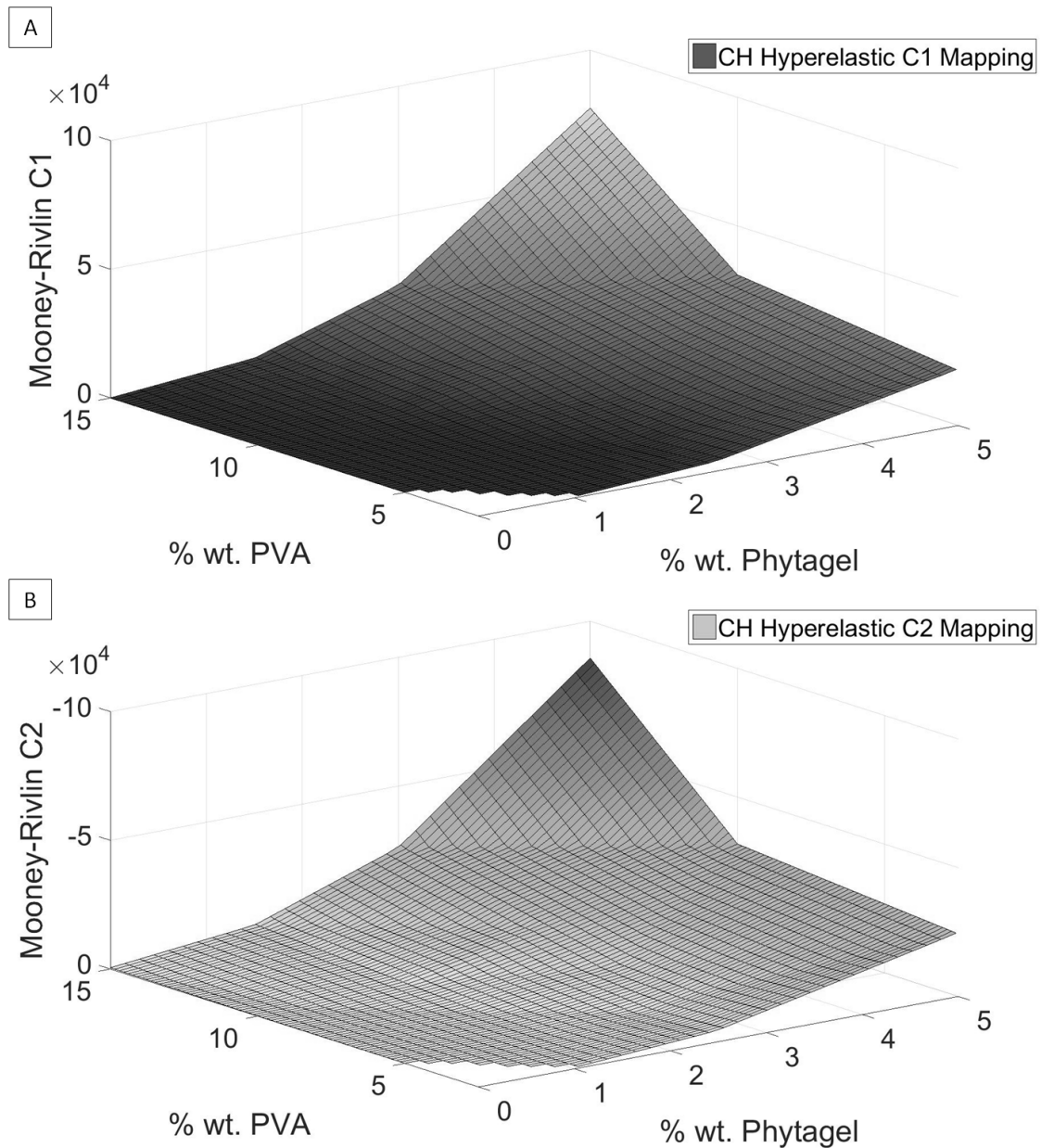


Figure 36: Map of Mooney-Rivlin model parameters (A) C1 and (B) C2 fitted to each hydrogel concentration at 0.01 s^{-1} strain rate.

As expected, the trend of the map is similar to the linear elastic graph obtained in Figure 34 (B) at 0.01 s^{-1} strain rate, which shows that a 1 wt% increase in phytigel concentration results in a stiffer hydrogel compared to an increase of the same concentration of PVA. The models for each hydrogel data point were not checked for stability at stretches beyond the results obtained (1-0.7 compressive stretches), as they were done for the biological tissues previously. Analysing the results shown in Figure 36, it is apparent that the magnitude of C1 and C2 is relatively similar across all hydrogel compositions, however the main difference is that C2 appears to be the inverse of C1 in terms of sign.

A parametric study was produced in Figure 37 to show the effect of positive and negative parameter sign on the shape of the stress-stretch curve. Figure 37 (A) shows the 4 possible parameter combinations when the signs of C1 and C2 are varied as described in the legend. The magnitude of C1 and C2 was set to a constant 100 to show the effect of sign combination only. First of all, hyperelastic parameters are derived by assuming compressive stresses are negative, as it is convention. Since the true stresses calculated at compressive strains in this work are reported as positive, the curve resulting from the hyperelastic parameter fitting should be inversed about the x-axis, but the parameters reported in this thesis follow usual convention. As there are 2 variables and each can have a positive or negative sign, there are 4 possible combinations. From Figure 37 (A) it is easily understood that the top two curves are the mirror of the latter two about the x-axis. This therefore results in only two sign combination options; either both C1 and C2 are the same sign or they are opposite. Next, Figure 37 (B) illustrates the difference between the two sign combinations. It was apparent from Figure 37 (A) that keeping a constant magnitude of 100 whilst changing the sign combination resulted in different stresses at 30% compressive strain. To achieve the same stress at 30% strain across both sign combinations, the C1 and C2 parameter magnitudes were tuned, as shown in Figure 37 (B). The graph clearly shows that when the hyperelastic parameters are of opposite signs, the curve is much more non-linear than when the signs for C1 and C2 are both the same sign.

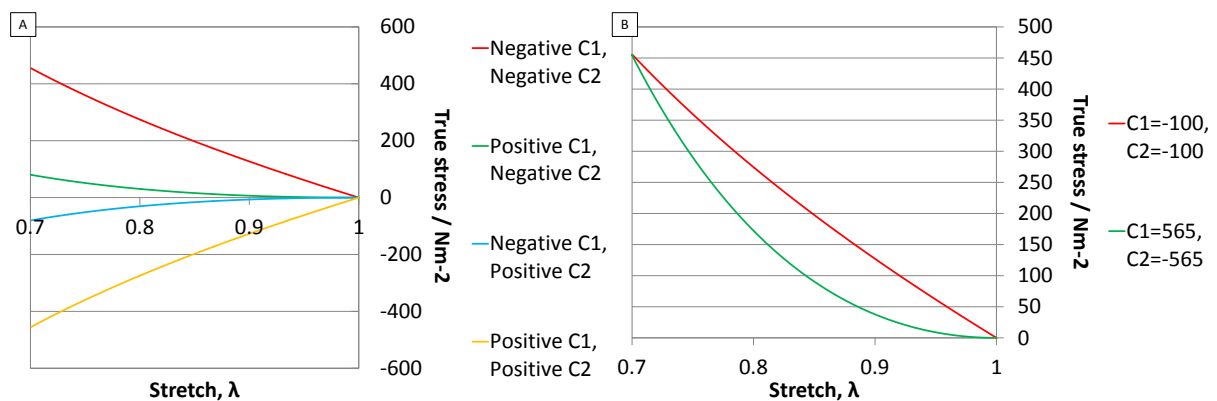


Figure 37: Graphical explanation of how the hyperelastic parameters magnitude and sign gives rise to the resulting stress-stretch curve shape.

Recalling the results from the hyperelastic fitting of biological tissues from Chapter 3, section 3.3.4, Table 12, the fitted hyperelastic parameters for brain and liver are of opposite signs compared to those of lung, which are of the same sign. This indicates that the brain and liver stress-stretch curves are more non-linear than lung, whose stress-strain curve was already shown to be the most linear out of the three biological tissues tested. Again, the extent of strain stiffening was especially evident in liver, which, as discussed previously, is so extremely non-linear that a first order MR fit was not able to fully capture its behaviour. Conversely for the hydrogel, the first order MR material model was able to fit all tested compositions with a combined average goodness of fit of 0.9983, indicating that this material model adequately represents the mechanical behaviour of the composite hydrogel.

Furthermore, the fitted parameters for compositions that were pure PVA concentrations (with 0 wt% phytagel) were of the same sign. As soon as phytagel was added to the hydrogel blend the fitted parameters expressed opposite signs, even from as little as 0.5 wt%. This indicates that phytagel plays an important role in increasing the degree of non-linearity of the resulting composite hydrogel and is therefore a necessary component in the development of a tissue mimicking material. The full list of values for C_1 , C_2 and R^2 can be found in the Appendix B.

When comparing these parameters to those found in literature, it should be checked whether firstly, the results are for tension or compression and secondly, if the results are reported with a positive or negative sign. Bracq et al.[253] fitted a first order MR model to positively reported compressive stress-stretch curve results of a polymer gel (styrene-ethylene-butylene-styrene SEBS) used to model biological tissue under ballistic impacts and reported C_1 and C_2 values of 43 and -5 kPa at 0.018 s^{-1} strain rate[253], which lie within the range of values found in this work also. The opposite signs of C_1 and C_2 indicates that the non-linearity of the SEBS gel is significant and hence describes how it is able to mimic soft tissues. The hyperelastic parameter values reported in the Appendix B may be used to define the material in computational simulations of the hydrogels tested here. Possible future work involving the use of these results are the computational modelling of hydrogel elasticity when cellular proliferation forces are applied to the hydrogel substrate in order to study the mechanobiological effects of the substrate on cell behaviour.

4.3.3. Viscoelastic Parameters

Relaxation Ratio

The relaxation ratio mapping is shown in Figure 38. A linear interpolation method is used to fit the three rates tested, 1, 0.01 and 0.0001 s⁻¹, and to show the trend in the overall relationship between the concentrations of PVA, phytagel and relaxation ratio. The relaxation ratio and the range of these ratios decreases as strain rate decreases hence the map becomes much flatter for the lowest (0.0001 s⁻¹) strain rate. This attests to the assumption that 0.0001 s⁻¹ is a quasi-static strain rate as the viscous effects are much less apparent in these results compared to the results obtained at 1 s⁻¹. The results show that generally, the relaxation ratio increases as the concentration of phytagel increases, which is especially evident at high concentrations of PVA. This agrees with the understanding that phytagel contributes to the viscous response of a material, as an increasing relaxation ratio can be related to increasing viscosity. This is particularly evident in Figure 39, from which the 2D view of the 3D map shows that the rate of increase in relaxation ratio is relatively constant as the phytagel concentration increases, despite the absolute values being higher for higher strain rates.

These results could possibly be explained by understanding that phytagel crosslinks with the PVA structure to form smaller microstructural voids that trap water more effectively in these pockets. This is evidenced by an SEM image taken of the composite hydrogel in Figure 40 (A) that shows a disorganised, homogenous web-like microstructure with pore sizes in the range 5-20 µm through which free water tries to pass when the material is deformed. The pore size and crosslinking morphology of the microstructure conforms with the SEM imaging results reported by Forte et al.[60], shown in Figure 40 (B). At high concentrations of phytagel, although there is a low free fluid to non-fluid mass ratio, more water is retained in the microstructure of the CH during the thawing process and this contributes to the high relaxation ratios measured. Trapped water in the porous microstructure causes high stresses in the material at high strain rates because the water does not have time to escape the composite hydrogel solid matrix (as is the case for most biphasic materials). This behaviour suggests that the composite hydrogel is to some degree a biphasic material, which means that the fluid phase contributes to the load bearing ability of a material in parallel with the solid matrix, which is a common characteristic for hydrogels[254–258]. A widely researched biological example of this is cartilage, where its viscoelastic properties are attributed largely to fluid pressurisation and hence, biphasic material models have been developed to characterise this phenomenon[259–262].

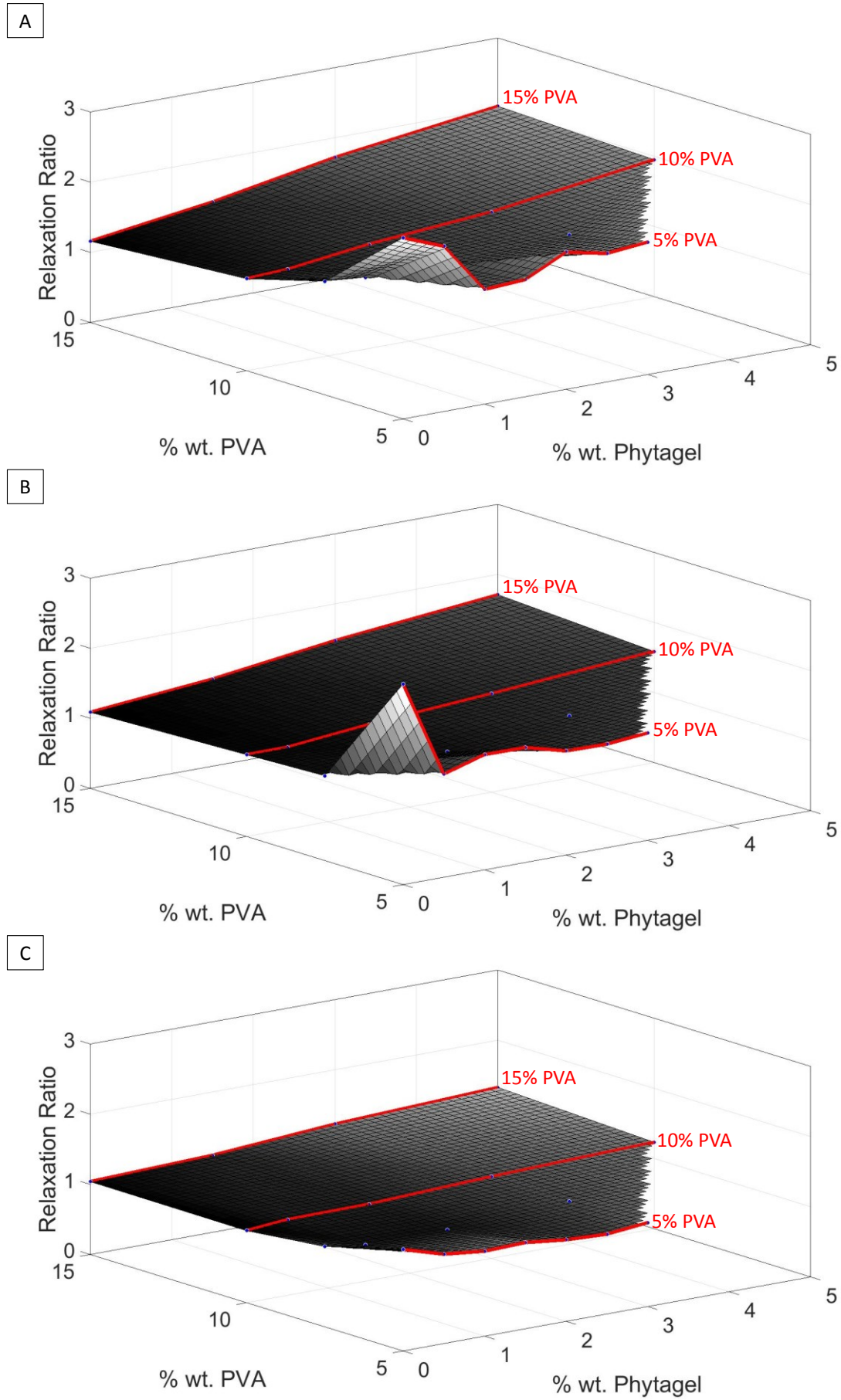


Figure 38: Relaxation ratio mapping of a) 1 s^{-1} , b) 0.01 s^{-1} and c) 0.0001 s^{-1} strain rate, with red lines of constant PVA concentration and varying PHY concentration to help guide the eye.

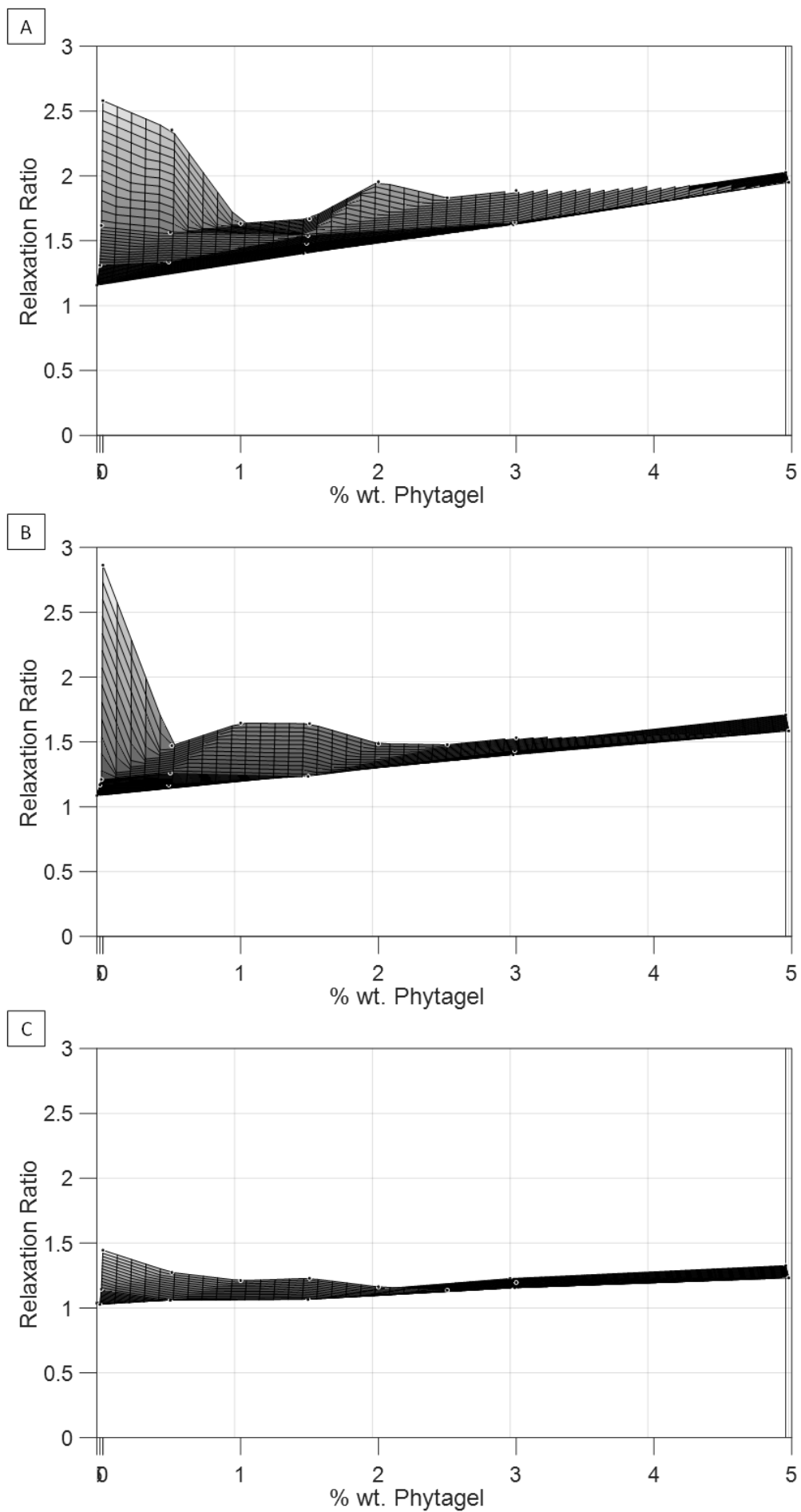


Figure 39: 2D view of map showing the dependence of relaxation ratio on phytigel concentration.

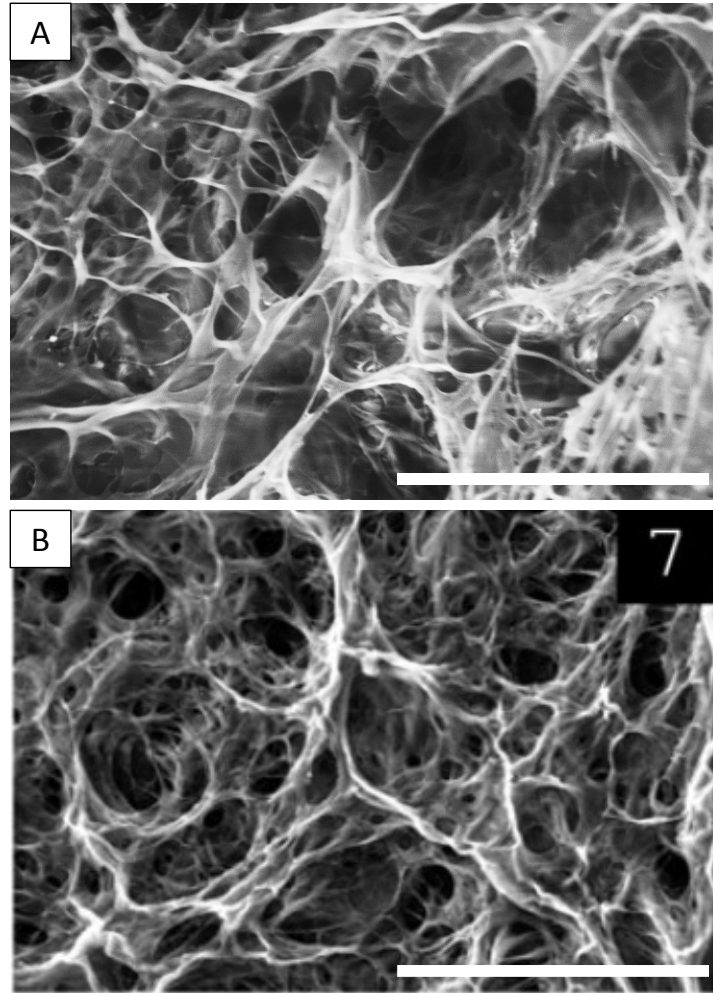


Figure 40: (A) SEM images of the hydrogel microstructure showing pore size and crosslinking morphology compared to those obtained by (B) Forte et al.[60]. Scale bars both 100 μm .

Having understood this, the maximum relaxation ratio is conversely found at the lowest concentration of PVA and phytigel tested, 5 wt% PVA 0 wt% phytigel. At this concentration of PVA, increasing phytigel initially decreases the relaxation ratio before increasing after a concentration of 1.5 wt% phytigel has been reached. This trend cannot be seen at concentrations of PVA higher than 7.5 wt%. This signifies that below 7.5 wt% PVA and 1.5 wt% phytigel, there is high free fluid content in the structure because there is little solid matrix mass. During tests, the free fluid is observed pool around the sample. This creates a water tension force that may reduce the compressive stresses, resulting in a lower equilibrium modulus, leading to a high relaxation ratio. Initially, as the concentration of phytigel increases, the ratio of free fluid to non-fluid mass decreases and the solid matrix becomes more stable. The trapped fluid content is not high enough to result in an increase in relaxation ratio so it decreases. This continues until the concentration of phytigel reaches 1.5% wt. after which the solid matrix has formed enough bonds for the fluid trapping behaviour to begin to increase the relaxation ratio. The relaxation ratio of real brain reported by Rashid et al. is around 3 [187]. The composition that best matches real brain is around 5 wt% PVA 0.5 wt% phytigel, which is in keeping with the tuning results in section 4.3.1.

A third order Maxwell-Wiechert model was used to find the viscoelastic parameters for the fast strain rate. Figure 4l shows the 3D mapping of the first term of the Prony series since they are more relatable to the viscosity, for strain rates of 1 s^{-1} . As established in the previous section, the results suggest that the composite hydrogel is a biphasic material owing to the evidence for fluid pressurisation at higher compression speed. The following discussion on the viscoelastic The characteristic time, τ_1 , describes the rate at which the instantaneous modulus decays to the equilibrium modulus. It is inversely proportional to the rate of molecular motion[21]. The results show that the rate increases (characteristic time decreases) when the concentration of phytagel increases, which means that the molecular fluid mobility increases. This is because the amount of trapped fluid increases so the fluid pressurization increases. The instant the change in strain stops, the pressure gradient of the water compared to atmospheric pressure forces the water to exit the matrix. The higher the fluid pressure at the instant maximum strain is reached, 30% strain in this case, the faster the water will exit the matrix because of the higher pressure gradient between the trapped water and the atmosphere. Therefore this again confirms that phytagel increases the viscosity of the CH.

Conversely, at the lowest concentrations of both PVA and phytagel, 5% wt. PVA 0% wt. phytagel, the results suggest that the CH has a low viscosity since the characteristic time is long. This appears to contradict the high relaxation ratio results at this concentration. This may be because the relaxation ratio does not provide information about effects of the fluid trapped in the microstructure. At low phytagel concentrations, there is little trapped water so the fluid pressurization is lower resulting in a slower rate of fluid mobility leading to a longer characteristic time. However, since the concentration of PVA is also low, the free water content is higher. Therefore, although there is a low rate of fluid mobility, a high relaxation ratio is still measured, possibly because of the effect of water tension as mentioned previously.

This thesis goes so far as to suggest the Prony series parameters that reproduce the 1 s^{-1} strain rate relaxation curves. Whilst the comparison of these parameters has provided insight into the role of PVA and phytagel in the overall composite material, this curve fitting exercise does not supply the full viscoelastic model parameters that describe the time dependency of the material's response. Future work should explore the use of one single viscoelastic model that is able to reproduce the relaxation curves across the three strain rates tested, 1, 0.01 and 0.0001 s^{-1} , for each hydrogel composition. Additionally, it is difficult to separate the purely viscous behaviour from the elastic behaviour by solely analysing the relaxation response.

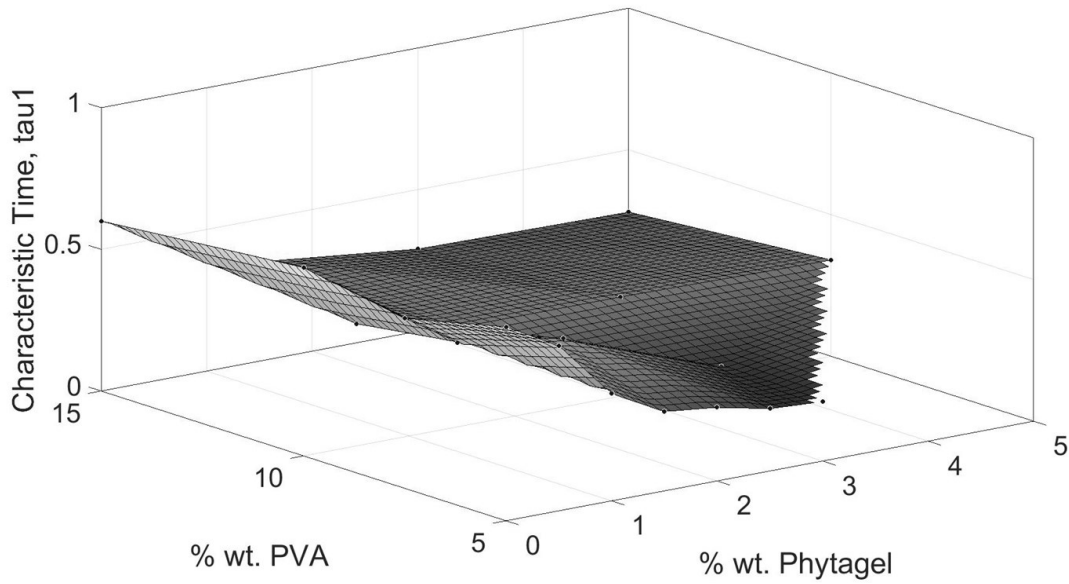


Figure 41: Dependence of characteristic time, τ_1 , on concentrations of PVA and phytagel for 1 s^{-1} strain rate.

As previously mentioned in the viscoelastic modelling of biological tissues, the relaxation stiffness term, k_1 , can be related to the material stiffness. Figure 42 displays an increasing trend with increasing phytagel and PVA concentrations. These results are compliant with those found in the maps for stress at 30% strain. These parameters may be compared with the Prony series parameters found in literature[23]. For future work, rheological methods should be used to separate the two effects by finding the storage and loss moduli. Then the purely viscous effects can be better understood and can be used to help explain the relaxation responses found here. Following this, a suitable biphasic material model that takes into account the surface permeability of the hydrogel should be explored and fitted to the experimental curves.

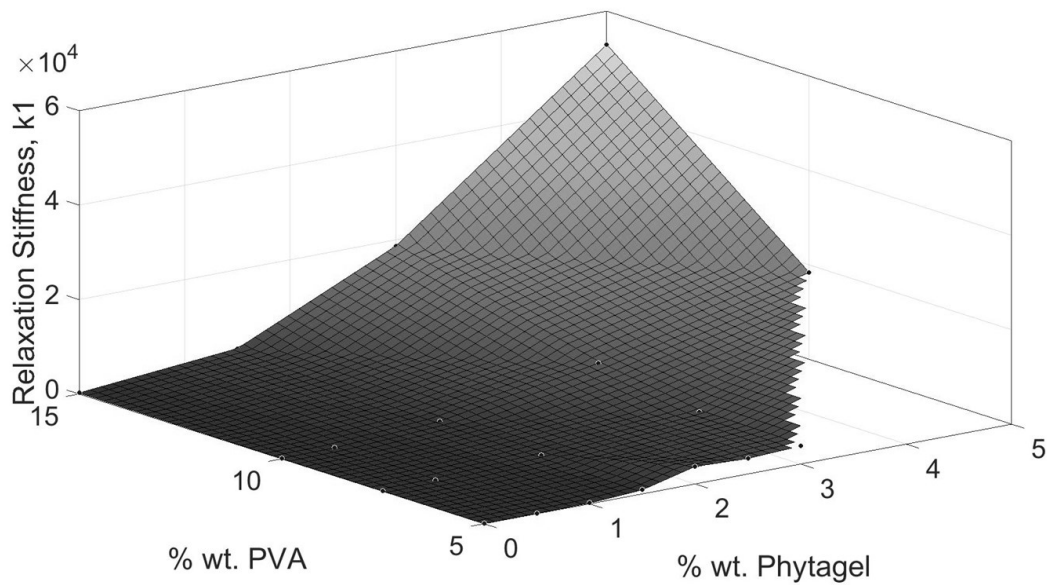


Figure 42: Dependence of relaxation stiffness, k , on concentrations of PVA and phytagel for 1 s^{-1} strain rate.

4.3.4. High Rate Tests

The results from the dynamic tests performed on hydrogels are plotted in the 3D map shown in Figure 43. Although softer compositions were tested, the results were non-distinguishable from the noise of the machine due to the sensitivity of the load cell. From the results that were able to be extracted, an increasing concentration of phytigel results in an increase in stress at 20% strain. However, comparing Figure 43 to the maps obtained at lower rates, the gradient of the slope was less steep. A particularly interesting result was that the stress experienced by the 15 wt% PVA 5 wt% PHY was approximately equal to those obtained at lower strain rates, after taking into account the difference in strain. This may be because an increasing polymer concentration is equivalent to a reduction of water content. This makes the hydrogel less strain rate dependent at the highest concentrations but highly viscoelastic at the compositions with the highest water content. More evidence for this is apparent when realising that the stress at 20% strain is increased between by 24.74 times for a hydrogel with 90 wt% water (10 wt% PVA 0 wt% PHY) compared to only 1.33 times for 80 wt% water (15 wt% PVA 5 wt% PHY) for strain rates of 1 s^{-1} to 60 s^{-1} .

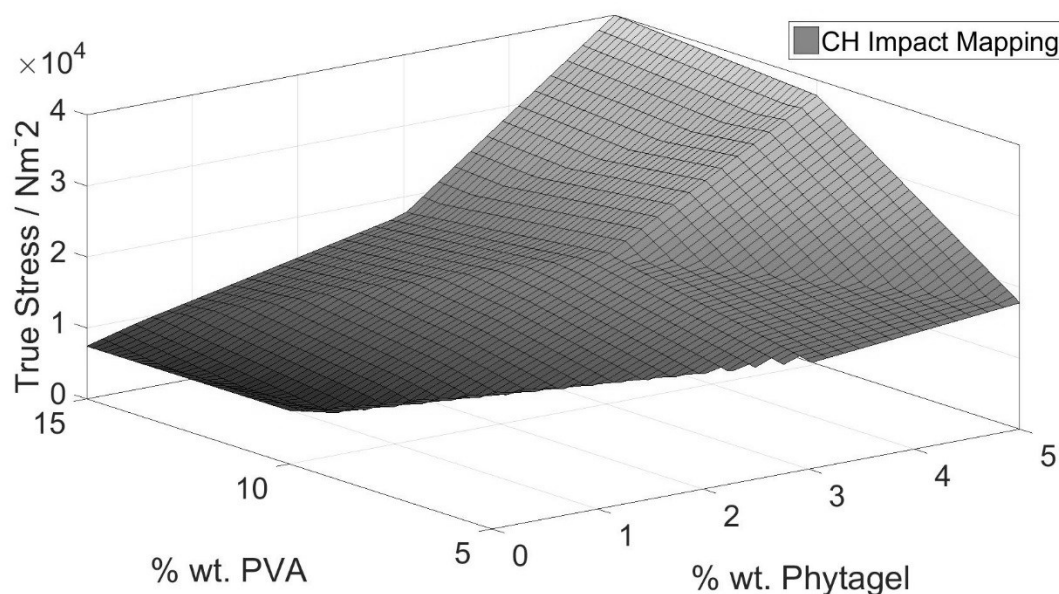


Figure 43: Impact stress at 30% strain with respect to hydrogel composition.

It is well known that biological tissues exhibit a highly viscoelastic behaviour due to its incompressibility and high water content. This is a particularly critical topic when discussing traumatic brain injury caused by an impact load to the head. This load may be in the form of a mechanical force, e.g. car crash, contact sports, or a pressure wave, e.g. explosions. A high loading speed imparts a combination of loading patterns onto the material. Tensile, compressive and shear loads are expressed simultaneously as the loading wave courses through the material, leading to a complex loading pattern that is not easily analysed. The mapping

results were only obtained for compression at one strain rate, 60 s^{-1} . In addition, only compressive test results were obtained. In future, the behaviour of the hydrogel at higher strain rates should be investigated to mimic blast injury loading conditions. The Split Hopkins Pressure bar can be used to characterise the mechanical behaviour of hydrogels and even biological tissues at high order strain rates of $O(1000\text{s}^{-1})$. [8]

For full material characterisation, tensile and shear testing should be conducted. It is noted that tensile testing is largely neglected in this work, however, the results of a Master's project, in which the author played a supervisory role, was able to map the stiffness of the composite hydrogel at the low, 1, 0.01 0.0001 and high, 60, rates in the tensile direction[213].

4.3.5. Fracture Behaviour

The highest average insertion force is achieved by the highest composition of PVA and phytigel. Conversely to the results of the stiffness mapping, increasing the PVA concentration, as opposed to phytigel, results in a greater increase in insertion forces. As mentioned in the methods section of this chapter, the fabrication of $>15 \text{ wt}\%$ PVA hydrogels is arduous and often requires additional equipment, such as an autoclave. The addition of phytigel to the PVA increases the strength of the hydrogel without increasing the difficulty of fabrication. The phytigel forms shorter chains between the long chained PVA structures, resulting in a less flexible but stronger and more load bearing material.

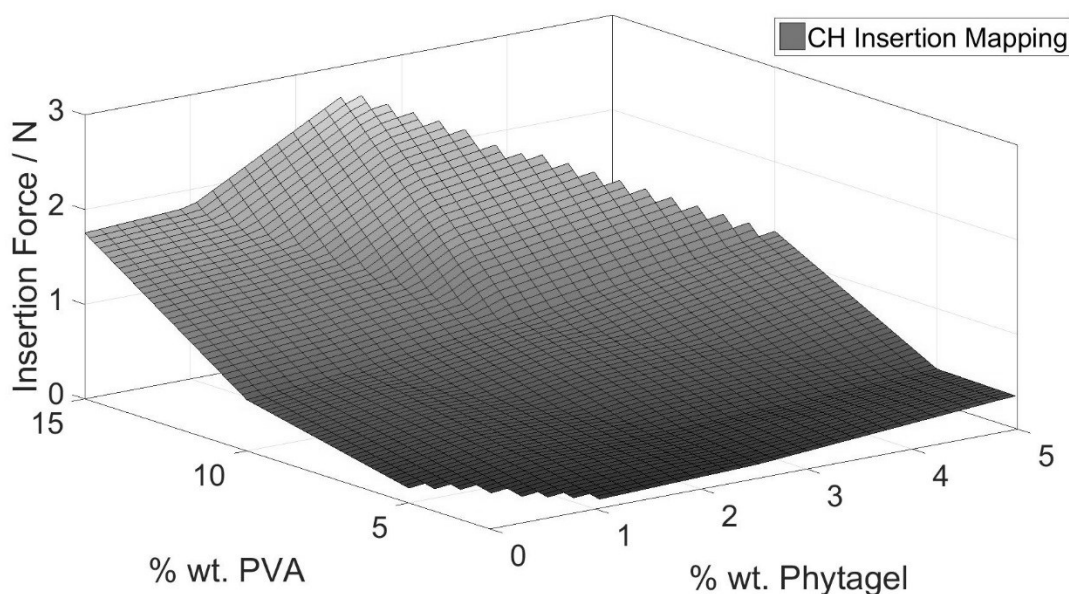


Figure 44: Map of insertion forces with respect to hydrogel composition.

However, it may not be entirely accurate to characterise the fracture toughness of a material by focusing solely on its average insertion force as this does not give information about the

development of the needle insertion profile. Ductility is another material characteristic that should be used to evaluate the fracture behaviour as it is linked to the amount of energy a material can absorb before material fracture. Ductility can be found from tensile tests by measuring the strain at which the samples fails. This work suggests that ductility can also be quantified by analysing the needle insertion depth at which the hydrogel breaks during the needle insertion tests. Similar methodology used to analyse super stretchy materials has been applied to other hydrogels in the field[263].

During testing, the insertion force profiles for some concentrations, notably 10 wt% PVA 0 wt% PHY, were found to demonstrate repeated rupture peaks. Furthermore, because these hydrogel compositions were so soft yet so resistant to fracture, the samples were dragged through the hole at the bottom of the box that was originally designed to allow the needle to pass through for a through-all needle insertion testing method. Because of this, the penetration depth at which no further cutting was observed was used to characterise the point at which the needle had passed through the entire hydrogel sample, which in some cases was greater than the original height of the sample. The map created showcasing the ductility of the hydrogel with respect to its composition is shown in Figure 45. The individual results, including all repeats for each test can be found in the Appendix C.

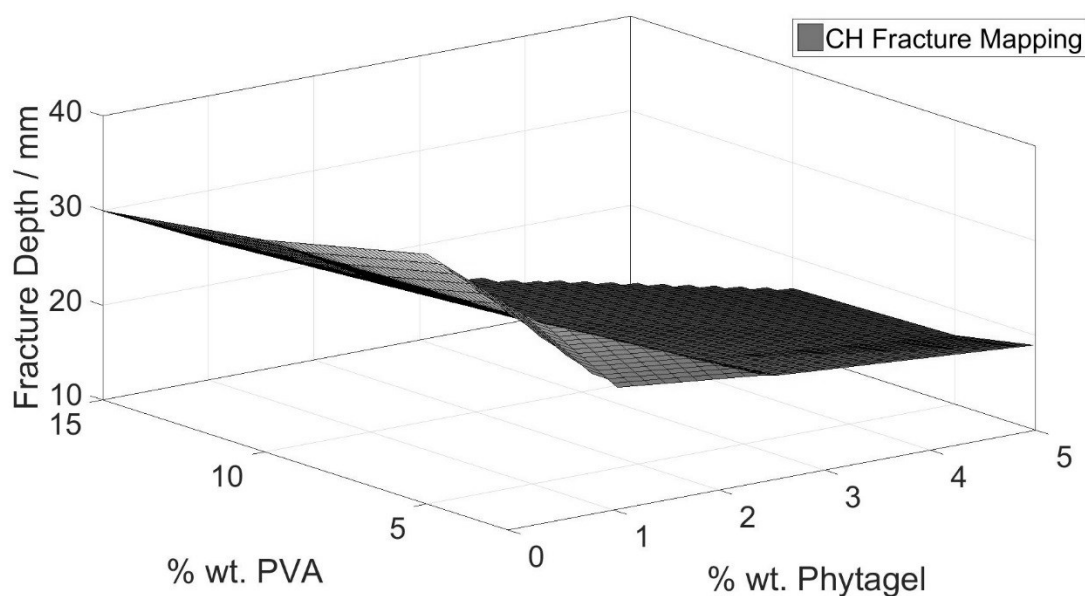


Figure 45: Map showing the needle insertion penetration depth at which fracture through the entire sample was observed, for each hydrogel composition.

Figure 45 shows that the greatest penetration depths sustained before fracture was achieved by pure PVA compositions. This suggests the long chains of the PVA are able to absorb more energy in order to resist fracture. Furthermore, the PVA chains are bonded by physical crosslinks that are weaker than chemical bonds, which form a complex web of entangled polymeric chains.

This may be what is allowing the microstructure to absorb the energy as the chains slide across each other resulting in a very ductile material.

The results suggest that ductility and brittleness work together to describe the shape of the needle insertion curve and one factor alone cannot fully describe or be correlated to the toughness of a material, which is a measure of energy absorbed by the material during fracture. To properly evaluate the toughness of the composite hydrogel, the area under the insertion force graph should be calculated, which is analytically found through integration of the function of the curve. However, the fitting of the insertion curve was not attempted in this work due to its complex non-linear features. In future, data analysis methods should be explored to compute the area under the curve, such as counting of all pixels below the insertion force line. Furthermore, algorithms that compute the number and gradient of the insertion peaks based on the second and first order derivatives of the curve could be used to characterise and quantify the insertion force profile. Once the fracture energy is computed, fracture properties, such as the fracture toughness J_{IC} , could be extracted and used to define computational models for surgical needle insertion applications.

To further analyse the fracture toughness of the composite hydrogel, different methodologies should be conducted to assess whether the results are in agreement with those discussed here, such as the wire cutting method that is used to characterise gelatine[107]. For future work, needles of various diameters and needle tip geometries should be explored as the influence of these parameters on the insertion force profiles have been studied by others in the field[167].

4.3.6. Frictional Properties

The average friction force results determined from the end of the needle insertion profile for each hydrogel composition are shown in the 3D plot in Figure 46. The frictional forces between the needle and the composite hydrogel are increased when increasing either the PVA or phytagel concentration. This trend is similar to those found in the stiffness mapping. To analyse the relationship between composite hydrogel stiffness and friction between the needle and hydrogel, the coefficient of friction was investigated. The normal force results from the FEA model were used to calculate the frictional force between the 3D printed needle and the surrounding tissue. Figure 47 shows an example of the computational model results, where the contours represent the von-Mises stress distribution in the representative hydrogel block. The material block can be seen to be deforming around the needle. The normal force, RT2, along with the friction mapping results were used to form a map for friction coefficient.

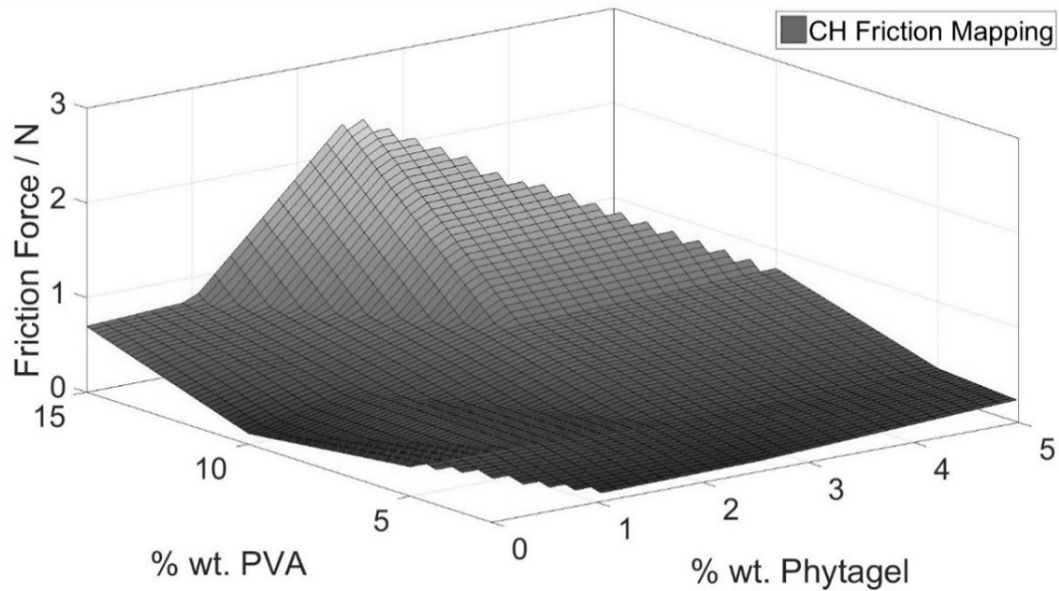


Figure 46: Map of friction force between needle and hydrogel, with respect to hydrogel composition.

A limitation of the finite element model (FEM) was that it did not set boundary conditions for the crack width. The hydrogel samples, and similarly for the biological tissues observed experimentally, were found to be closed around the needle so that the crack width was essentially the diameter of the needle. When implementing this geometry in the FEM, the elements around the crack corner were severely distorted and led to extremely high and improbable reaction force values. Therefore, whilst the final model used in this work is limited in terms of real life geometrical accuracy, the results obtained were deemed more accurate. In future, a 1D radial model could be a more accurate alternative, when only the reaction force needs to be modelled, and it would be able to avoid issues related to element geometry.

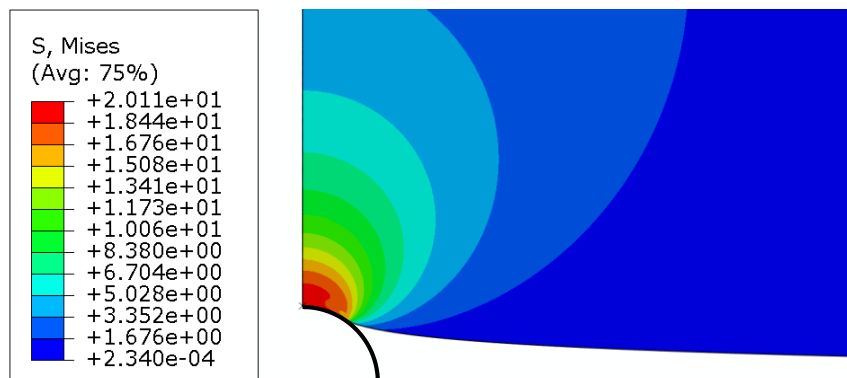


Figure 47: Quarter symmetry FEA model results for 5 wt% PVA 0 wt% PHY showing the von Mises stress field. The normal force in the Y direction, RT2, was 0.03051 N.

The results presented in the friction coefficient map in Figure 48 show that despite the PVA and phytigel concentration both creating an increase in the material stiffness and the friction force between the needle and the hydrogel, the friction force at low concentration is not low enough

to make up for the extreme softness of the hydrogels without phytigel. Whilst the individual maps for stiffness and friction force show that the increasing PVA and phytigel concentrations results in increasing forces, the friction coefficient map shows that as the phytigel concentration decreases, the rate of decrease in normal force is greater than the rate of decrease in friction force. This results in a high coefficient of friction at low concentrations of phytigel. The coefficient of friction is essentially the first order derivative of the rate at which the stiffness and friction force change with respect to one another, assuming linear elasticity.

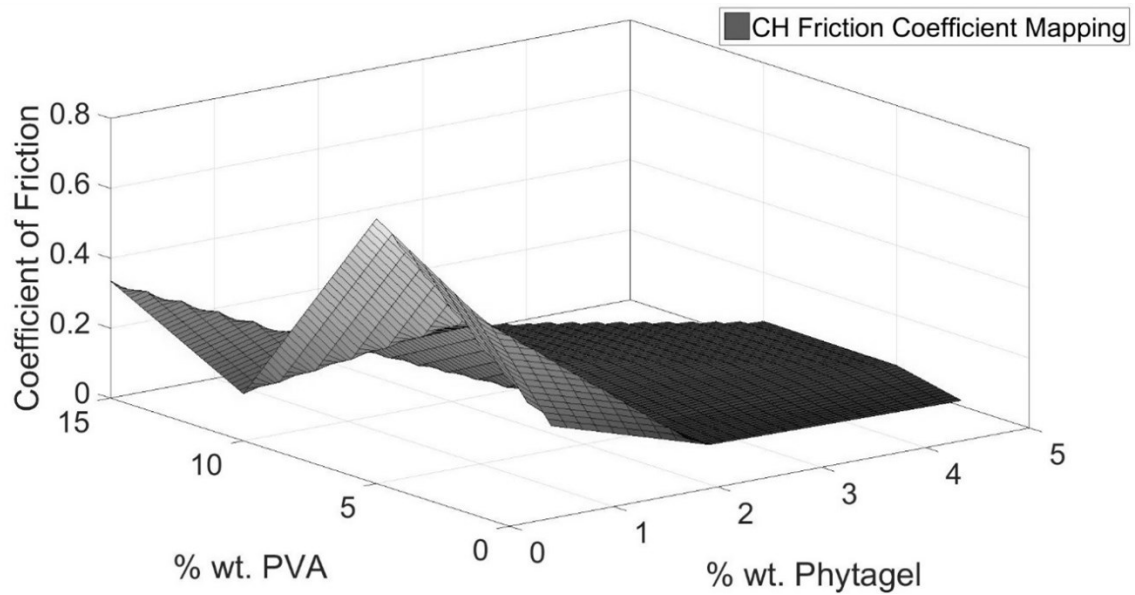


Figure 48: Map of coefficient of friction evaluated using needle insertion test results and FEA modelling methods for each hydrogel composition.

Friction was also assessed between the composite hydrogel material and stainless steel, as traditional rigid surgical needles, graspers and other instruments are made from medical grade stainless steel. In order to try to assess the frictional behaviour independently from the material stiffness, three hydrogels of similar stiffness but with varying PVA and phytigel concentrations were formulated. These hydrogel compositions were obtained from the stiffness map, which was discussed in Section 4.3.1. The hydrogel compositions that matched a stiffness of 10 kPa at 30% strain were chosen. These were 5 wt% PVA 3.55 wt% PHY, 7.5 wt% PVA 3.08 wt% PHY and 10 wt% PVA 2.5 wt% PHY.

Firstly, Figure 49 shows that the axial force was very similar for each of the three different hydrogel compositions. This reflects the results of the stiffness mapping and validates its use for finding hydrogel compositions that match a specific material property to a reasonable degree of accuracy. The results for coefficient of friction shown in Figure 50 indicate that there is little difference between the hydrogel samples submerged in water and those tests that were performed dry. It should be noted that the definition of a dry condition here means that no

additional lubricant was provided to the surface of the samples. However, due to the fabrication method of the composite hydrogel, some water may be exuded during the freeze-thaw cycle. Upon observation, at the time of testing, it was clear that the 5 wt% PVA 3.55 wt% PHY and 7.5 wt% PVA 3.08 wt% PHY hydrogels were coated with the water from their own matrix. Hence, during the frictional sliding tests, these samples were self-lubricating. This explains the insignificant difference in results between the dry and wet conditions of the 5 wt% PVA 3.55 wt% PHY and 7.5 wt% PVA 3.08 wt% PHY hydrogels.

Self-lubricity was not observed for the 10 wt% PVA 2.5 wt% PHY samples, whose results show a marked difference between the submerged in water and dry conditions. The coefficient of friction for the wet condition was half that of the dry condition. The surface of the samples were observed to be of a sticky consistency, which may be a contributing factor to the higher coefficient of friction calculated. It is thought that the self-lubricity of the hydrogel may be due to the matrix allowing free water to be exuded through due to its microstructural porosity. Increasing the concentration of both PVA and phytigel has already been discussed to increase the solid matrix crosslinking, resulting in a stronger composite hydrogel. The fact that self-lubricity occurs at low concentrations of PVA yet high concentrations of phytigel suggests that this phenomenon is dominated by the concentration of PVA as an increase in PVA concentration decreases the free water flow more than a decrease in phytigel concentration can account for. This is again because less phytigel is required to achieve the same stiffness as an equivalent concentration of PVA.

These results suggest that hydrogel matrix is not self-lubricating at concentrations greater or equal to 10 wt% PVA 2.5 wt% PHY. At this critical composition, the matrix of long PVA chains have formed a more dense and entangled matrix. Even though there is a lower concentration of phytigel, there are enough bonds formed to result in a matrix of equal stiffness to the other compositions. Also, just by comparing the concentration of water across each other the compositions we see that there is only 87.5 wt% water in the 10 wt% PVA composite hydrogel compared to 89.42 wt% in the 7.5 wt% and 91.45 wt% in the 5 wt%, meaning that there is less water in the system in the first place resulting in less free water being available to act as a surface lubricant. Ultimately, it is more so the PVA network as opposed to the phytigel that is able to trap the water inside the solid matrix.

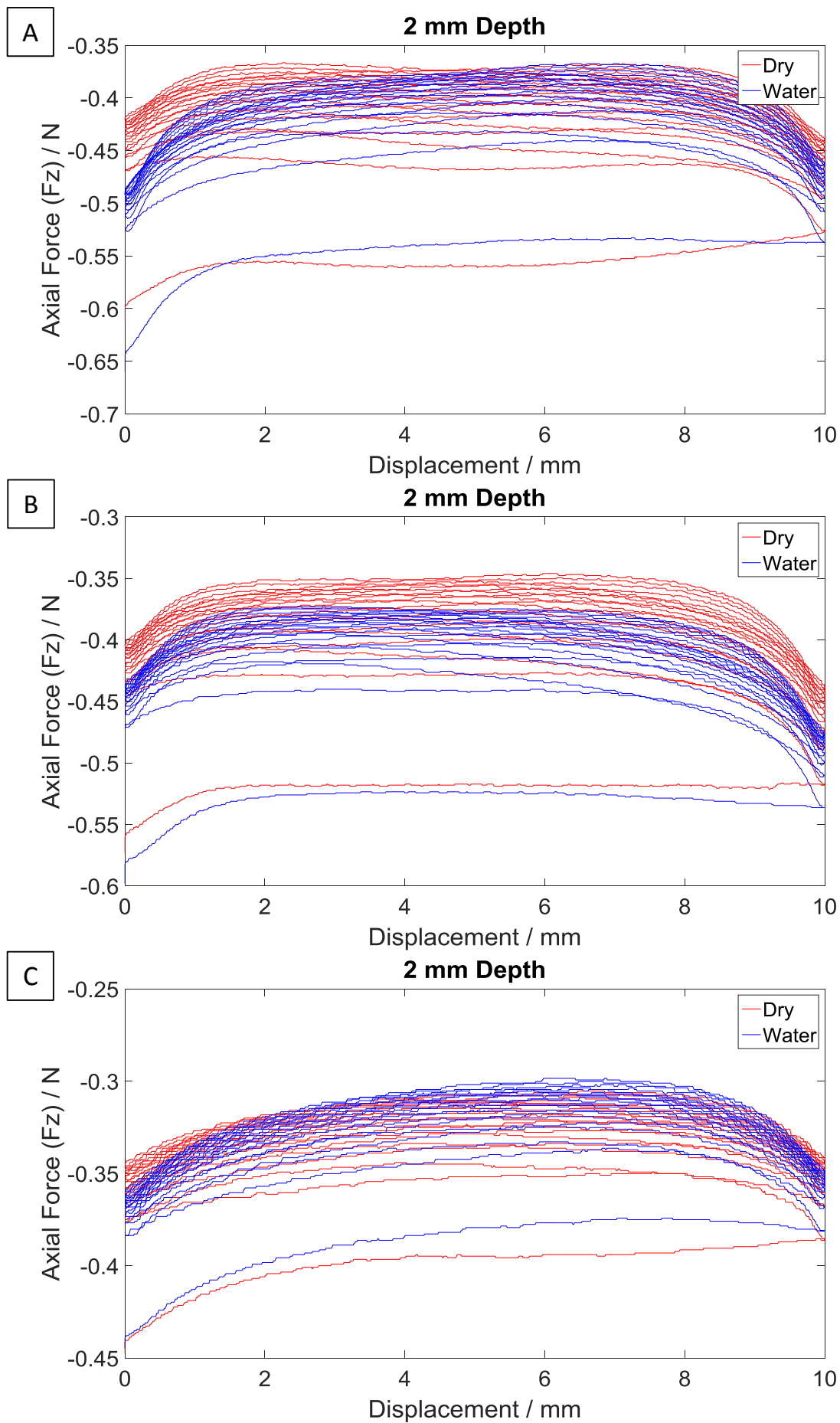


Figure 49: Axial force showing the stiffness of (A) 5 wt% PVA 3.55 wt% PHY, (B) 7.5 wt% PVA 3.08 wt% PHY and (C) 10 wt% PVA 2.5 wt% PHY over a 10 mm stroke length at a sliding speed of 1 mm/s and an indentation depth of 2 mm.

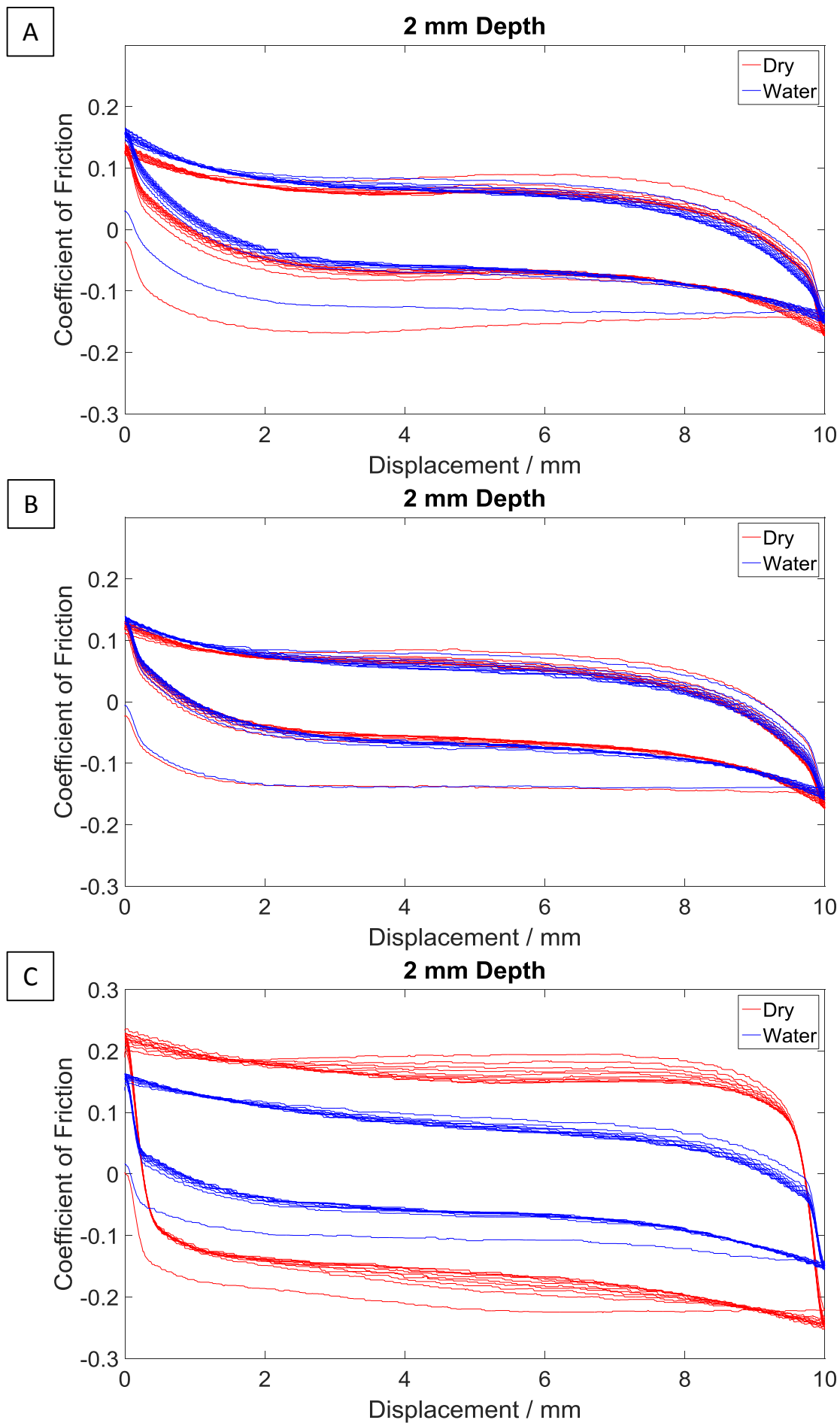


Figure 50: Coefficient of friction between stainless steel and hydrogels (A) 5 wt% PVA 3.55 wt% PHY, (B) 7.5 wt% PVA 3.08 wt% PHY and (C) 10 wt% PVA 2.5 wt% PHY over 10 mm stroke length.

Two testing methods of evaluating the friction coefficient were carried out in this work. A comparison of the friction coefficients found by each method can be found in Table 15. Many testing conditions are known to affect the frictional behaviour between two materials. The testing conditions; 1 mm/s needle insertion speed and 1 mm/s indenter sliding speed along with 2 mm diameter needle and 2mm indentation depth, were kept constant between the two methodologies to allow the comparison of friction coefficient to be due to the difference of materials only. The results show that the CH/stainless steel system consistently experiences friction coefficients twice as great the CH/3D printed needle system, for the same hydrogel compositions. This indicates that the friction force between CH/stainless steel was greater than CH/3D printed needle. An explanation for this behaviour should be investigated in further detail in future.

The CH/stainless steel system also exhibits the trend that was reported in the needle insertion friction coefficient map whereby, in the dry condition, the coefficient of friction doubles when a composition of 10 wt% PVA 2.5 wt% PHY is reached. This suggests that the fracture of the hydrogel during needle insertion does not release water on a macroscopic scale, and therefore the crack path retains a dry condition throughout the insertion procedure.

Table 15. Comparison of friction coefficient between results found in this work and literature results of similar interactions involving hydrogels.

System	Hydrogel Composition	Friction Coefficient
CH/3D printed resin (dry)	5 wt% PVA 3.55 wt% PHY	0.05
	7.5 wt% PVA 3.08 wt% PHY	0.06
	10 wt% PVA 2.5 wt% PHY	0.09
CH/stainless steel (dry)	5 wt% PVA 3.55 wt% PHY	0.1
	7.5 wt% PVA 3.08 wt% PHY	0.1
	10 wt% PVA 2.5 wt% PHY	0.2
Gelatine/3D printed resin[264] (dry)	6 wt% gelatine	>0.2
PVA/stainless steel[265,266] (dry)	15 wt% PVA	>0.06

Also listed in Table 15 are literature values of other related systems for comparison. PVA is often likened to cartilage and touted as a cartilage replacement material[57,267,268]. However, a brief look at the literature shows that the frictional coefficient of cartilage/cartilage is still much lower than PVA/cartilage can achieve. Since the key characteristic and function of cartilage replacement material is to mimic the biological cartilage/cartilage frictional behaviour, better mimicking materials should be developed. Additionally, the standard test for any artificial cartilage replacement material should be against real cartilage using human synovial fluid as a lubricant as it has already been proven that the frictional behaviour of materials against stainless steel is very different to real tissue.

These results highlight the importance of understanding that frictional behaviour is a system property and should be accurately characterised before used to define a computational model. The results have shown that the frictional interaction between CH/stainless steel, CH/3D printed needle and more generally between hydrogels and rigid materials is significantly different. For a more in depth investigation into the tribological behaviour of hydrogels, future work should include the investigation of factors such as surface microstructure and roughness, contact area and pressure, sliding speed and path geometry, needle surface coatings and the effect of other physiological lubricants, such as the cerebral spinal fluid in the brain, bovine calf serum (BCS), human synovial fluid (HSF) and mucin.

In summary, increasing PVA and PHY concentration, increased the stress at 30% strain. Also, an increase in PHY concentration resulted in a greater degree of strain rate dependency and non-linearity as expressed by the fitted first order Mooney-Rivlin model parameters. The combined examination of relaxation ratio and 3rd order Prony series parameters showed that the relaxation response was most severe at low concentrations of PVA and PHY but that an increasing PHY concentration increased its viscoelasticity. For the results of the insertion tests, increasing PVA concentration led to bigger increases in both the average insertion and friction force than PHY. In addition, increasing PHY at any PVA concentration resulted in lower ductility. Studies on the friction between hydrogel and stainless steel revealed greater friction coefficients than between the hydrogel and the 3D printed needle. Future work should be focused around characterising other key mechanical properties, such as the use of rheometry to find the shear, storage and loss moduli. The ability of the constituent hydrogels to affect different mechanical properties, sometimes independently of each other, will aid its tissue mimicking ability, evaluated in the next chapter, 5, as Chapter 3 has also shown that the mechanical properties of biological tissues are independent to each other.

5. Composite Hydrogel as a Soft Tissue Phantom for Biomedical Applications

This chapter has been partly published in Materials and Design[153] and Scientific Reports[228].

5.1. Introduction

The mechanical characterisation of real soft tissues, such as brain, lung and liver, is important as it allows us to understand how they deform during critical scenarios e.g. in surgical operations. Hence, ways to reduce damage to living tissues during real life situations can be identified and developed. However, real biological tissues are difficult to obtain and test due to accessibility and ethics. Therefore, there is a clear advantage in designing a mechanically accurate synthetic tissue (also known as a tissue phantom) that is easier to procure and produce.

A key example of a destructive test that would be unethical or difficult to perform on real biological tissues is the testing of novel surgical technologies. In this field, the effort required to progress a novel surgical technique, equipment or therapeutic agent from laboratory research to clinical level is arduous, and for good reason. A number of rigorous animal, pre-clinical and clinical trials are required before the technology is even considered to be used on real patients. Even after the treatment is proved to be relatively safe, its efficacy continues to be researched as side effects could emerge years later. A recent technological development is the implementation of permanent neuro-catheters for convection enhanced drug delivery (CED) in the treatment of brain cancer[269]. The use of an accurate surgical phantom to test the safety and efficacy of these high risk technologies would be another tool in addition to *in-vivo* models and could even reduce the number of animal trials required, especially in the preliminary stages of prototype evaluation, or make it easier to try any number of designs since there are no ethical limits.

Another application in the topic of surgery where tissue phantoms would be useful is surgical training. Surgeons are required to reach high dexterity standards and perform complex technical tasks in a short training period [270]. However, in the last two decades the number of dedicated training hours has been reduced in the US and Europe along with an increasing attention on safety in the operating theatre [271]. Therefore, trainees have less time to practice, and new training methods (including tissue-mimicking materials) are needed to assure a faster learning process. In fact, many studies have shown that trainees who train on physical models have superior skills in guiding percutaneous needle positioning, among many other clinical procedures, compared to trainees who did not have the opportunity to train on phantoms [272–

274]. As such, there is strong motivation to develop a material that can mimic the mechanical properties of biological tissues.

The properties involved in surgical scenarios include compressive stiffness, surgical tool insertion forces and frictional forces between the tool and surrounding tissue. These properties are independent of each other so whilst some biological tissues may have similar stiffness, they can behave very differently during a needle insertion process, as was shown in the results of Chapter 3. Many authors have developed hydrogels to mimic a specific mechanical property, but the accuracy of these mimics can be improved by attempting to simultaneously matching multiple mechanical properties [60,63,275–279]. Hence, this chapter reports the development of a material able to mimic the three mechanical properties (stiffness, toughness and needle-tissue frictional interaction) of real biological tissues most relevant to surgical applications. For the specific application of convection enhanced delivery of therapeutic agents in solution, the diffusive properties of the hydrogel were also investigated to analyse the potential of using the composite hydrogel as a surgical phantom with the ability to mimic the microstructure, porosity and permeability of real biological tissues. Matching multiple mechanical properties will increase the fidelity of the tissue mimicking material. In addition, the final aim of the work is to use different compositions of the same material to mimic the three different soft tissues characterised in Chapter 3; brain, lung and liver, to demonstrate the flexibility of the composite hydrogel. Therefore, a mandatory requirement for the chosen material is that it should be highly tunable so that multiple mechanical characteristics can be reproduced.

Another highly important application that desperately requires the development of a more accurate tissue mimicking material is the study of traumatic brain injury. Due to the complex loading patterns associated with the propagation of the impact waves, it is difficult to isolate the individual mechanical components acting on the material. Hence, it is more feasible to use a testing setup that simulates the impact load. Due to impossibility of *in-vivo* experiments, studies in this field are mainly based on *ex-vivo* tissue samples and cellular scale *in-vitro* tests. Another method to study the loading patterns is to create a computational model, for which a large amount of literature exists. However, as is a common issue, the accuracy of these models depend on the constitutive material properties, which means they are limited to the accuracy of *ex-vivo* experimental data.

In this realm, an artificial material that is able to mimic the mechanical properties of brain at high speed loads would be an alternative study mechanism to the field. Although this material would be limited in accuracy, as again it would be tuned to mimic *ex-vivo* tissue properties, it would be easier and more ethical to procure, store and test. Most importantly, a tissue phantom

would allow for a more detailed study of impact wave propagation and related stress levels to understand how our tissues are damaged. Following this would be the development of safety standards and equipment that could use the tissue phantom to test and validate their products.

If the tissue mimicking material is also biocompatible, it will offer the additional advantage of providing larger scale, more realistic *in-vitro* tests to investigate how impact loads cause primary (immediate) and secondary (longer term) biological damage. Studies that induce damage onto individual or a small collection of cells are already being carried out by authors such as Ma et al.[280] who showed that an application of the protein neurotrophin-3 (NT-3) to a culture of damaged cerebellar granule neurons (CGN) offered neuroprotection to the cells and increased their vitality. The characteristics of primary injury that should be quantified when evaluating *in vitro* TBI models are as follows: changes in metabolism, followed by excessive release of excitatory neurotransmitters, leading to cell membrane degradation, resulting in necrosis or apoptosis, as summarised by Werner and Engelhard[281]. Secondary damage can be characterised by the following three mechanisms: (i), excitotoxicity[282], which damages cells by allowing excess calcium ions to enter the cell, (ii) free radical generation[283], which damages proteins, and (iii) the neuroinflammatory response[284], which can be measured by quantifying the level of raised inflammatory markers[285]. However, other physiological symptoms of TBI, such as the effect of swelling and edema on intracranial pressure, can only be investigated at the larger real-size level, which a cell seeded artificial brain phantom would allow researchers to achieve[286].

For the past half a century, ballistic gelatine, which was introduced and developed by Fackler[287], has been regarded as the standard tissue mimicking material for blast injury studies due to its ease of use and ethicality. However, ballistic gelatine was formulated to mimic porcine muscle tissue, and whilst it is a good mimic for the average stiffness of the human body, it cannot be used to study the intricacies of impact wave propagation through particular biological organs that exhibit large degrees of strain stiffening, such as the liver as shown in this work. Furthermore, experimental results obtained using ballistic gelatine cannot be used to validate computational models, which have evolved to now include hyper-, visco- and even poroelastic material models, which ballistic gelatine does not share the characteristics of, presenting a huge problem. Thus, there exists a strong case for the development of material that is able to mimic the mechanical response of biological tissues at dynamic $O(10) \text{ s}^{-1}$ to ballistic $O(1000) \text{ s}^{-1}$ loading rates. Leading on from the work in previous chapters, the use of the composite hydrogel as an artificial tissue mimicking material is extremely feasible due to its stiffness tunability, hyperelasticity and viscoelasticity. Consequently, the development of this

composite hydrogel as a brain mimicking material for traumatic brain injury loads is one of the goals for this work.

Finally, the development of a biocompatible tissue mimicking material is also a crucial requirement given the state of the art. It is now widely known that cells respond differently when subjected to mechanical loads; a discovery that has opened up the field of mechanobiology. A classic example of this is the ability of stem cells to differentiate into different lineages when seeded on substrates of different stiffness[6,288–290]. There is also evidence of stem cells express specialised signalling when experiencing mechanical loads that stimulate , An incredible example of this is the work by Grier and co-workers who showed that human mesenchymal stem cells (hMSCs) show signs of differentiating into tenocytes (tendon cells) when subjected to cyclic tensile strain loads that simulate the movement of the gross tissue[291]. Hwang and co-workers have also shown that chondrocytes (cartilage cells) differentiated from mouse embryonic stem cells show different biological behaviours if cultured in 2D or 3D environments[292]. Since the role of cell seeding substrate material is so crucial in the field of tissue engineering, any opportunity for a biomaterial to join that world would significantly increase the impact of the work. Therefore, the final important piece of work investigated in this chapter is to validate the biocompatibility by evaluating the viability of cells seeded on the printed material in order to confirm the potential for future mechanobiological studies.

The work completed in Chapters 3 and 4 has laid the foundation for the determination of specific tissue mimicking compositions reported and evaluated in this chapter. The mechanical characterisation of biological tissues, which acts as the ground truth for any tissue mimicking efforts, has been reported in Chapter 3, and Chapter 4 has shown justification for why the composite hydrogel this is a suitable material to be used as a tissue mimicking material due to its highly tunable nature. The results of Chapter 4 introduced the concept of how 3D maps can be used to showcase the mechanical properties of the composite hydrogel, where the PVA and PHY concentrations are on the x and y axes, respectively, and the material characteristic is on the z axis. This provided a visual representation of the mechanical behaviour dependence on the composition of the CH. The work in this chapter shows how those mapping results are used to determine tissue mimicking compositions that best match brain, lung and liver for surgical applications and traumatic injury studies. And finally to open up the possibility of using the composite hydrogel as a cell seeding substrate in future tissue engineering and mechanobiological studies by evaluating cell viability.

In summary, the aims of this chapter are as follows: (i) to determine and evaluate the best surgical mimicking hydrogel composition for brain, lung and liver, from the compilation of the

three separate maps for stiffness, insertion force and friction force, (ii) to determine the best matching brain mimic for traumatic brain injury loads and (iii) to evaluate the biocompatibility of the composite hydrogel as a cell seeding substrate based on cell viability analysis.

5.2. Materials and Methods

5.2.1. Surgical Phantom Composition

The mechanical characteristics of the porcine brain, lung and liver obtained from the results of Chapter 3 were overlaid onto the map for each mechanical property related to surgery (stiffness, insertion and friction force) obtained in Chapter 4. The intersection between the biological results and the map revealed the matching CH compositions. Since the maps are 3D, the result of the matching will be a line of intersection, hence there were a range of different hydrogel compositions that match each mechanical property. The best mimicking composition can be determined by choosing a single composition that is able to match as many of the required mechanical properties as accurately as possible. To do this, all possible matching compositions for stress at 30% strain, average insertion force and average friction force were collated onto a single 2D graph. The individual hydrogel compositions which best matched brain, lung or liver tissue were determined by finding the single value which was the best compromise to satisfy all three mechanical properties. To evaluate and validate the tissue mimicking accuracy of these interpolated compositions, samples of the determined compositions were produced and tested and the results were directly compared to the biological tissue results.

5.2.2. Fluorescent Diffusion Tests

One of the purposes of deep tissue needle insertion is the locoregional delivery of therapeutic drug solutions. An important example is the perfusion of chemotherapy agents directly to the tumour site, also known as convection enhanced delivery (CED)[269]. The definition of convection encompasses the diffusion of the therapeutic agent due to concentration gradient and advection of the particles from external needle injection forces. In this case, it is imperative to know the extent of the drug diffusion through the tissue. This calculation will depend on the mechanical resistance of flow (permeability of substrate, size of agent molecule) and the chemical resistance of the flow (concentration gradient, osmolality). Thus it was complementary to investigate the potential of the composite hydrogel as a tissue phantom for diffusion.

CED is often tagged with substances, like gadolinium, that enhance under MRI so that the fluid flow can be tracked[293–295]. However, the scans often take place after the infusate has been injected, providing no real-time imaging of diffusive drug progression whilst the injection is being administered. Since intra-operative and dynamic MRI are state of the art technologies, they are not always feasible for use due to high cost and setup expertise. As such, a fluorescent imaging method was used to investigate the diffusive properties of the composite hydrogel as it was a low cost and more easily attainable alternative. Traditionally, fluorescent microscopy techniques are used to characterise cellular processes by tagging specific molecules that are only expressed in certain conditions[296–299]. A standard use of fluorescent microscopy is to stain live and dead cells with different fluorescent markers to quantify cell viability[300–302]. Fluorescent techniques were adapted to track the movement of fluids since fluorescent agents are normally dissolved in physiological fluids, such as water, saline or PBS. This has led to the use of the fluorescent dye, fluorescein, as a diagnostic tool for vascular and eye disorders[303–306]. The feasibility of the fluorescent tracking technique in this work was mainly permissible by the fact that the composite hydrogel is translucent. As such, it was evaluated that a fluorescent tracking and imaging technique could reveal the diffusive patterns of a solutions being perfused under at positive pressure gradient by providing real-time imaging.

To study the forced convective flow of the drug delivery, a hollow needle, 31 gauge, connected to a syringe, 1 mL, was inserted into the centre of a CH phantom, cast moulded in a 50 mm diameter, 10 mm height petri dish. The fluorescent dye, 0.05 wt% sodium fluorescein dissolved in water, was used to mimic the therapeutic agent and injected into the phantom at 1 μ L/min flow rate, which is typically used in CED *in vivo* studies[293]. The molecular weight of fluorescein is 376, which is much smaller than the pore size of hydrogel contact lenses and has therefore been reported to be able to pass through hydrogels with ease[307]. Doughty et al.[308] suggests the use of a more negatively charged fluorescein derivative with alkyl side chains to reduce the chance of staining. However, in this work the sodium fluorescein solution was observed to easily rinse out of the composite hydrogel after a few cycles, which suggests that the fluorescein molecules do not adsorb onto the hydrogel and should therefore be appropriate for the purposes of these experiments. The reason for this may be because both the sodium fluorescein molecules and hydrogel matrix are weakly negatively charged and therefore will slightly repel each other[308].

The peak absorption wavelength of fluorescein is 494 nm, which is of the blue spectra, and the peak emission wavelength is 512 nm, which is green. Therefore, fluorescent light microscopy equipped with a blue light source and a green microscopic lens filter was used to track the flow of the dyed water. Figure 51 shows the equipment setup. The results were recorded for 50 mins

at 10 min intervals, giving rise to a total injected volume of around 50 μL . The needle is placed into the centre of the hydrogel volume and since the hydrogel is translucent and not fully transparent the fluorescent area is only observed once the solution has reached a certain proximity to the top surface of the hydrogel sample, as illustrated in Figure 51.

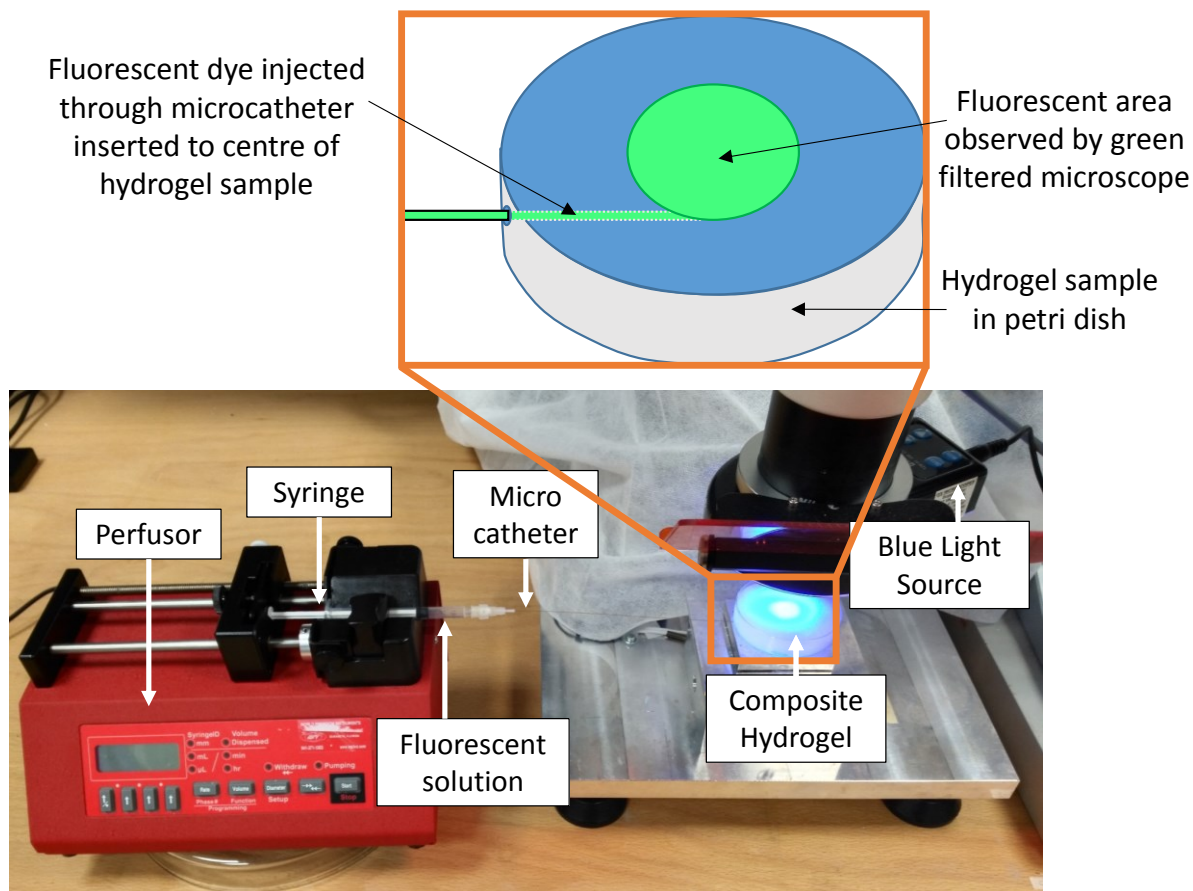


Figure 51: Setup for injection of fluorescent solution into brain mimicking composite hydrogel.

In this work, the fluorescent solution aims to mimic the diffusive behaviour of a therapeutic drug being delivered during a CED procedure. The diffusive behaviour of a solution into a biphasic material, as the composite hydrogel was shown to be in Chapter 4 and that brain is also reported to be [309], will be affected by factors such as osmotic pressure, ionic concentration, solution viscosity, chemical reactions and, where applicable, complex biological interactions. The *in vitro* artificial model in this work will focus on investigating the isolated effect of solution viscosity on its diffusive behaviour in a controlled hydrogel environment to examine the conclusion reached by Mardor et al. [293], who suggested that an increased therapeutic agent viscosity improves the efficacy of CED. Therefore, the effect of solution viscosity was investigated by dissolving sucrose into the fluorescent dye solution, as it has been shown that sucrose can increase the viscosity of a solution [293]. 30 μL mixtures of 0 wt% and 50 wt% sucrose concentration were perfused into the hydrogel phantom sample to show the effects of a drastic increase in viscosity. Furthermore, the perfusion rate was increased to 5 $\mu\text{L}/\text{min}$ to observe a greater effect of advection on the convective system.

Post processing of the fluorescent images was carried out by the RGB colour thresholder available in MATLAB, where the threshold intensity is set by selecting a value in the range of 0-255, where 0 results in all RGB pixels passing the threshold, hence indicating that a value of 255 would mean no pixels pass the threshold. The selection of an appropriate threshold was difficult because of the diffusive nature of the fluorescent dye coupled with the translucency of the hydrogel. In fact, this mimics similar difficulties that arise clinically when diagnosing the size of a brain tumour due to the infiltrating and diffusive nature of the glioma cells[310,311]. An assessment of the effect of different thresholds is shown in Figure 52, where thresholds of 0, 25, 50, and 75 are displayed to illustrate the difficulties in selecting the most appropriate threshold that is able to fully represent the spread of the fluorescent solution. Therefore, an arbitrary threshold of 50 was chosen and applied to each red, green and blue filter. The filter was applied to all images and the evolution of fluorescent area was calculated and plotted against time.

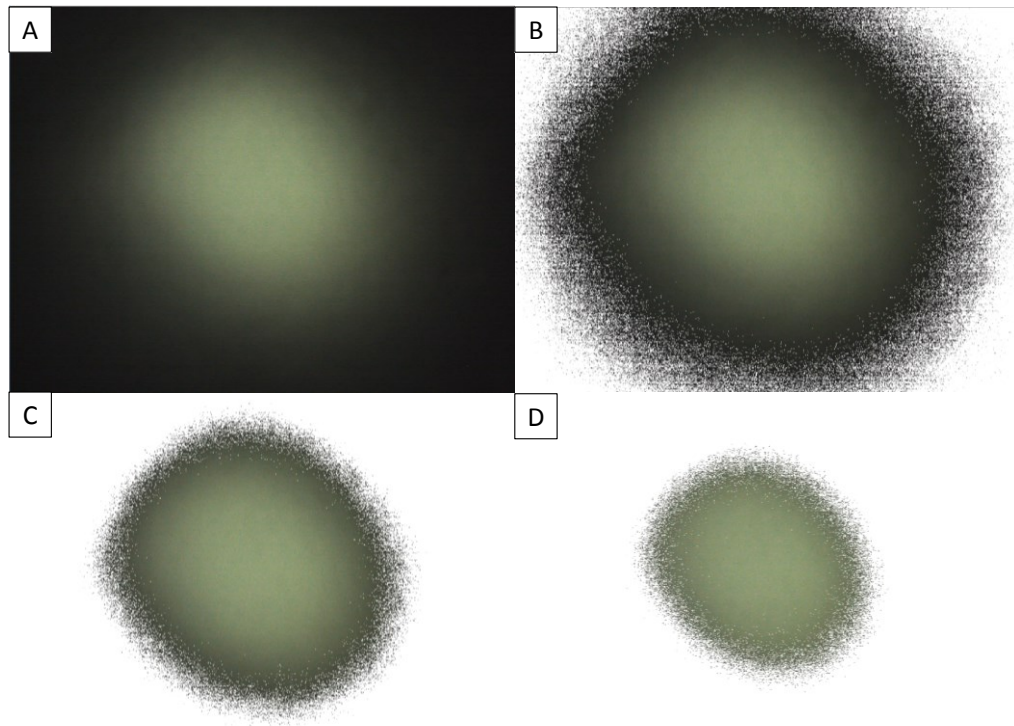


Figure 52: Demonstration of how the fluorescent area measurement is affected by the RGB intensity threshold values set at (A) 0, (B) 25, (C) 50 and (D) 75.

5.2.3. Traumatic Brain Injury Phantom

The development of a highly accurate tissue phantom for super soft biological tissues such as brain and lung in the field of traumatic and blast injury is essential. The results obtained in Chapter 4 for stress at 20% strain at 60 s^{-1} strain rate were used to determine a tissue mimicking composition that corresponds to real brain. Since real brain results at 60 s^{-1} strain rate were not obtained in this work, literature reported values obtained from Rashid et al.[187] were used as

the ground truth for tissue mimicking. The endeavour was focused on compositions that mimicked brain and lung as these biological tissues are among the softest in the body and therefore the furthest away from being represented accurately by ballistic gelatine; they thus present a greater need for a high fidelity tissue phantom to be developed.

5.2.4. Composite Hydrogel Cell Seeding Substrate

Primary Normal Human Dermal Fibroblasts (NHDF, Promocell, Germany) were seeded onto the 3D printed 5 wt% PVA 0.59 wt% Phytigel hydrogels, at a density of 4×10^4 cell/cm². Samples were previously coated with collagen, poly-L-lysine or gelatine to enhance cell attachment. Collagen has been used as a coating for cell attachment by Engler et al. [6] to study the effect of substrate stiffness on cell differentiation, which therefore indicates that the cells have the ability to feel the underlying substrate stiffness through the collagen coating. Gelatine is often used as an alternative tissue scaffold material to collagen due to its greater availability and cost effectiveness. Poly-L-lysine has also been used as a coating as it forms a cationic layer that attracts the anionic sites on the cells surfaces [80,312]. Whereas the collagen and gelatine coating rely on absorption into the pores of the hydrogel where they crosslink and attach to the substrate, the poly-L-lysine is electrostatically adsorbed onto the surfaces owing to the -OH functional groups present in the PVA and phytigel chains. This creates a stronger bond that cannot be physically removed. Cell viability was assessed using Live/Dead staining. Samples were kept in culture for 72 hours and then stained using calcein for live cells and ethidium homodimer-1 (EthD-1) for dead cells. Samples were immersed in a phosphate buffered saline (PBS) solution, containing 2 µl of 4 mM Calcein AM (acetoxymethyl) and 1 µl of 2 mM EthD-1 for each ml of solution, and then incubated for 15 minutes at 37 °C. Live (green, FITC) and dead (red, TRITC) cells were then observed under a fluorescence microscope.

5.3. Results and Discussion

5.3.1. Composite Hydrogel as a Surgical Tissue Phantom

Due to the complexity of the biological organs, mechanical characterisation is a difficult task, especially when studying and attempting to replicate multiple mechanical properties and tissue response characteristics. In this study, the aim is to use different compositions of the same composite material to mimic organs in the body that have wide-ranging mechanical properties. The organs; brain, lung and liver, were chosen because they cover a wide range of stiffness, they are often subjected to surgery and there is limited knowledge on their response to surgical insertion tests. The focus of the present study is to create a tissue mimicking material for surgical planning and training. Therefore, we concentrated on tuning the material to match the compressive stiffness and needle-tissue interaction forces.

Figure 53 shows the range of compositions that match the mechanical properties of brain, lung and liver characterised in Chapter 3, which are represented by the highlighted lines on the surface of each map. For the purposes of general tissue mimicking, the stress-strain characterisation used as a mimicking parameter was the stress at 30% strain as this allows for easy comparison between different biological tissues. Similar tissue mimicking analyses could be completed using the maps for the Mooney-Rivlin and the Prony series models to obtain a phantom that closely matches the hyperelastic and viscoelastic tissue properties derived in Chapter 3. However, the stress at 30% strain was initially deemed to be descriptive enough for the purpose of creating a tissue mimic for surgery as the insertion force and friction forces are also very important mechanical behaviours to be matched. Only the stress-strain map at 0.01 s^{-1} was used as mimicking criteria as this is the most relevant speed in the context of surgery.

The results for the matching compositions for stress at 30% strain, insertion force and friction force for each soft tissue were combined to identify a single composition that best matched all three mechanical properties. The CH compositions found to match the three mechanical properties of brain, lung and liver are summarised in Table 16 with the determination process of these compositions detailed for each tissue separately in the rest of this section.

Table 16. CH compositions matching range of soft tissues.

	CH composition in wt%
Porcine Brain	2.5 wt% PVA 1.1 wt% PHY (CH_Brain)
Porcine Lung	11.5 wt% PVA 0 wt% PHY (CH_Lung)
Porcine Liver	14 wt% PVA 2.1 wt% PHY (CH_Liver)

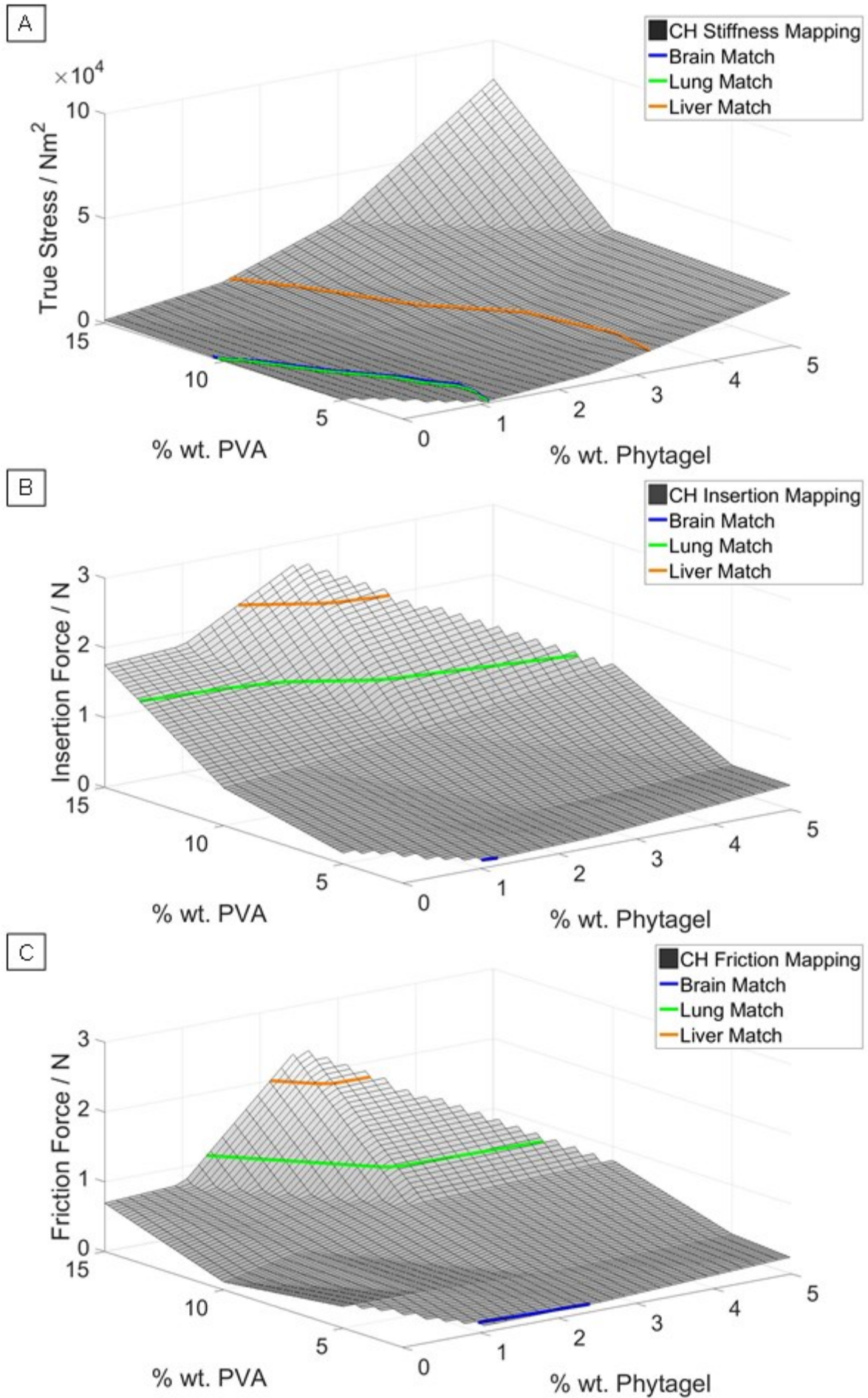


Figure 53: Composite hydrogel mapping of three mechanical behaviours: (A) stress at 30% compressive strain, 0.01 s^{-1} strain rate, (B) average insertion force and (C) average friction force.

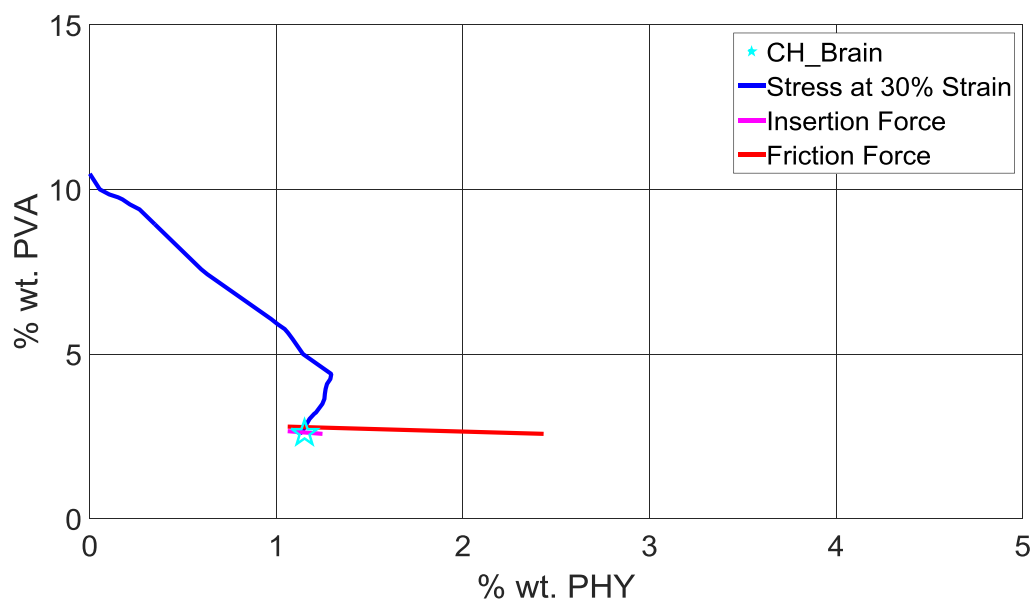


Figure 54: Determination of best tissue mimicking composition based on three mechanical behaviours for brain.

Samples of the tissue mimicking hydrogel compositions reported in Table 16 were created, tested and compared directly to results from the real porcine soft tissue, which is shown in Figures 55, 57 and 59. The CH composition of 2.5 wt% PVA and 1.1 wt% PHY, named CH_Brain, was able to closely match the stress at 30% strain of brain up to 30% strain. Whilst the end stress is well matched, it is apparent that the shape of the stress-strain curves show slightly different behaviours. At 30% strain, the porcine brain continues to increase in stiffness, whereas the hydrogel stiffness is greater in the initial 5% strain but remains almost linear from 10-30% strain. In addition and crucially, this composition was able to achieve an insertion force profile that closely resembled that of brain in terms of both the average insertion force and also the shape of the insertion force peaks, although these peaks sometimes did not occur in the same position as in the brain results, as shown in Figure 55 (B).

An explanation for this is that in a few samples, the needle was not able to cut through the entire material by the point it reached the hole at the bottom of the sample box. This led to the hydrogel being slightly dragged into the hole before through all sample penetration was achieved, resulting in final insertion force peaks occurring around 20 mm insertion depth. This suggests a variability in the elasticity of the hydrogel samples, which could be due to the uncontrolled freezing that results in a variability in sample microstructure. Aside from this, the friction forces at the end of the insertion phase are well within the results for brain.

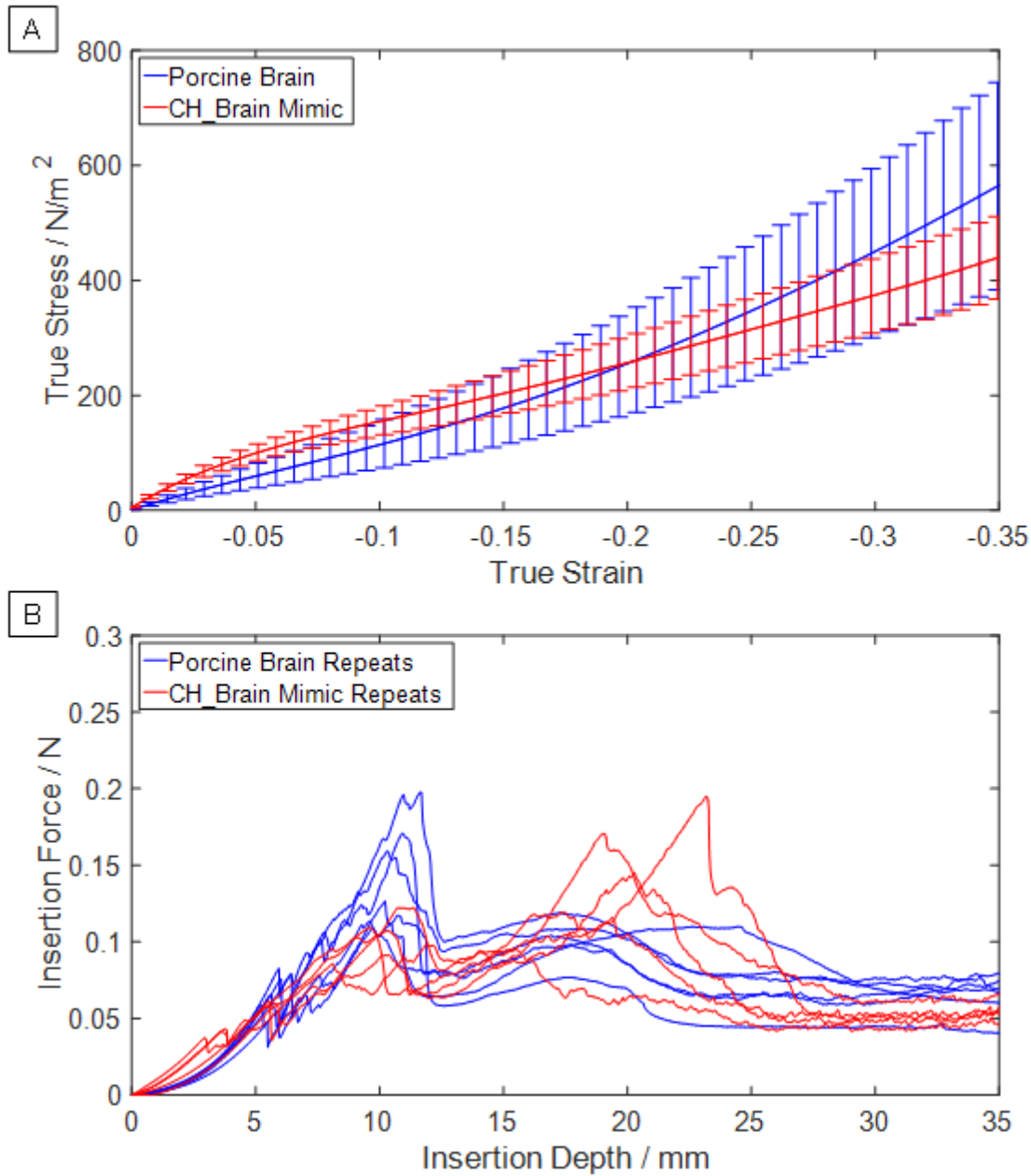


Figure 55: Comparison between CH_Brain, 2.5 wt% PVA 1.1 wt% PHY, and porcine brain for (A) stress-strain curve and (B) needle insertion tests.

Comparing the results for the brain mimicking concentration, CH_Brain, to the porcine brain results in Figure 55, we see that not only is the stiffness well matched, but the location, size and number of force peaks mimics the behaviour of brain. The microstructure of the CH is web-like, as evidenced by Forte et al. [60] and Tan et al. [228], so the fracture of this matrix causes sharp and repeated puncture peaks as the needle travels through the material, mimicking the fracture of the cerebral veins and any membranous and vesicular features that occur in brain[313–316].

Determination of Lung Mimicking Composition

When determining the best mimicking composition for porcine lung, it was clear that a single composition of CH was not able to exactly match porcine lung for all three mechanical properties. In this situation, the reader is able to pick a composition that best mimics the mechanical properties that are most important for their application. In the case of a surgical tissue phantom, a composition of 11.5 wt% PVA and 0 wt% PHY, named CH_Lung, was chosen as the stiffness and insertion forces were deemed to be the more crucial parameters to mimic. In future studies, should there arise a context that places the greatest importance on mimicking the insertion and friction forces of lung, a composition of 12 wt% PVA and 2.5 wt% phytagel could be used.

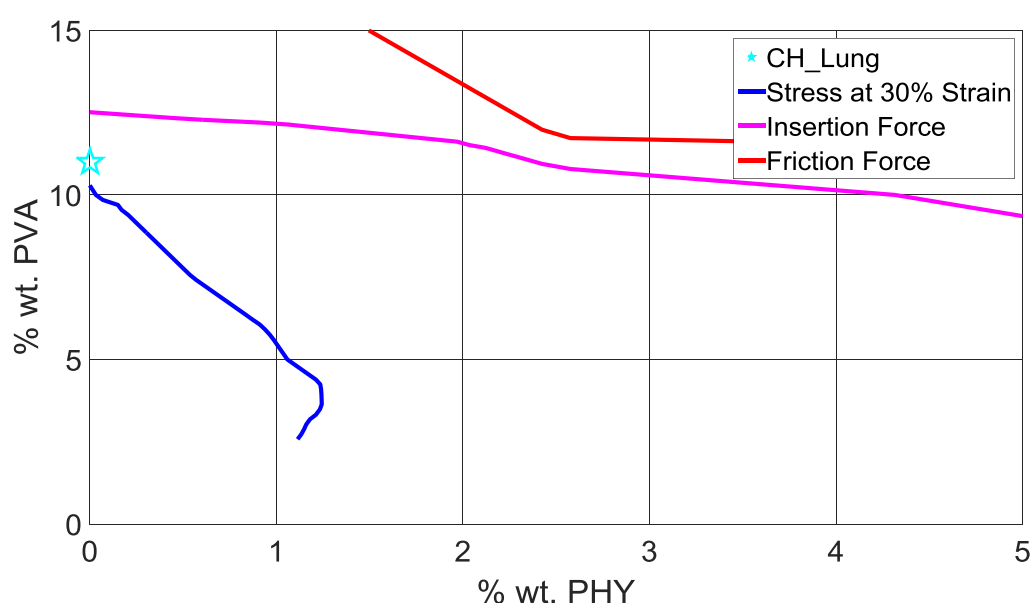


Figure 56: Determination of lung mimicking hydrogel composition.

The comparison between the CH_Lung mimic and porcine lung is shown in Figure 57. Firstly, the stiffness is very well matched to the results for porcine tissue. The insertion forces reached by the CH_Lung are also within the range of the porcine lung results. The number of puncture peaks are also well matched between the CH_Lung and porcine lung, however the maximum insertion force peak exhibited by CH_Lung is consistently lower than porcine lung. This can be understood by referring back to Figure 56, where the best mimicking composition was chosen to lie below the composition that would ideally match porcine lung insertion forces. The result of the compromise means that whilst the CH_Lung insertions forces are lower its stress-strain behaviour mimics porcine lung more accurately.

The shape of the CH_Lung results graph in Figure 57 clearly shows a similar behaviour to that of porcine lung, a soft material with many sharp and steep puncture peaks. The overall concentration of CH_Lung is 5.75 wt% PVA, as PHY concentration is 0 wt%, resulting in the

low stiffness observed. Since the PVA cryogel is physically cross-linked during a freeze-thaw cycle, the chains are weaker than chemical bonds, letting the chains unravel and slide under mechanical loads. This may therefore contribute to the elasticity and toughness of the pure PVA hydrogel, allowing it to mimic the insertion behaviour of porcine lung.

The average frictional forces exhibited in the CH_Lung results are lower than the porcine lung, which confirms the results expected from the mapping, although there is a large spread in both sets of results. This may be due to the fact that there is a high volume of interstitial water. This means that when the needle compresses or fractures the hydrogel, water is released, which lubricates the interface between the needle and the surrounding hydrogel. Thus, the accuracy of the lung mimic could be improved in future by mimicking the interstitial fluid of real lung.

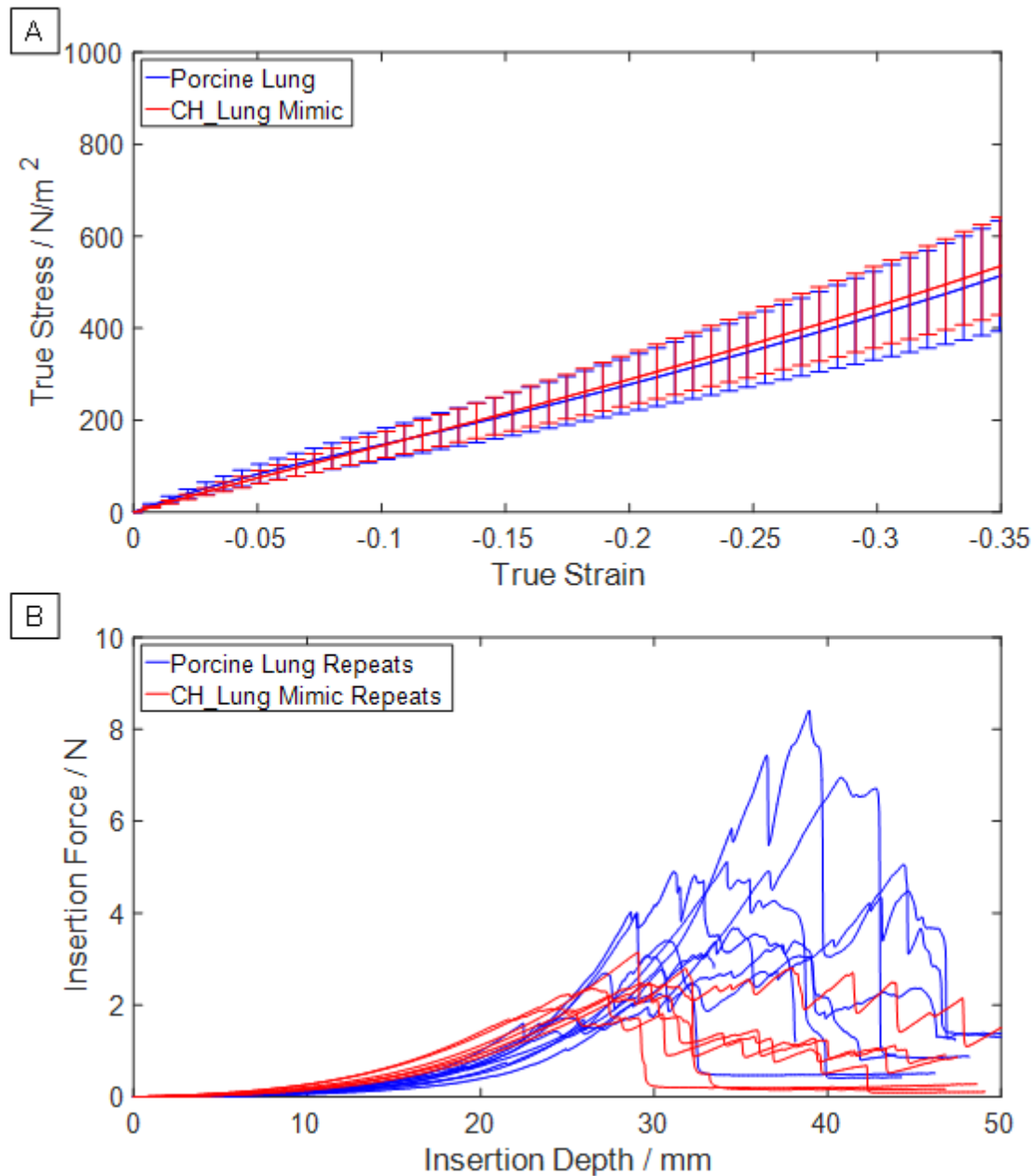


Figure 57: Comparison between CH_Lung, 11.5 wt% PVA 0 wt% PHY, and porcine lung results in the transverse direction for (A) stiffness and (B) needle insertion tests.

Determination of Liver Mimicking Composition

A composition of 14 wt% PVA 2.1 wt% PHY, named CH_Liver, was determined to mimic porcine liver. The mechanical comparison between biological liver and CH_Liver shows a good match in stress at 30% strain with porcine liver samples taken in the central region of the liver, however the porcine liver shows a greater strain stiffening behaviour than the CH_Liver, as shown in Figure 59. Similarly to the CH_Brain phantom, the CH_Liver is also initially much stiffer than porcine liver. Due to its reduced non-linearity, it is able to match biological results for the stress at 30% strain mimicking criteria. The results show that whilst the composite hydrogel exhibits a greater degree of non-linearity than PVA and gelatine, it is not able to fully mimic the extremely strong strain stiffening behaviour that is exhibited by liver tissue. The criteria for the insertion mapping is the average insertion force. By comparing the insertion results for CH_Liver and porcine liver it is clear that the criteria is more complex. While the average insertion force is very well matched, the number of puncture peaks in the porcine liver is much more than that of CH_Liver. Nevertheless, the friction force is generally well matched.

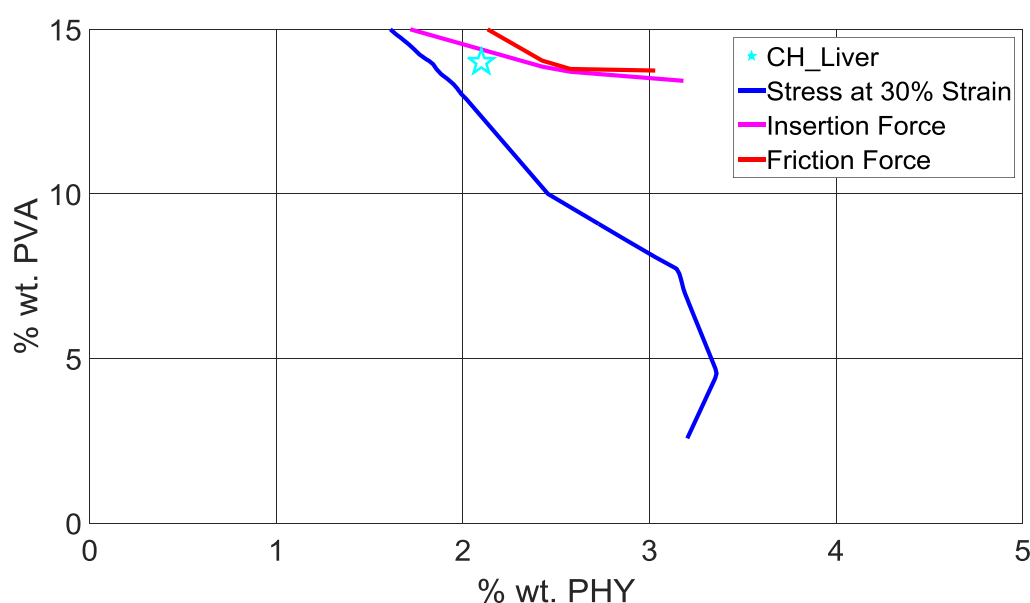


Figure 58: Determination of lung mimicking hydrogel composition.

The CH_Liver is able to match the stress at 30% strain, average insertion forces and average friction forces well. However, the shape of the CH_Liver needle insertion results graphs after the initial surface puncture is featureless compared to the real porcine liver, as shown in Figure 59. This is possibly due to the microstructural differences. As mentioned previously, the composite hydrogel microstructure is web-like whereas the biological liver is comprised of parenchymal lobules held together by connective tissue, creating an organised microstructure, on the millimetre scale [221]. Future investigations into the cause of the numerous small insertion force peaks should be carried out. This will allow the development of a tissue mimicking material that is able to match the microstructural details of the liver.

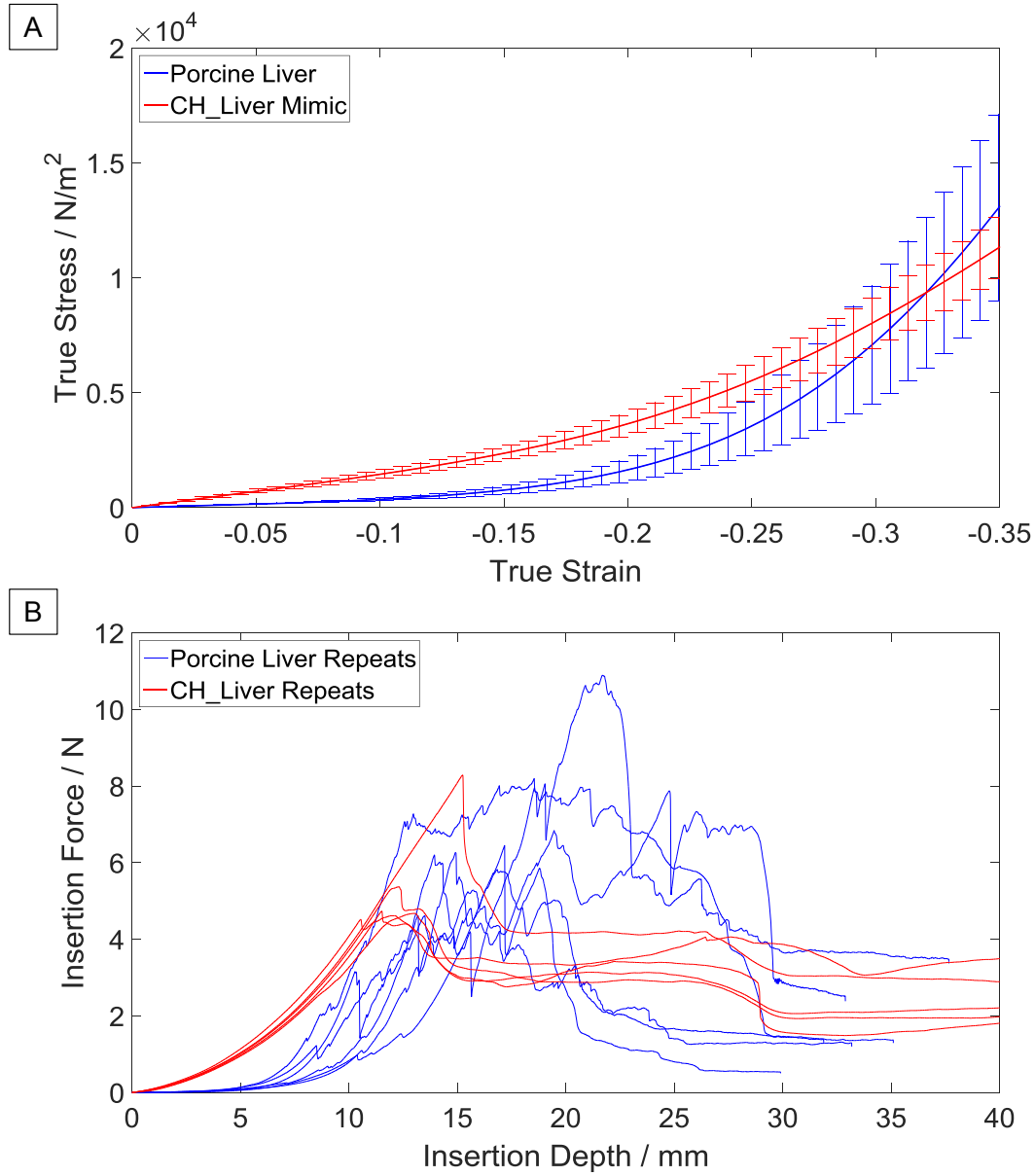


Figure 59: Comparison between CH_Liver, 14 wt% PVA 2.1 wt% PHY, and porcine liver in the transverse direction for (A) stiffness and (B) needle insertion tests.

Biological tissues have a complex architecture that make the mechanical evaluation of these soft tissues very difficult. Whilst the composite hydrogel is homogeneous, its flexibility and tunability has allowed it to mimic three different mechanical properties of three completely different soft tissues, which is already an achievement. Overall, for the purposes of tissue mimicking for surgery, the tissue phantoms that arise from the compositions of CH_Brain, CH_Lung and CH_Liver presented in this work provide a greater, more ethical and easily procurable, tactile training experience than any other material known to the author. The can be improved by incorporating more accurate mimicking criteria, such as the hyper- and viscoelastic model parameters and a quantitative measurement for the number of insertion force peaks and their evolution. In addition, other physical properties such as optical, thermal and diffusive properties should be investigated in future and integrated with the existing results reported here to continue improving the fidelity of these tissue mimicking materials.

5.3.2. An in vitro Model for Convection Enhanced Delivery

A crucial mimicking criteria in the design of a surgical phantom used to study convection enhanced delivery is the diffusive properties of the hydrogel. Earlier in this chapter, a composition of hydrogel named CH_Brain was shown to mimic the mechanical properties most related to surgical tool-tissue interaction. The results detailed in this section builds upon the mimicking capabilities of CH_Brain by evaluating its diffusive mimicking potential.

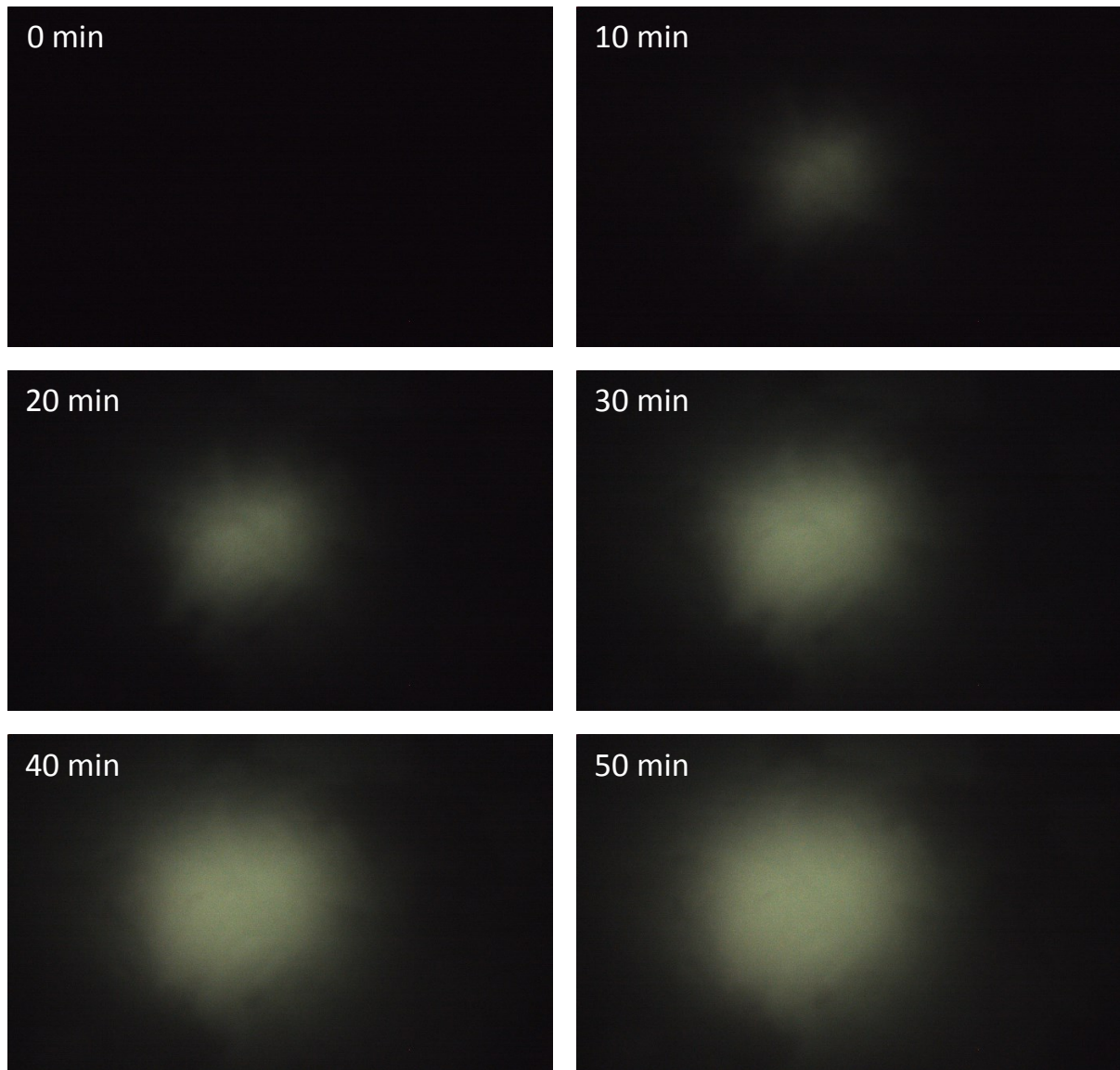


Figure 60: Diffusive progression of fluorescent solution images taken every 10 mins at a flow rate of 1 μ L/min.

At this point, it is assumed that the diffusion is governed by concentration gradient and positive pressure gradient from the fluid flux of the needle. Since fluorescein dissolves in water to form an aqueous solution and not a mixture of fluorescent particles, it is assumed that tracking the fluorescent volume is equivalent to tracking the diffusion of the entire solution. However, it is unknown whether the fluorescent particles would interact with the hydrogel matrix by chemical

or physical adsorption. If adsorption was occurring, it would remove the fluorescent molecules from the solution, affecting the concentration gradient and therefore affecting the diffusive test results.

Figure 60 shows the development of the fluorescent solution as it diffuses through the hydrogel at 10 min intervals. At this flow rate of $1\ \mu\text{L}/\text{min}$, the distribution appears to be evenly distributed and diffusion, as opposed to convection, is the most likely key mechanical driver. These results suggest that the hydrogel microstructure is very homogeneous as there have been no efforts so far to try to control the directionality of the microstructure, unlike *e.g.* the inherent axonal microstructure of real biological brain. Although there is a positive pressure gradient, the results suggest that it is not great enough to cause the solution to behave in an inhomogeneous fashion, unlike what is observed at higher flow rates that will be discussed later on.

The average and standard deviation in fluorescent area observed by the microscope is shown in Figure 61. The microscope was only able to view the top surface of the sample and although the hydrogel was semi-transparent, it was apparent that the microscope was only able to detect the fluorescent solution once it had already diffused to the top surface of the sample before spreading circularly. Thus, the results reflect a linear development of fluorescent area over time.

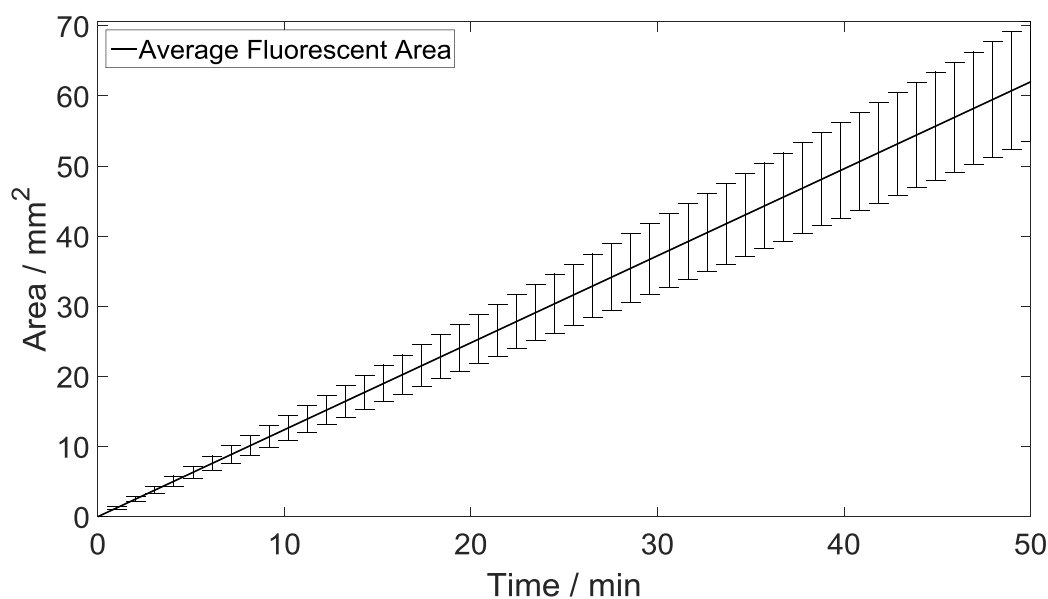


Figure 61: Average observed fluorescent area from top surface of sample.

These results were also confirmed separately by a computational model developed in collaboration with Dr. Wenbo Zhan. According to Darcy's law of diffusion, in 3D, the spherical diffusive volume should increase linearly with time if the mass flow rate is constant and permeability is constant. The fact that these results show that apparently area increases linearly with time does not refute Darcy's law, instead it indicates that when the fluorescent solution

approaches the surfaces of the sample, it spreads radially under the hydrogel surface. This is validated by the boundary conditions used to mimic this system in the computational model whose results show the same trend.

In future, the spherical diffusion characteristics could be explored by using a deeper hydrogel sample or a spherical sample with enough volume so that the injected solution will not reach the surface. However, attempting to image the 3D volume using such an optical technique may be difficult given the opacity of the hydrogel being greater than expected. Instead, the best case scenario would be the use of intra-operative MRI methodology[233], which is not always feasible.

The average CED radius and volume after 50 mins was calculated to be 4.4 mm and 367 μL , respectively. Since the flow rate was 1 $\mu\text{L}/\text{min}$, the injected volume was around 50 μL , therefore the final CED volume indicates that the fluorescent solution diffused greatly through the hydrogel material to cover 7.3 times its original volume. The results were compared to the finding reported by Mardor and co-workers, who performed similar experiments on rat brain models, and reported a CED volume of 27 μL after injecting 90 μL of therapeutic solution[293]. The comparison of the results found here and in literature highlight the major issues that arise during CED procedures in real biological tissues, which are mainly due to the ineffective delivery of the therapeutic agent to the tissue. Mardor and co-workers explicitly reported the observation of major infusate leakage to the ventricles and suggested, with supporting evidence, that CED efficiency could be increased by increasing the viscosity of the solution[293]. However, this comes at the cost of higher localised pressures exerted on the brain tissue, which may lead to damage in those areas.

The results of the investigation into infusate viscosity on diffusive behaviour, shown in Figure 62, illustrates the effect of viscosity and also injected flow rate on the progression of diffusion. Firstly, comparing Figure 62 (A), where the only difference is that the flow rate is 5 times higher, to the image in Figure 60 at 30 mins, we see that a higher flow rate results in a less homogenous diffusive pattern due to the higher pressures imparted by the increased flow rate. These pressure seek to be relieved as quickly as possible by spreading along the regions in the microstructure that offer the least resistance, i.e. the largest pores in the microstructures. Although the hydrogel is described as being homogenous, it actually consists of a random web of pores that range from 5-40 μm diameter. When the pressure of the injected infusate is high enough, the solution will tend to travel down the paths of the microstructure that have the largest pores. This effect is observed especially in Figure 62 (B) where the higher infusate viscosity increases the pressure even more, resulting in a sudden and branching diffusive pattern resembling tendrils as opposed to the smooth cloud formation observed at the lowest pressures.

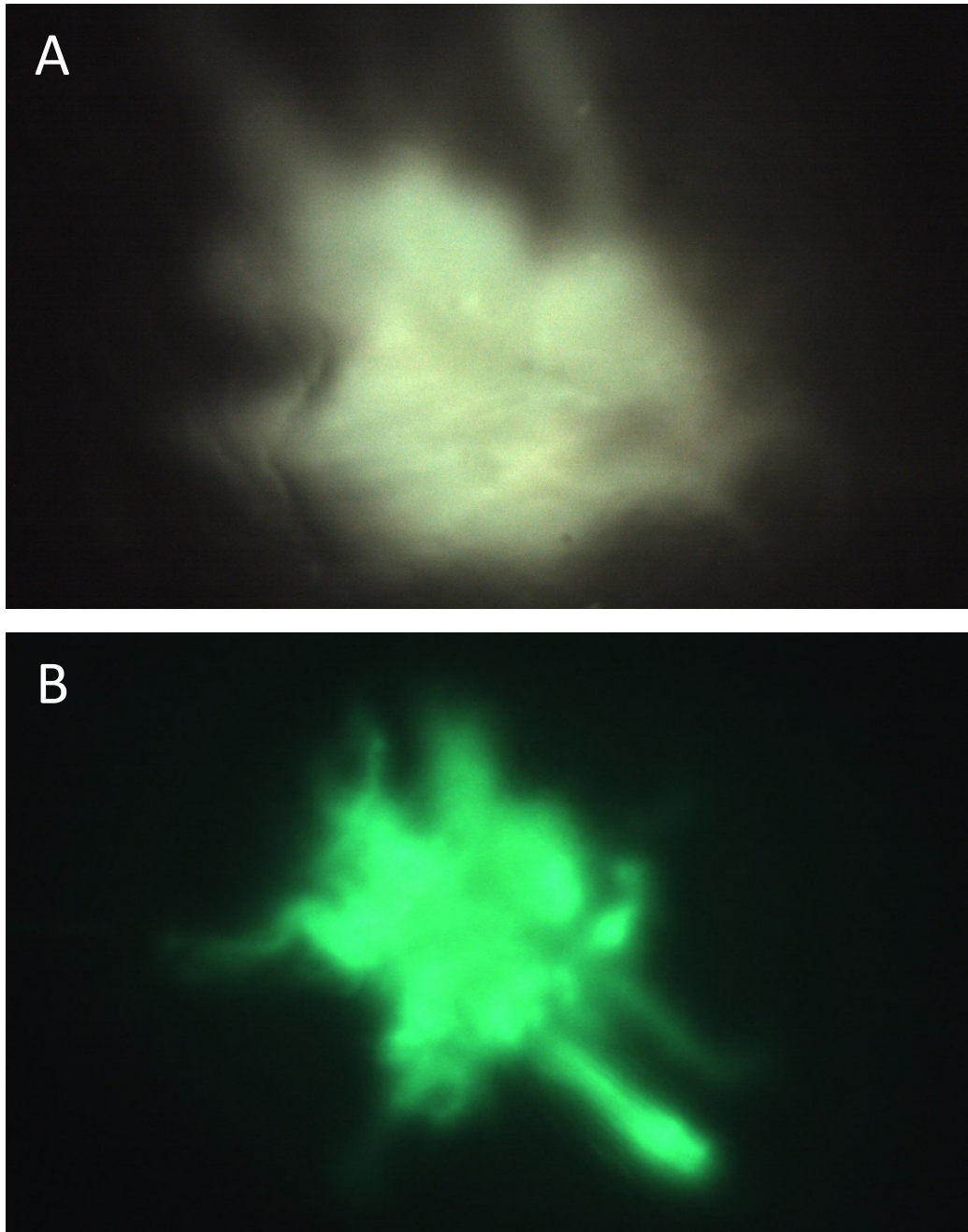


Figure 62: Diffusive progression of (A) 0 wt% sucrose and (B) 50 wt% sucrose fluorescent solutions at a flow rate of 5 $\mu\text{L}/\text{min}$.

To summarise, CED is an emerging field where mechanical engineers can help in the fight against cancer. The main advantage of these systems in the treatment of brain tumours is that effective cytotoxic agents, which cannot cross the blood-brain barrier when administered systemically, are locally perfused into the tumour tissue. Conventional chemotherapy must administer high doses of cytotoxic agents to the whole body just so that a therapeutic level can be achieved in the brain tissue, which is devastating to the well-being of the patient. CED would allow lower doses to be administered directly to the tumour and even if higher doses are necessary, they would not pass into the blood to affect the rest of the body since they would be contained within the brain by the blood brain barrier. Additionally, a permanent neuro-catheter would also reduce the number of craniotomies and hence lower the chances of infection.

However, the success of this technique depends as much on the mechanical behaviour of the materials as the biological mechanisms. An expert review by Debinski and Tatter discusses many issues that could be solved by better understanding the mechanical interactions in the system[317]. The major issues include, reflux, leakage into the ventricles and sulci. Whilst the development of a high efficacy anti-cancer drug is a chemical and biological issue, mechanical engineering skills can be utilised in this multidisciplinary effort to beat cancer by providing knowledge about surgical tool-tissue interactions, biological tissue mechanical properties and the diffusion of therapeutic solutions into tissues. Engineering skills, such as systematic mechanical testing and computational modelling, that have been applied to all other industries can be used to provide analytical insights into the mechanical behaviour of real biological tissues and to help elucidate fundamental material behaviours. This work has suggested and provided evidence of an *in-vitro* method of analysing real-time CED progression using a mechanically accurate brain phantom in order to understand the mechanisms of effective diffusion against leakage. Other important factors such as osmolality, chemical and biological interactions could also affect the diffusion behaviour. Therefore, future developments of the hydrogel as a CED tissue phantom should look to implement more accurate chemical gradients, for example functionalisation of the hydrogel in combination with solutions that interact chemically with these functional groups.

5.3.3. Composite Hydrogel as a Traumatic Injury Phantom

Whilst the use of ballistic gelatine has been standard practise for decades, it simply does not mimic real biological tissues as closely as this new biomaterial development now allows us to do. Meaning that the propagation of shock waves through a material with a high water content will be substantially different to that of gelatine and will result in better hyperelasticity and viscoelasticity mimicking. The range of CH compositions matching the mechanical properties of brain are highlighted in Figure 63 by the blue line of intersect. These combinations will be able to mimic the real brain results of 7.5 kPa at 20% strain, 60 s⁻¹ strain rate found by Rashid et al.[187] The results in this work have suggested a range of traumatic brain injury phantom compositions that can mimic the tensile properties of brain. Future work involving the characterisation of other material properties that are important for the application of traumatic injury, such as the compressive and shear moduli, will help to narrow down the most suitable composition out of the range reported here. The introduction of new materials means that old practises should be updated and move towards those which may help advance the understanding in the field.

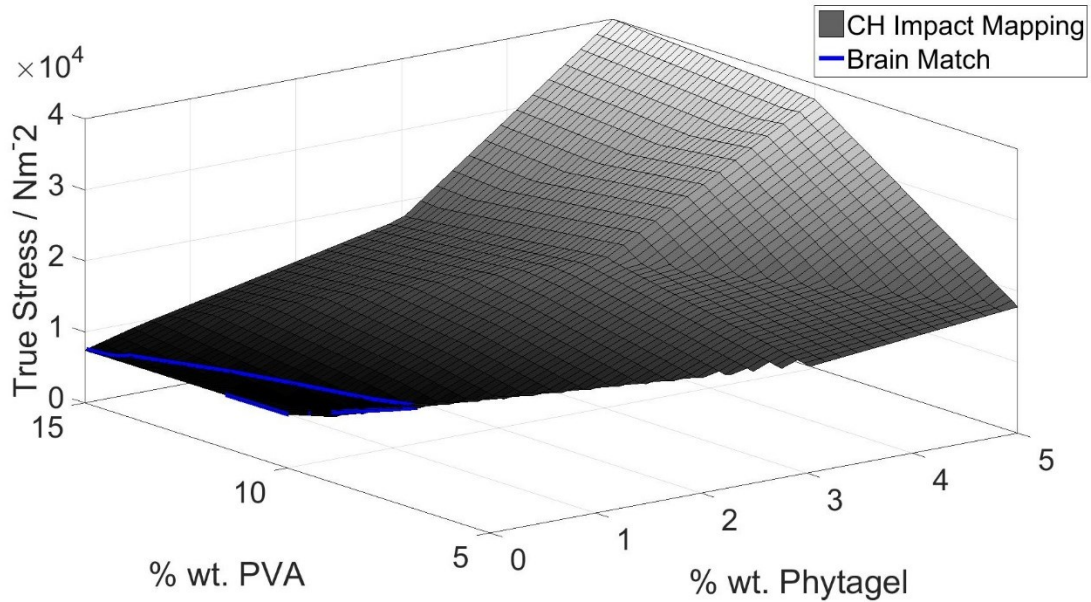


Figure 63: Mapping showing the concentrations of PVA and phytigel that match the stress of brain at 20% strain, 60 s^{-1} strain rate.

A life size cast moulded composite hydrogel brain was created, shown in Figure 64, to demonstrate the use of the tissue phantom for macroscale testing. Due to its larger volume, the brain phantom requires two days to create, including one freeze-thaw cycle, and because there are no ethical issues involved with this artificial phantom, as many brain phantoms can be made as possible.

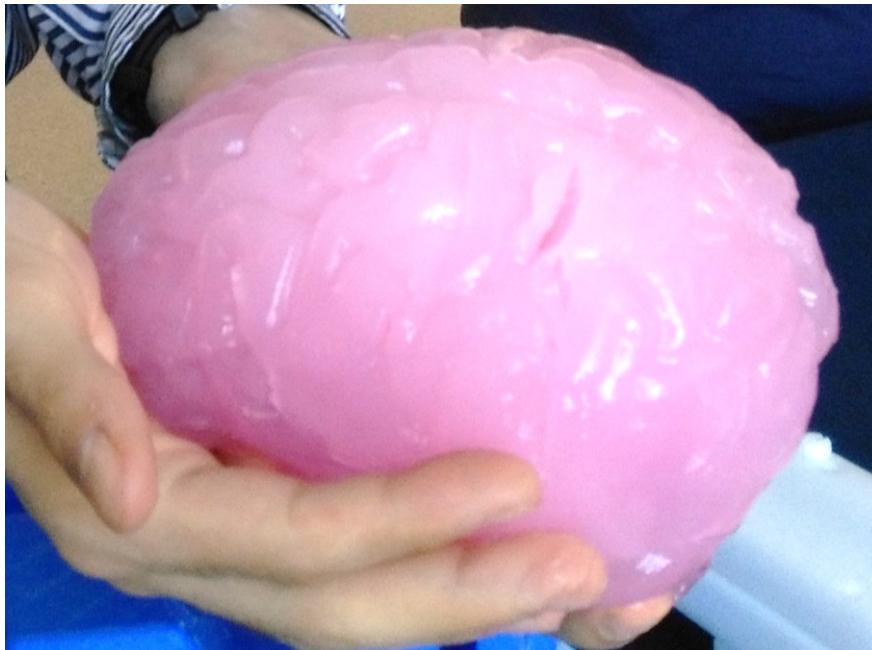


Figure 64: Life size brain phantom made from cast-moulded composite hydrogel, dyed pink with food colouring.

The brain phantom can be used to investigate the propagation of tensile, compressive and shear waves that the material experiences during destructive impact tests to elucidate the mechanical

behaviour of the tissue. These forces could be measured by placing accelerometers and strain gauges on the surface and integrated throughout the volume during the casting stage. Another method of measuring deformation would be to disperse particles all over the brain phantom surface, similar to the technique Forte et al.[233] applied to study low strain rate brain shift during neurosurgery, and capture the deformation using digital image correlation (DIC). To better mimic the boundary conditions of the brain during impact, the brain phantom could be placed inside a sealed 3D printed skull and submerged in a pressurised fluid to mimic the natural intracranial pressure inside the skull. In addition, pressure sensors can be applied all around the phantom-skull interface to measure the local pressures during impact as well as the intracranial pressure after impact.

The application of the composite hydrogel as an impact tissue phantom for other soft tissues could also be expanded in future work, similar to what has been shown in this work for low strain rates. Phantoms constituting of different hydrogel compositions that represent different organs could be created to represent the full body more accurately. These could then be subjected to destructive tests that affect the whole body, such as car crash incidents, to analyse which organs are at highest risk of injury and to develop safety measures to reduce damage.

As a final point, the development of an accurate tissue phantom opens up the possibility of investigating large-scale in vitro tests involving biological cells seeded throughout the structure. Different methods, including 3D bioprinting, that aim to deposit cells throughout the volume of the substrate as opposed to just on the surface are being developed[124,149]. This is very important because the boundary conditions affecting cells on the surface of a substrate are very different compared to those that are in the centre of the volume. Furthermore, it has been shown that cells cultured in a 3D scaffold behave differently to when they are seeded on a 2D surface, like a petri dish[318–321].

Therefore, a mechanically accurate tissue phantom with cells successfully seeded throughout the 3D volume would allow researchers to study how boundary conditions affect cell damage during impact loads, which could be correlated to the damage done to grey and white matter since grey matter is the brain surface. Further investigations have the potential to elucidate how brain injury influences the development of certain neurological diseases or how bone growth after blast injury manifests[322–324]. For these reasons, the biocompatibility of a tissue mimicking material is essential to be able to unlock the possibility of investigating these extremely important topics. Hence, the results and discussion of a preliminary study to assess feasibility of conducting cell studies on the composite hydrogel material is detailed in the next section.

5.3.4. Cell Viability of Composite Hydrogel

An initial assessment of biocompatibility is to conduct a cell viability assay on the composite hydrogel to show that cells are able to attach, survive and proliferate. Figure 65 shows cell viability of fibroblasts on 3D printed CH previously coated with collagen gel (a), poly-L-lysine (b) and gelatine (c), after 72 hours in culture. Live cells were more than 97% on collagen-coated CH, confirming a good biocompatibility of the materials. Collagen gel coating showed the best results in terms of cell attachment and viability. Compared to the collagen coating, live cells attachment on the poly-L-lysine and on the gelatine coated hydrogel were respectively, 76% and 8%. Cell viability was greater than 98% on both the poly-L-lysine and gelatine coating. Cell morphology was different for each coating. Cells on the gelatine-coated substrate attained a more rounded morphology. 80% of the live cells observed were well-spread on the poly-L-lysine coated hydrogel, and all cells were well-spread on the collagen-coated materials. Although the cell viability and spreading were similar on both poly-L-lysine and collagen coatings, there were more distinct regions of tightly packed cell clusters observed on the poly-L-lysine coated hydrogel. This indicated that cell attachment was less homogeneous, and the cells favoured a more densely packed configuration than on the collagen coated hydrogel. Cell attachment for poly-L-lysine and gelatine coated 3D printed CH has been achieved with optimized coating processes, which are currently under further investigation.

The cell viability studies on collagen and poly-L-lysine coated CH substrates were carried out to assess the potential of the composite hydrogel and printing method for future use in a wide range of bioengineering applications. Additionally, gelatine coatings were studied, as it is a more affordable alternative to collagen. Cell viability and attachment were excellent on collagen-coated hydrogels. This was expected considering that collagen is widely known as a favourable material on which cells thrive since it provides them with sustenance to grow[325]. Compared to the collagen coating, cell attachment was lower by 26% on poly-L-lysine coated CH. Only rounded cells were found on gelatine-coated samples.

Furthermore, the poly-L-lysine coating is a very promising alternative as it functionalises the hydrogel through adsorption providing a more robust and durable coating. Cells exhibited a good attachment, although the morphology was slightly inconsistent, with large spreading of some cells and low spreading of others. Small regions of tightly clustered cells were observed, creating localised areas with high cell attachment. Despite this, the poly-L-lysine coating has demonstrated great potential as a coating that greatly enhances the cell attachment of the substrate. It is expected that advancing the adsorption process will improve the homogeneity of the coating and therefore enhance cell attachment, comparable with collagen-coated hydrogels.

Future work will focus on improving the adsorption of poly-L-lysine onto the hydrogel, which may be done by using a solution of pH 11 and radiating under UV[312,326,327].

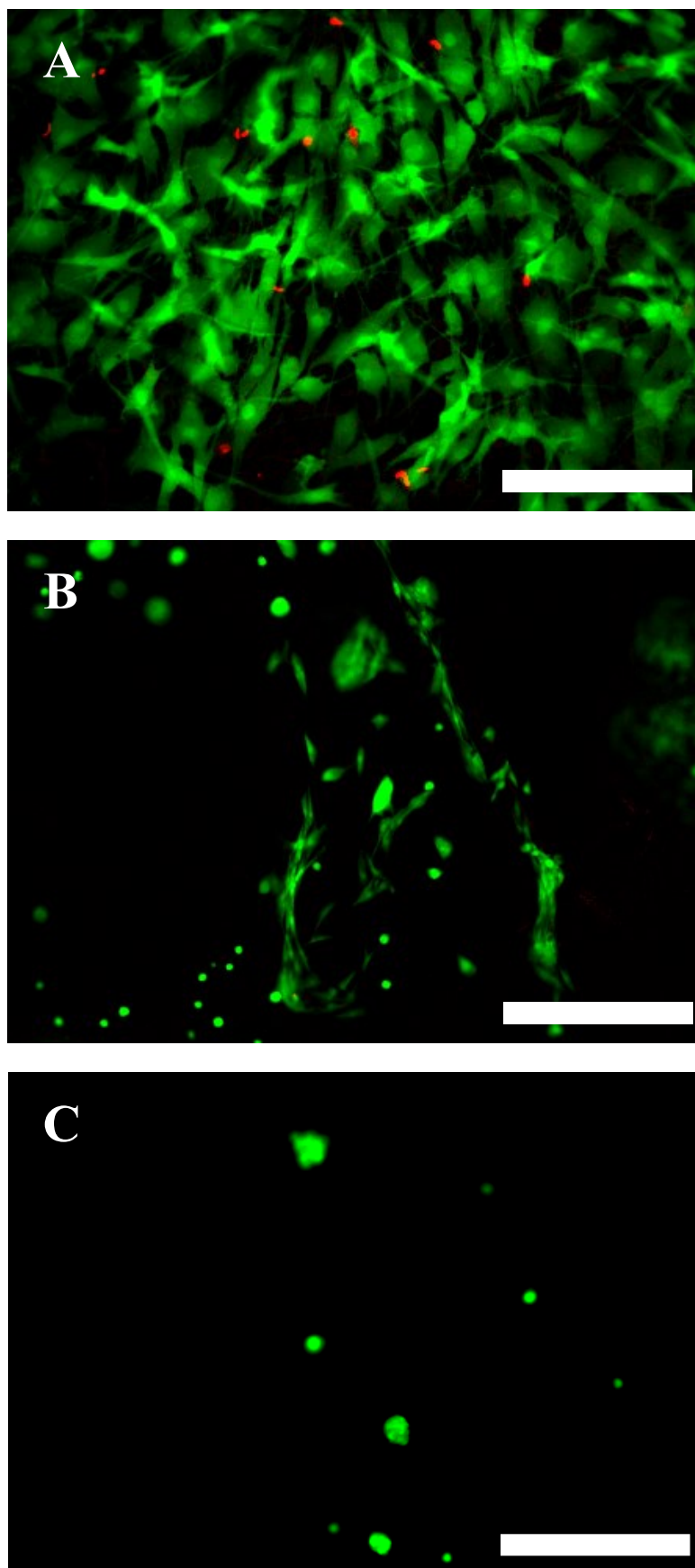


Figure 65: Cell viability on (a) collagen, (b) poly-L-lysine and (c) gelatin coated 3D printed CH. All scale bars are 100 μm.

The gelatine coating was unable to provide substantial results due to the thermal reversibility of gelatine at the incubation temperature of 37 °C, where the coating, which was a gel at room temperature, turned into the liquid state. Nevertheless, further work into the thermal stabilisation of gelatine using UV radiation will be considered in future studies.

Dermal fibroblasts were used to evaluate the biocompatibility of the printed constructs. This was a deliberate choice as the cellular evaluation was an initial screen to determine the general biocompatibility of the newly designed scaffold, rather than a specific biofunctional study. Furthermore, considering the wide range of applications of these super soft hydrogels, with great potential for closely mimicking various soft tissues, fibroblasts were chosen since they showed to adapt to various material stiffness[328]. Moreover, it has been shown by Volckaert and De Langhe[329] that fibroblasts are involved in lung development, and fibroblasts growth factors (FGFs) pathways are crucial for the regulatory fibroblast-epithelial cell cross-talk. A full comparative study involving an in-depth analysis of coating efficiency has not been performed in the current work since was not in the scope of this investigation. This is a limitation of the present study and will be carried out as a follow-up investigation in the near future.

Collagen has been commonly used as biomaterial for 3D scaffolds in neural tissue engineering, thanks to its versatility, biocompatibility, low antigenicity, inflammatory and cytotoxic response[330]. It has also been shown that neuronal cells are able to survive for more than 42 days and maintained high cell viability in collagen scaffolds[331]. All these evidences pave the way for future in-depth biofunctional studies of our 3D printed scaffolds coated with collagen in combination with various cells types, including neural cells, to determine a more functional 'tissue-like' response of the material, mimicking natural soft organic tissues.

In summary, the work in this chapter has combined the mechanical characterisation results of previous chapters, 3 and 4, to determine the most mechanically accurate tissue mimicking compositions based on the key mechanical properties relevant to each tissue mimicking scenario, including surgery, drug diffusion and traumatic brain injury. Furthermore, for the application of surgery, the proposed tissue mimicking formulations were produced and evaluated to reveal a high accuracy of mimicry for brain, and medium levels of accuracy for lung and liver, although these formulations still offer the greatest tissue mimicking ability for surgical applications known to the author.

In addition, the CH_Brain composition was used to elucidate the diffusion of a therapeutic agent mimic for convection enhanced drug delivery procedures. This topic is immensely complex, as diffusion relies on a number of complex interactions, which require a large number of tests

involving sacrificial animal models to be performed. Instead, the brain phantom suggested here will allow researchers to carry out any number of tests to evaluate the most effective delivery techniques, which would hopefully reduce the number of animal models.

Furthermore, the biocompatibility characteristic of the composite hydrogel is crucial to allow this material to be used in the field of mechanobiology, in order to investigate the effect of mechanical loads on cell behaviour. Thus, the cell seeding ability of the composite hydrogel was assessed and cell viability was shown to be good with the most successful attachment occurring on the collagen coating. This sets the basis for a variety of fascinating cell studies that will be carried out in future work. From exploring how mechanical cues direct stem cell differentiation to how traumatic injury loads induce primary, secondary and tertiary damage in neural cells leading to neurological diseases, the opportunities that arise from creating a mechanically accurate biocompatible tissue mimicking material are vast and exciting.

A significant theme investigated heavily in Chapter 2 but not largely addressed in this chapter, was the directionality of soft tissues and, in particular, the effect of the internal structures on its mechanical properties. As was discussed previously, PVA fibres or nanotubes could be implemented during the cast-moulding fabrication process to induce anisotropy. Whilst this may be able to replicate the largely radial directional nature of axons in the brain, the geometry of the internal structures in lung and liver are often branching and therefore cannot be mimicked with linear fibres. Thus, the development of an innovative fabrication method, involving 3D printing technology that will allow researchers greater freedom to create complex geometries out of hydrogels, will be described in the next, and final, chapter.

6. Cryogenic 3D Printing of Super Soft Hydrogels

This chapter has been partly published in Scientific Reports[228].

6.1. Introduction

In the past three decades, 3D bioprinting has become one of the leading techniques for the *replication* of real tissue geometries, with the potential to mimic the soft tissue microstructure. Hence, bioprinting is currently the focus of several rapidly developing research fields. Recent applications include printing full human organs to contribute towards the shortage of organ donors[332]. With the development of new soft tissue materials that can be used as printing inks, the field of biological 3D printing has grown exponentially, giving rise to the extrusion of living cells suspended in the printing ink[140,145,146,333,334]. A wide range of bioinks used for 3D printing tissue scaffolds have been summarized in a recent review carried out by and Munaz et al.[125], although there is no quantification of stiffness past the qualitative distinction between soft and hard tissues.

It has been shown that the stiffness of the majority of human tissues lies within the order of a few kPa[335]. Furthermore, in specific cases, cell differentiation and regeneration is promoted in tissue scaffolds that exhibit mechanical properties similar to those of the real tissue[173,174,176,336,337]. Therefore, a 3D printing technique that is able to produce geometrically and mechanically accurate scaffolds could hold enormous potential in regenerative medicine and biomimetics[338]. This reinforces the importance of soft 3D printing. To the best of our knowledge, there is a lack of studies focusing on bioprinting very soft materials with stiffness $O(1)$ kPa. One of the causes of this is the inability of extremely soft materials to withstand their own weight: the printed structure is usually too soft to hold its shape or allow further layers to be built on top of it. A few methods that have been developed by other researchers are reported here.

Hinton et al.[124] have developed a technique for free-form extrusion-based 3D printing of biological structures (e.g. arterial branches) using alginate, collagen and fibrin gels as printing inks and a gelatine slurry as a support bath[124]. The technique was able to achieve a resolution of $\sim 200\ \mu\text{m}$ demonstrated through the printing of a scaled down human brain using an alginate bioink. However, the stiffness of the alginate ink was reported to be $O(10)$ kPa, and therefore not comparable with that of super soft tissues, such as human brain or lung ($O(1)$ kPa[15,18,124,150]). In another study, Lozano et al.[141] used a RGD modified gellan gum 1 wt% hydrogel bioink with encapsulated cortical neuron cells. The authors were able to demonstrate the ability to print soft 3D cell-laden constructs. However, the printing process was achieved

through a hand-held device, hence lacking precision, and the material stiffness was not characterised.

Adamkiewicz et al.[152] introduced a novel cryogenic 3D printing method using liquid nitrogen. The conceptual idea behind the cryogenic method is that it allows inks in a solution state to transform into a solid state, thus allowing stable structures to be built in 3D using a layer-by-layer approach, without the need for a support bath. However, the stiffness of the hydrogel ink was not reported and the precision of the printing method was not discussed[152]. The cryogenic method was also used to create 2D constructs for implants by Wang et al.[339], who utilised a substrate cooled by coolant flow to create the cryogenic stage. Again, mechanical characterisation of the printed structure was not reported.

Therefore, this article demonstrates the fabrication of mechanically accurate 3D printed composite hydrogels that mimic the stiffness of super soft tissues through the use of a novel printing setup based on cryogenic theory. Solid carbon dioxide (dry ice) and an isopropanol thermal conductive bath was used to achieve the cryogenic stage, which is a safer alternative to liquid nitrogen. The ink used in this work is a composite hydrogel of poly(vinyl) alcohol (PVA) and Phytigel, which has been pioneered by Leibinger et al.[63] and Forte et al.[60,229,233] to mimic soft tissues, such as brain, with stiffness of $O(1)$ kPa. A further advantage of this novel 3D printing technique over traditional cast moulding methods resides in the possibility to produce hollow structures of super soft hydrogels. Interconnected holes make soft hollow structures impossible to extract from a mould using traditional cast moulding techniques.

The aims of the study are as follows: (i) to provide mechanical evidence showing the 3D printed material mimics real brain tissue, providing the same response as the casted material[60], through unconfined compression tests, (ii) to demonstrate the capabilities of this printing technique by achieving hollow 3D printed structures, whose continuity through the layers has also been assessed using Scanning Electron Microscopy (SEM) analysis.

6.2. Materials and Methods

6.2.1. Composite Hydrogel Ink Preparation

PVA (molecular weight 146,000-186,000 Da), Phytigel and deionised water were supplied by Sigma Aldrich, USA. A concentration of 2.5 wt% PVA and 0.295 wt% Phytigel (obtained by dissolving 5 wt% PVA and 0.59 wt% Phytigel powder separately, see below) was used for the ink as this is the composition used by Leibinger and co-workers to match the stiffness of porcine brain up to 95% strain[63]. A detailed descriptions of the constituent hydrogels is provided in the previous work by Forte et al.[60].

The CH ink solution was prepared by dissolving 5 wt% PVA and 0.59 wt% Phytigel powder separately in deionised water for 1 h at 90 °C[60]. The separate solutions were then combined together in a 1:1 weight ratio and kept at 70 °C under constant stirring for an additional 30 min. The mixed solution was then allowed to cool to room temperature to be used as ink for the printing process, where it remained in liquid phase.

6.2.2. Cast-moulded Samples

For mechanical comparison with the 3D printed samples, control samples were prepared using a standard cast-moulding technique. The CH solution, which was prepared from the same batch as the printing ink, was transferred into a plastic mould and frozen at -25 °C for 15 hours. The material was then thawed at room temperature and tested. Samples of 10.65 ± 0.16 mm diameter and 6.39 ± 0.26 mm height were cut using a biopsy punch.

6.2.3. 3D Printing Process

A commercial 3D printer, Ultimaker2 (Ultimaker, Netherlands), was modified for the purposes of 3D printing soft structures. The 5 wt% PVA 0.59 wt% Phytigel solution does not solidify at room temperature, thus allowing the ink to be extruded. An extrusion based printing method was developed using needle of gauge 21 (0.514 mm internal diameter) connected to a syringe perfusor (B. Braun, Germany) by PTFE tubing. The material extruding rate was determined as part of the print optimisation process detailed in Section 6.2.4. A stainless steel plate, chosen for its good thermal conductivity of 12.44 W/(mK) [340], was kept in contact with dry ice, which has a sublimation temperature of -78.5 °C. The contact was kept constant across the plate using isopropanol, with a melting point of -89 °C, as a thermal conductive fluid with thermal conductivity of 0.14 W/(mK) at 21 °C and atmospheric pressure [341], which therefore remained

in liquid phase when in direct contact with the dry ice pellets. The dry ice and isopropanol bath were contained in an insulating polystyrene container, with thermal conductivity of 0.03 W/(mK) [342], which had an outlet to vent the sublimated CO₂. Due to the high water content of the hydrogel, the solution froze immediately upon contact with the conductive stainless steel plate. This solid state allowed a stable structure to be built by means of a layer-by-layer approach. A schematic demonstrating the setup of this cryogenic printing method is shown in Figure 66. The highest resolution this setup was able to achieve was investigated by printing a single line on the lowest volumetric flow rate perfusor setting of 0.6 mL/hr and imaging the line using a white light interferometer to evaluate the line thickness, which is displayed in Figure 73 (A).

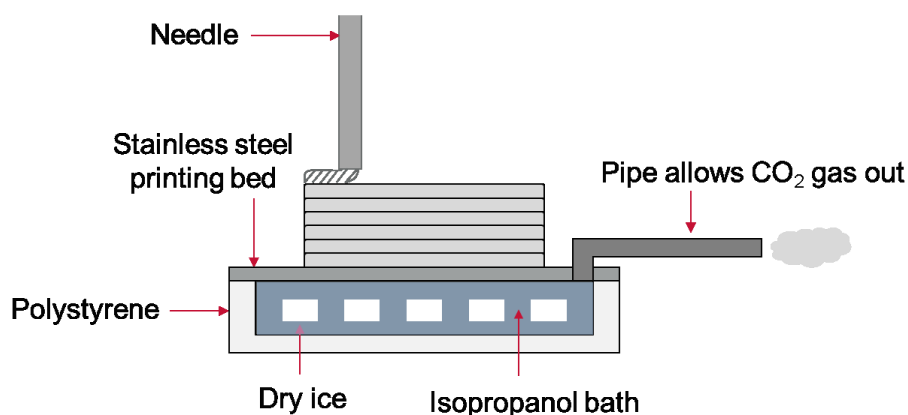


Figure 66: Schematic of the cryogenic 3D printing procedure and set up (not to scale).

6.2.4. Evaluation of Optimal Printer Parameters

Determination of the best printing parameters was assessed by trialing a range of different ink widths, printer head speeds and extrusion flow rates. The optimal printing parameters were chosen based on the smoothness of the initial printed layer weighed against the time taken to complete one layer, which is related to the time required for the depositing hydrogel to freeze. Whilst printing parameters has a large effect on print quality, it was also observed early on that the print geometry also effects print quality. This is because, if the flow rate of the hydrogel ink and the print speed are both constant, then the hydrogel will accumulate at points in the geometry where the print path changes direction rapidly, i.e. sharp corners.

To investigate the significance of this, the effect of printing path angles on the quality of the printed structures was investigated. Three different patterns; re-entrant, orthogonal and hexagonal, were chosen to investigate as they feature acute, right-angled and obtuse angles respectively, as shown in Figure 68 (A)-(C). Additionally, the re-entrant structure is auxetic, meaning it has a negative Poisson's ratio, which is a fascinating mechanical attribute that has also been found in specific parts of the body such as skin and bone[259,343].

6.2.5. Printed Cylindrical Structures

Using the CH ink solution, cylindrical samples were printed to match the same dimensions as the cast-moulded samples. The print settings are shown in Table 18. The printing path was obtained by importing a stl file made in Solidworks (Dassault Systemes, France) into the 3D printer software (Cura, Ultimaker, Netherlands) in order to generate the toolpath (gcode file), readable by the 3D printer. Following the completion of the print, the printed samples were immediately stored at -25 °C for 15 hours, to be as consistent as possible with the conditions of the cast samples. Before testing, the samples were thawed at the same rate as the cast-moulded samples since it has been shown that the mechanical characteristics of the PVA component depend highly on the thawing rate, as the slower the rate the more time is allowed for a physical network to form[344]. The dimensions of the final printed samples were 10.25 ± 0.1 mm diameter and 6.75 ± 0.13 mm.

6.2.6. Printed Complex Structures

To evaluate our cryogenic 3D printing method for the fabrication of soft tissue scaffold constructs and to demonstrate its precision, structures with complex geometries were printed. Therefore this method also demonstrates the viability to print these complex structures that would be difficult to fabricate by cast-moulding and can be used as cell scaffolds for future mechanobiological studies.

The hollow structures were printed based on a cylindrical pore scaffold microstructure, shown in Figure 74 (A), suggested by Hollister et al.[345] in order to have the porosity and stiffness required for cells to successfully thrive. A schematic of the 3D structure to be printed is shown in Figure 74 (B). These structures were achieved by introducing deionised water as a support material. At the beginning of the process, the CH ink was deposited using the same cryogenic setup described previously. After the completion of each layer, the supporting liquid water was deposited layer-by-layer using a needle and syringe guided by hand.

The liquid water changed phase to solid ice when it contacted the printing plate and frozen material from previous layers, thus providing a stable surface. This allowed subsequent layers of CH ink to be deposited on the supporting solid ice layer. Upon print completion, water was applied across the entire structure as an ice encasement to make the structure more stable and encourage even thawing. The structure was removed from the printing plate and allowed thawed to room temperature over 4.5 hours. The supporting ice melted away, leaving the hydrogel network intact.

6.2.7. Mechanical Characterisation

Unconfined, uniaxial compression tests, up to 30% strain, at 0.01 and 0.0001 s⁻¹ strain rates were carried out on both cast and printed cylindrical solid samples ($n=6$). A Mach-1™ mechanical testing system (Biomomentum, Canada) with a 1.5 N load cell (Honeywell, USA) was used to carry out the tests as it is designed towards soft material studies. A 100 Hz sampling rate was used and the data was filtered using a software integrated low-pass filter of order 2 and cut-off frequency 50 Hz. Samples were kept well hydrated prior to testing and immediately transferred onto the machine platen. Each test took 30 s, which is a sufficiently short time to confidently rule out dehydration effects on the samples. Stress-strain curves were plotted and compared for the cast and printed samples to show that the cryogenic technique produces a mechanically similar material compared with the cast-moulded technique.

As mentioned in section 6.2.5, cryogels, hydrogels that crosslink through the physical phase change process of a freeze-thaw cycle, which is what happens in the composite hydrogel, are affected by the thawing rate. To analyse this further and confirm the behaviours researched in literature, the effect of the thawing rate was also studied by conducted unconfined axial compression tests on samples thawed at different rates. The thawing rate was controlled by allowing the 3D printed frozen samples to thaw in two different environments, at room temperature and in a refrigerator at 4°C, corresponding to thawing rates of 2.1 and 0.3 °C/min respectively.

The compressive stiffness was characterised by the stress-strain curves calculated from the axial force and displacement data obtained from the experimental tests. The diameter and initial height of each sample was recorded in order to calculate the true stress and strain. Given the very large water content, incompressibility was assumed for the CH in the calculations; this is also conventionally used when modelling real brain, which the CH has been shown to match in compression up to 30% strain [17,60].

6.2.8. Analysis of Printed Microstructure Morphology

SEM analyses of various features of the printed material microstructure and surface morphology were conducted for further validation of the cryogenic printing method. The cross-section of the 3D printed structures were prepared for microscopy using a freeze-fracture method[60]. Since the hydrogel requires to be thawed in order to form its microstructural crosslinks, the CH samples for SEM analysis were subjected to one complete freeze-thaw cycle in addition to a subsequent freeze step in order to prepare the samples for the freeze-fracture method.

The cross-sectional surface was then gold-sputtered using the Auto Sputter Coater (Agar, UK) to allow for the conduction of the electron beam and to help preserve the microstructure. A scanning electron microscope (SEM) S-3400N (Hitachi, Japan) was utilised with a 15 kV electron beam at 50 pA in a variable pressure mode of 100 Pa at a working distance of 10 mm. The regions investigated were the internal homogenous microstructure, the bonding between consecutive layers and the sample surface with the intention of validating the cross-linking of the hydrogel throughout the entire printed material. For completeness, the impact of the freezing rate was also studied by analysing the crosslinks formed between different layers, as the freezing rate decreases with layer height.

6.3. Results

6.3.1. 3D Printing Parameter Optimisation

The accuracy of the cryogenic printer was first assessed to determine the optimal printing parameters. Figure 67 shows initial straight line geometry printing trials that investigate the successful build-up of layers. It can be seen that for a straight line, the surface of the second layer is still quite smooth, although it did not solidify immediately. The printing of more complex geometries in the following results showed more inaccuracies.

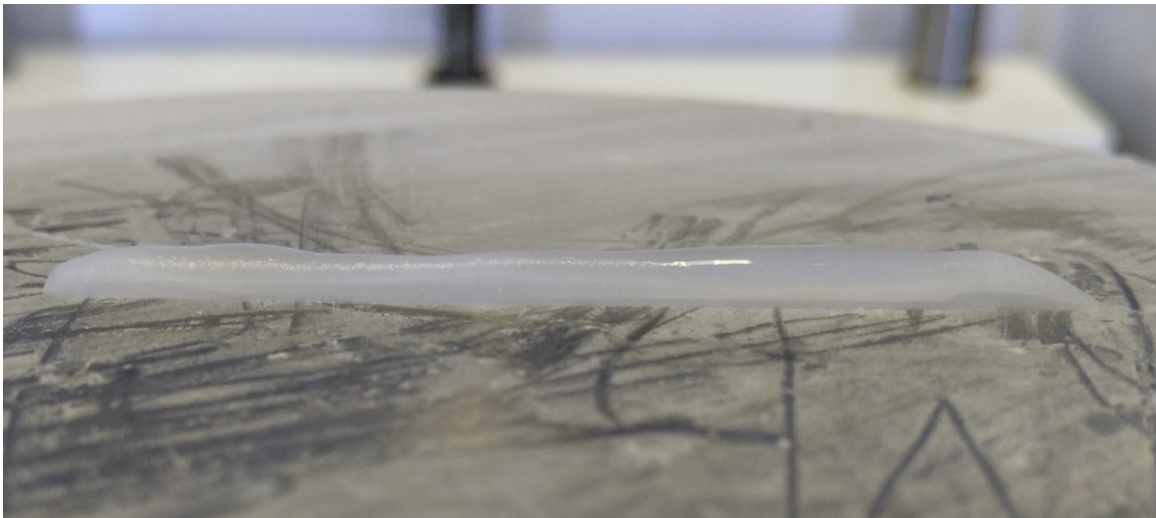


Figure 67: 3D printed line created using optimal printing parameters, 2 layers, showing relative smoothness of print for a simple geometry.

The finest resolution achieved by the cryogenic printing method was approximately 262 μm at 2 ml/h flow rate, 5 mm/s print speed, shown in Figure 73 (A). This resolution was comparable to values found in literature by Hinton and co-workers who report a resolution $\sim 200\ \mu\text{m}$ [124]. However, print settings are required to be optimised for different printed geometries. The settings that produced the most stable results for the 3D hollow structures produced in section 6.3.4 are shown in Table 17. The toolpaths for these structures were generated using a proprietary MATLAB script (Mathworks, USA).

Table 17. Structure dimensions and corresponding printer settings for complex hollow structures.

Unit Cell Dimensions (mm)	Ink Width (mm)	Print Speed (mm/s)	Print Flow Rate (ml/h)
5x5	0.5	5	6

6.3.2. Effect of Path Geometry on Print Quality

The results in Figure 68 (D)-(F) show that the geometry of the printed structure affects the quality of the print. The use of a constant printing speed and flow rate results in the build-up of material at certain regions of the geometry, such as the corners. The number of layers achieved before print failure due to the build-up of imperfections in the printed surface was decreasing in the following order: hexagonal honeycomb, orthogonal and auxetic as is shown in Figure 68 (G)-(I). In future, the MATLAB code should be further developed to increase the speed of the printing head when going round a corner as a function of the angle, faster speed for sharper angles, to achieve smoother layers.

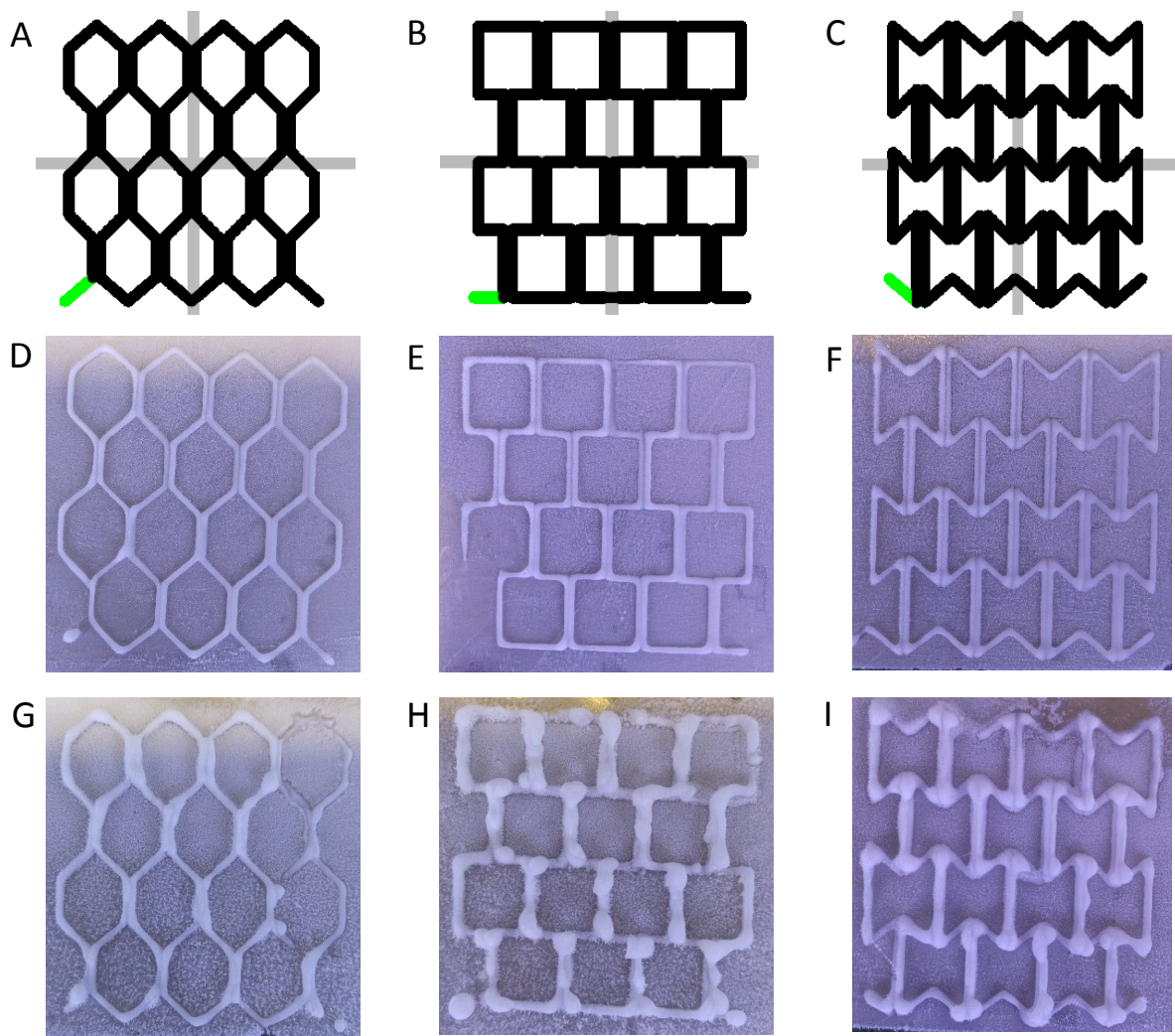


Figure 68: Gcode visualisation of complex printed structures (A) hexagonal, (B) orthogonal, (C) re-entrant auxetic; first layer prints of (D) hexagonal, (E) orthogonal and (F) re-entrant auxetic; 3 layer prints of (G) hexagonal, (H) orthogonal and (I) re-entrant auxetic structure. All scale bars are 5 mm.

6.3.3. Printed vs. Cast Mechanical Properties and Comparison with Brain Tissue

During the fabrication of solid cylindrical samples, it was observed that a printing ink width of 1 mm was more suitable to produce solid cylindrical samples. A comparison of the initial layer printed with an ink width of 1 mm compared to 0.5 mm is shown in Figure 69. The ink width is calculated from the print head speed and volumetric flow rate of the hydrogel ink, which were adjusted according to Table 18.

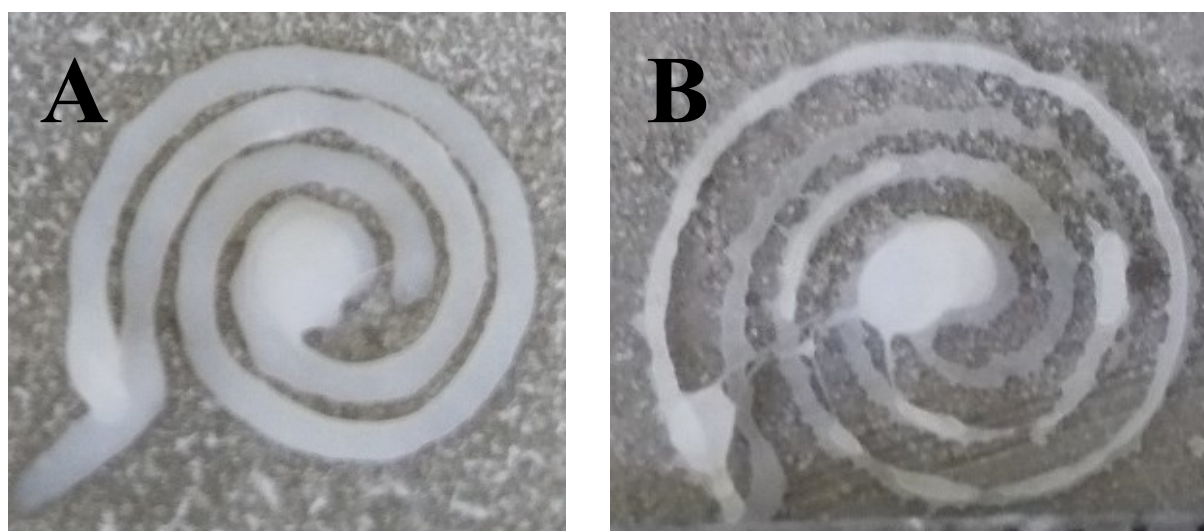


Figure 69: Evaluation of optimal printing width for 3D printed solid cylindrical structures being either (A) 1 mm compared to (B) 0.5 mm.

Table 18. Structural dimensions and corresponding printer settings for solid cylindrical samples.

Diameter (mm)	Height (mm)	Ink Width (mm)	Print Speed (mm/s)	Print Flow Rate (ml/h)
10	6.5	1	5	12

The 3D cylindrical samples produced were slightly conical in shape, as shown in Figure 70. This was likely because of a misalignment of layers since the layer height was custom modified for each layer as it was observed that needle displacement from previous layer was crucial in ensuring successful and even deposition. Also, since the geometry of the printing path was not completely solid, some material in the middle layers undoubtedly filled any gaps in the previous layer. Additionally, as the layer height increases, the freezing rate decreases, leading to a situation where the penultimate layer was not fully frozen before the final layer was applied. This resulted in an accumulation of material in the top layer which merged into one thicker and apparently bulging top layer.

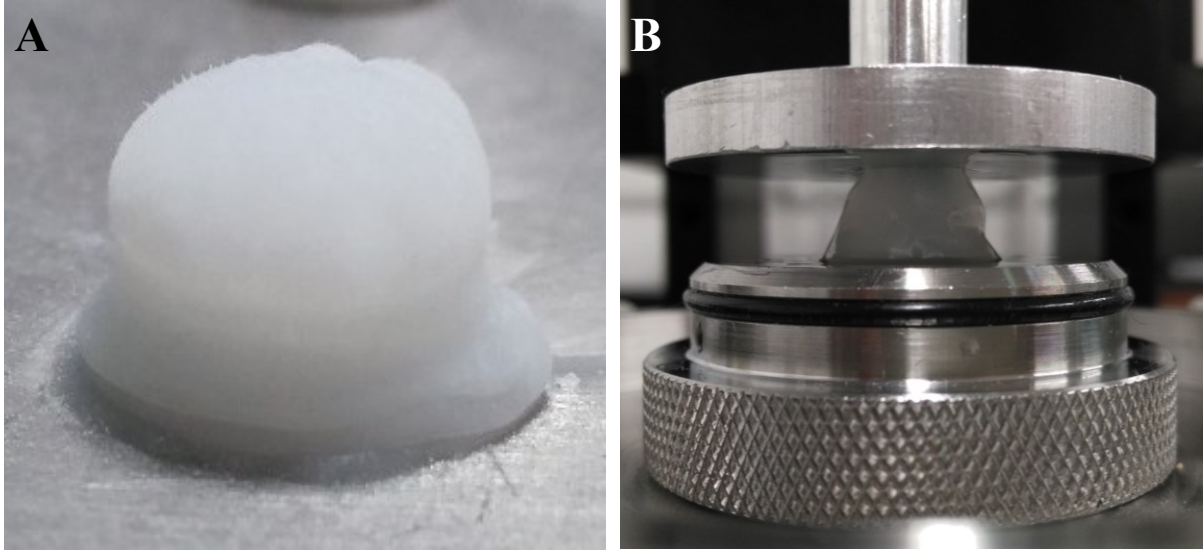


Figure 70: 3D printed cylindrical samples in (A) freshly printed frozen sample and (B) under unconfined uniaxial compression test.

The results from the compression tests of the printed samples compared to the cast samples are shown in Figure 71 (A). The stress-strain curves show a good agreement between the mechanical properties of the samples produced by the two different methods. The average true stress at 30% engineering strain at 0.01 and 0.0001 s⁻¹ strain rates for cast moulded and printed samples are summarised in Table 19. The average stiffness of the printed samples was greater than that of the cast samples for both strain rates; however, the findings are well within one standard deviation of each other. The viscoelastic nature of the composite hydrogel is also exhibited by the 3D printed method, as the stiffness is greater at higher strain rates, demonstrating strain rate dependency. In addition, a comparison between the mechanical response of the printed composite hydrogel and real brains tested in literature at the same strain rates is shown in Figure 71 (B).

The results for 0.01 s⁻¹ strain rate show that the printed CH is within the wide range of real brain results obtained from Forte et al.[346], Prevost et al.[210] and Vappou et al.[211]. For the 0.0001 s⁻¹ strain rate, the stress-strain curve has a wider error range than those previously reported by Forte et al.[346], but still well within the range of brain tissues results reported in the literature, and especially comparable to the results at 5% strain reported by Cheng and Bilston et al.[347].

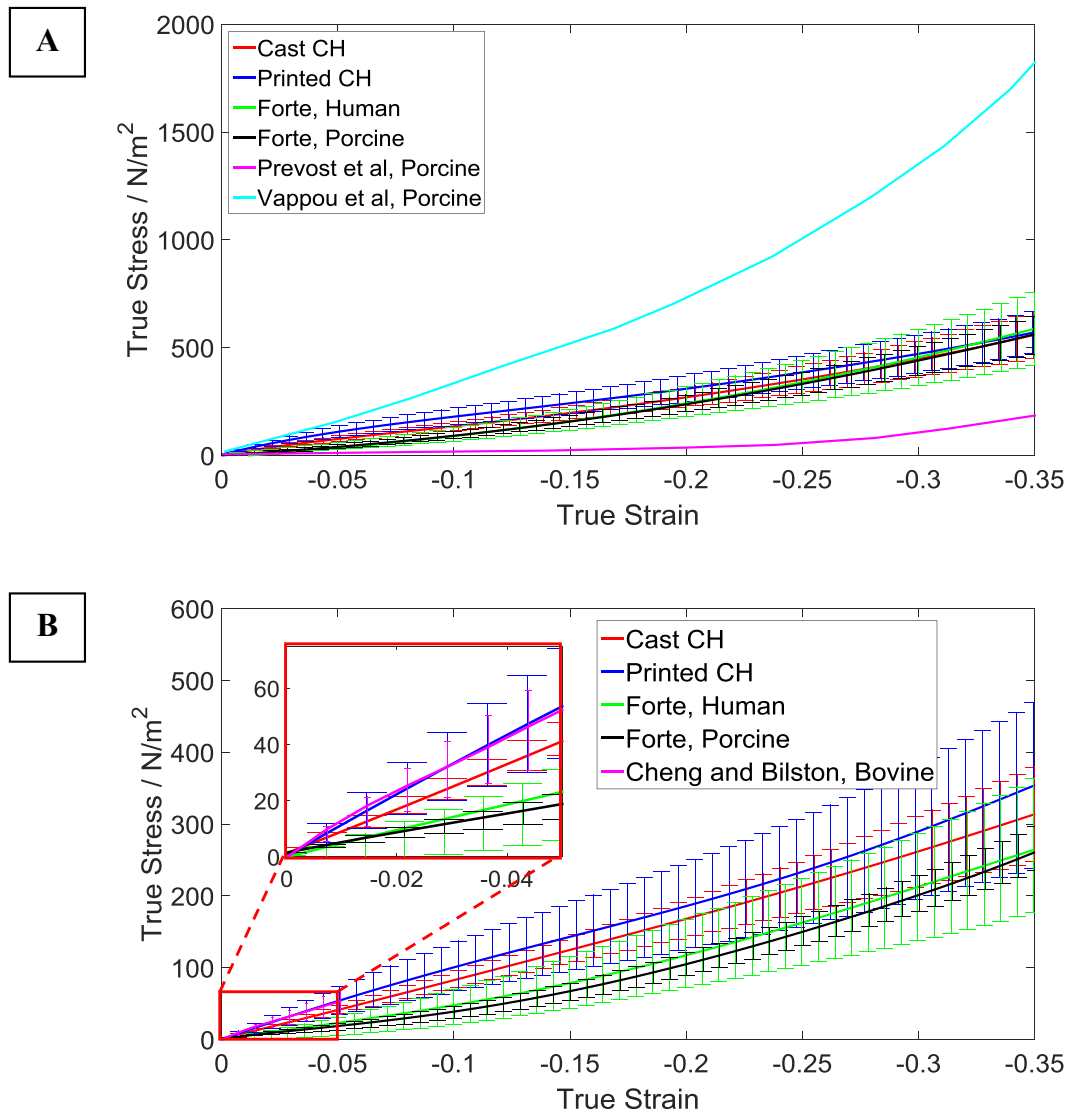


Figure 71: Stress-strain curves of printed and cast 5 wt% PVA 0.59 wt% Phytigel at (a) 0.01 s^{-1} and (b) 0.0001 s^{-1} strain rate [210,211,346,347].

Table 19. True stress at 30% engineering strain.

Fabrication Method	True Stress (Pa) $\pm$ Standard Deviation	
	0.01 s^{-1}	0.0001 s^{-1}
Cast Moulded	577.75 ± 111.12	321.34 ± 68.53
3D Cryogenic Printed	585.49 ± 97.33	363.10 ± 119.23

The effect of thawing rate on hydrogel stiffness is elucidated in Figure 72. For samples with a higher thaw rate of $2.1 \text{ }^{\circ}\text{C}/\text{min}$ compared to $0.3 \text{ }^{\circ}\text{C}/\text{min}$, the bonding between layers was weaker and layers were observed to separate from each other. In addition, the mechanical response of samples thawed at these two different rates was recorded and the result show that the higher thawing rate results in a significantly lower mechanical stiffness.

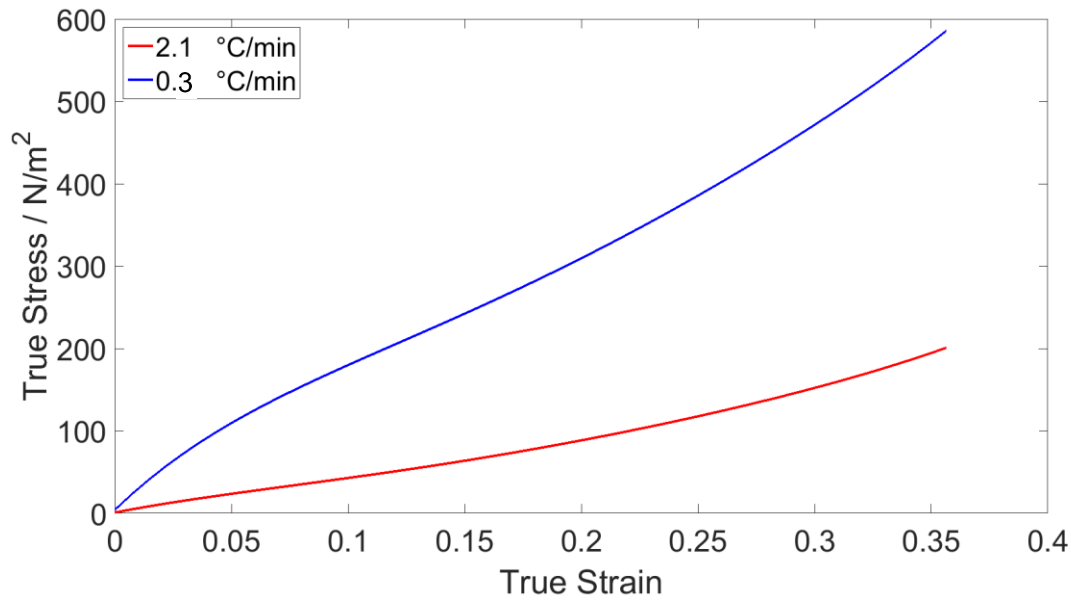


Figure 72: Mechanical response of 5 wt% PVA 0.59 wt% phytigel at 2 different thawing rates.

The temperatures were measured using an electronic thermometer with ± 0.1 °C precision. Time was measured to the nearest minute. Therefore, the precision of the thawing rates has been quoted to 1 decimal place. In reality, the outer surfaces of the samples will thaw first and the central volume will be the last to thaw. Therefore these thawing rates approximate average time taken to thaw the entire sample when exposed to a certain temperature condition (4 °C fridge vs. 21 °C room temperature).

6.3.4. Scanning Electron Microscopy Images

SEM on 6 samples that underwent one freeze-thaw cycle were conducted to reveal the morphology of the CH microstructure, as shown in Figure 73. The general microstructure of the material was homogeneous, with a pore size of approximately 10-20 μm , as shown in Figure 73 (B), which is consistent with what has been previously shown[60]. In addition to Figure 73 (C)-(D), the SEM images in Figure 73 (E)-(F) show that the pore size is consistent throughout all layers of the cryogenically printed sample, at around 20 μm . This suggests that the freezing rate, which decreases slightly as layer height increases, has little effect on pore size.

The scans also revealed the branching morphology of the 3D printed material microstructure. Figure 73 shows two different magnifications of the layer connection between layers 3 and 4 (scale bar of 1mm and 100 μm respectively). SEM analysis was also conducted on the connecting regions between consecutive printed layers and these are shown in the two sets of images in Figure 73 (C)-(D) and (E)-(F), where physical cross-linking across different layers is clearly demonstrated. Figure 73 (G)-(H) reveals bubbles of around 100 μm diameter trapped between layers due to imperfections in the surface of the previous layer.

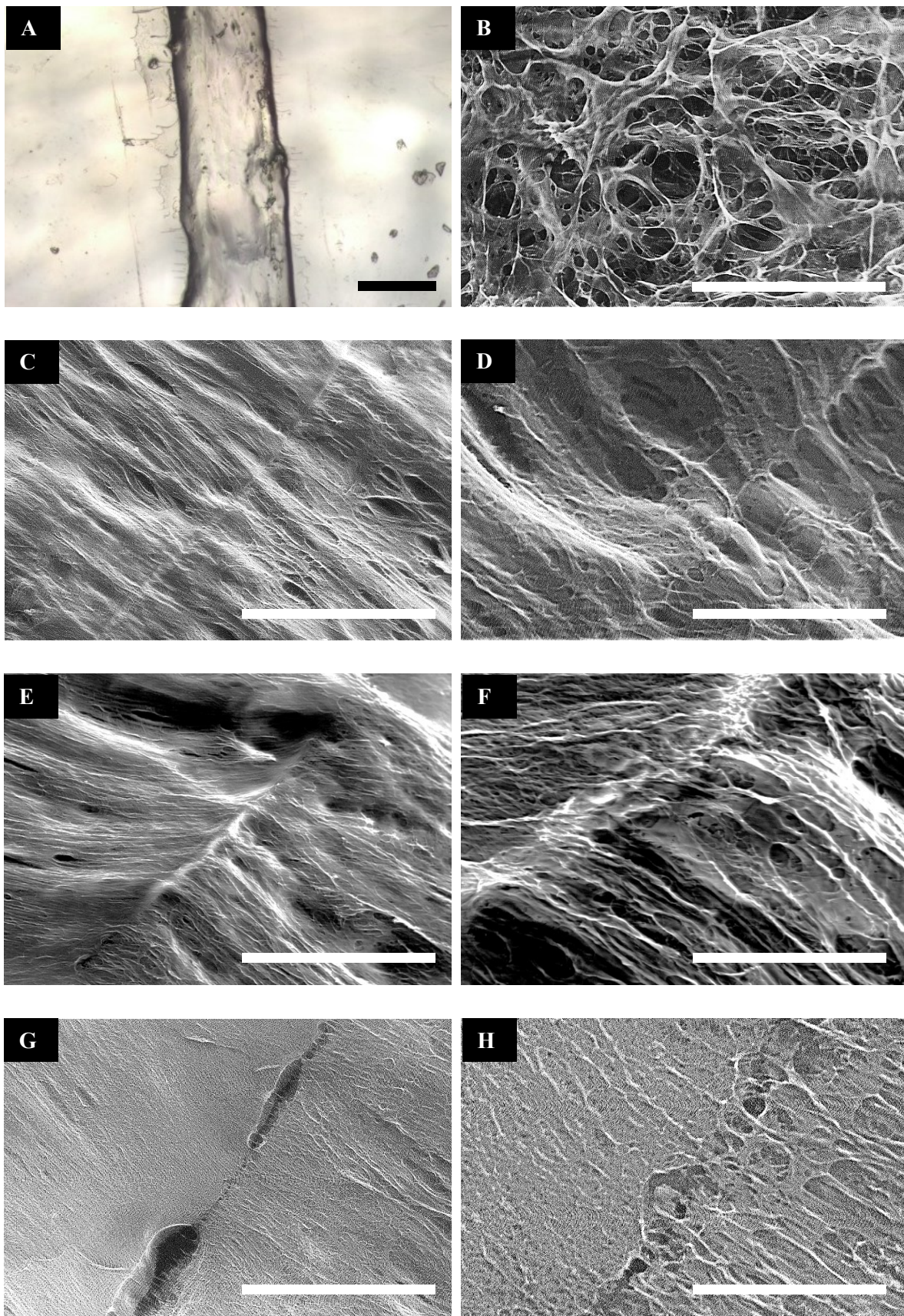


Figure 73: (A) white light interferometer image showing highest precision of printed material; SEM images of (B) general microstructure, (C)-(D) crosslinking between layers 1, left, and 2, right, (E)-(F) crosslinking between layers 3, left, and 4, right, at different magnifications, (G)-(H) flaws between layers. Scale bars, (A) 200 μm , (B) 100 μm , (C) 500 μm , (D) 100 μm , (E) 500 μm , (F) 100 μm , (G) 500 μm , (H) 100 μm .

6.3.5. Printed Complex Structures

Figure 74 (C) and (D) show the hollow printed structures. A structure of 10 mm height featuring two layers of cubic hollow geometry as shown by the schematic in Figure 74 (A) was successfully printed using 5wt% PVA 0.59wt% Phytigel composite hydrogel ink. Figure 74 (C) and (D) shows the printed structure in the frozen state and the thawed state respectively. Upon thaw, the voids in the centre of each unit cell are able to retain water and therefore, the structure remains hydrated for a longer period of time. The presence of frozen water trapped inside the central void of the cubic structure also structurally supports the hydrogel pillars. The pillars of the unit cells were 3 mm in diameter so the printed structure was still prone to collapsing under gravity once thawed. Nonetheless, the structure appeared uniform and homogenous throughout and did not exhibit any failure due to poor crosslinking. The distinction between liquid water and cross-linked hydrogel can be seen clearly in Figure 74 (C). The images revealed that the printing method was able to successfully produce the desired multi-layered complex structure with super soft bulk stiffness.

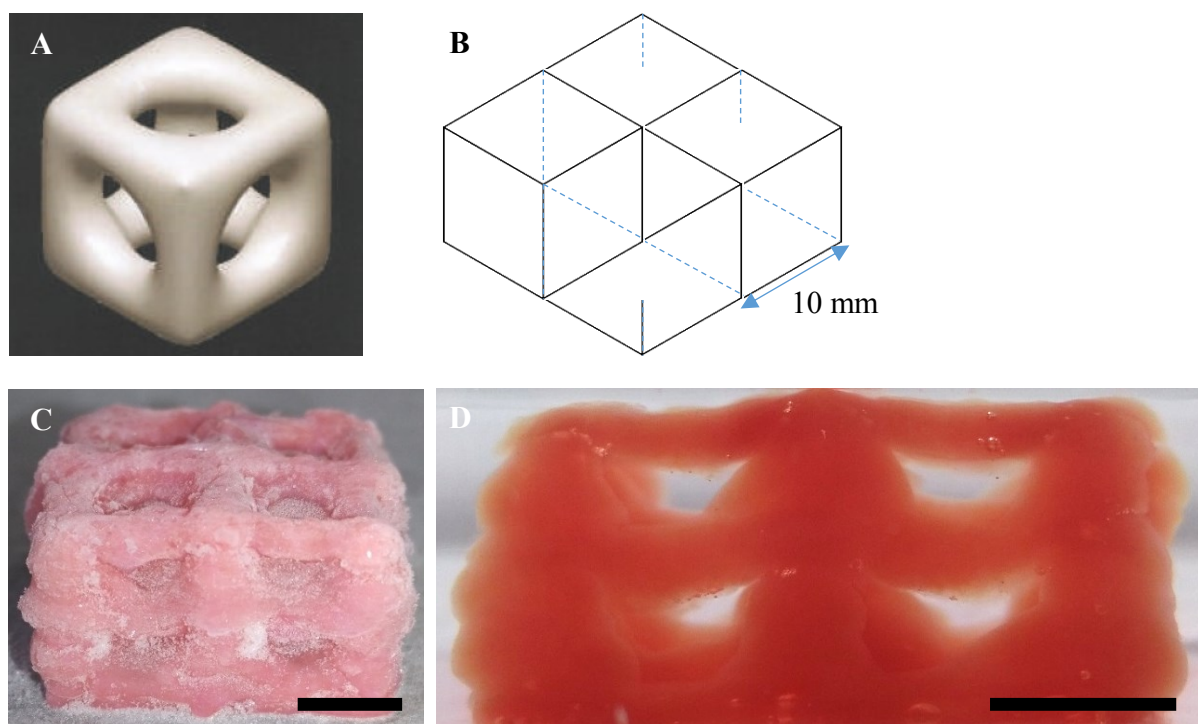


Figure 74: (A) Cylindrical pore microstructure, adapted from [345] and (B) 8 unit cells printed; thawed printed 8 cell structure in (C) isometric view and (D) side view. Scale bars, (C) 10 mm and (D) 5 mm.

6.4. Discussion

A composite hydrogel, of a composition that mimics soft tissues, was used as the ink material for cryogenic 3D printing. The use of dry ice, which is a safer alternative to liquid nitrogen, in an isopropanol bath to create the cryogenic stage was adequate at providing a sustained thermal sink, allowing the ink to freeze on contact with the stainless steel plate and preceding layers. The thermal energy required to freeze the deposited ink was negligible compared to the rate of energy removed by the dry ice, so new layers were not able to raise the temperature of the plate to the point of melting the material. Up to ~20 mm printing height, the samples were able to freeze upon contact with the prior layer. As the height increased, the time taken for a layer to freeze completely increased, which was taken into account when designing the code for the printer in terms of ink flow rate and speed.

The printing of the solid cylindrical samples were very repeatable and failed prints were not common, leading to a printing success rate of 95%. More repeats were executed to obtain a good print for the 2 layered 3D structure, due to the complex 3D geometry. This was mainly due to imperfections in the printing of each layer. The unevenness was then amplified in subsequent layers as the ink flow was caught on any protuberance in the preceding layer. When plastics are used as ink in commercial 3D printing, the printer is able to smooth down any protrusions in the previous layer to remove this issue. However, it was difficult to apply the same smoothing technique with the cryogenic technique presented in this article as the hydrogel froze immediately when deposited. Once the build-up of material at any point on the printed structure reached a critical level, the print failed, as investigated in Figure 68. In future, the print quality consistency can be improved by ensuring an extremely constant ink extrusion flow rate and limiting the inaccuracies caused by condensation through improved environmental control.

The 3D printed scaffolds demonstrated the concept of using a separate material to support the printed hydrogel to be able to create structures that would be extremely difficult to cast-mould. The support material was water, chosen for its similar freezing point to CH and biocompatibility. The water support did not affect the cross-linking of the hydrogel and, upon thaw, fully intact 3D structures were left once the supporting water had drained away. Owing to the nature of water, no harsh chemicals are required to wash the supporting material away. Therefore, this simple and biocompatible technique highlights the potential of this method to be used in the fabrication of very soft complex 3D scaffolds, which could be exploited for tissue engineering purposes.

The low viscosity of the supporting water resulted in a rapid spreading of the liquid before solidification. This reduced the precision of the deposited water and therefore, the use of other materials with tunable viscosity may improve the quality and should be investigated in future work. A candidate for a support material would be gelatine as it is biocompatible, has tuneable viscosity, inexpensive and removable when heated to a cell-appropriate temperature of 37 °C, as Hinton et al.[124] have shown in their work. An advancement of the cryogenic stage would be to build a setup that allows precise control of the temperature of the plate so that the material may be deposited at a temperature that allows the ice crystals to form slowly so that the nozzle is able to smoothen out the deposited layer before continuing to print further layers on top. Once this has been achieved, the temperature of the plate should be controlled so that only the previous layer is frozen, whilst leaving future deposited material semi-solid. Consequently, the temperature of the plate should be decreased as the printing height increases.

The ink used in this work is a composite hydrogel of poly(vinyl) alcohol (PVA) and Phytigel, which has been pioneered by Forte et al.[60] to mimic soft tissues with stiffness of O(1) kPa. This polymer blend is also able to reproduce the relaxation response of brain over other mimicking materials (such as agar gel, gelatine and polyacrylamide (PA))[63,107]. PVA hydrogel requires a freeze-thaw cycle to form physical hydrogen bonds that crosslink the material matrix. Therefore, this ink synergises with the cryogenic method and provides a simple solution that incorporates the crosslinking of the composite hydrogel in a single step.

Mechanical compression analysis of the stress-strain relationship between the solid samples of printed and cast 5 w% PVA and 0.59 wt% Phytigel showed good agreement, which indicates cryogenic printing is an effective method for the fabrication of super soft hydrogels. The average stress at 30% strain for the printed samples were greater than the cast molded samples by 1.3% and 13.0% at 0.01 and 0.0001 s⁻¹ strain rates, respectively. This result may be due to a double thawing process as the 3D printed samples experienced 1 minute of thawing as they were transported from the printing platform to the storage freezer. During this time, a small degree of crosslinking may have formed, which would contribute to the slight increase in stiffness of the 3D printed samples compared to the cast moulded samples. However, the compression results are within one standard deviation of the average stress.

Furthermore, the results for the stress-strain curves of the printed samples showed no evidence of fracture between layers, although shear tests should be performed in future to further validate this. The 3D printed constructs exhibited a larger standard deviation from the mean curve, which could indicate that the micro bubbles formed between layers caused by this method have some influence on the mechanical behaviour of the material. The findings demonstrate that the

printed material is very well matched to the work published by Leibinger et al.[63] and Andrikakou et al.[18] and hence well matched to very soft tissues, such as brain and lung. Furthermore, the printed material also matches the mechanical response of brain at two different strain rates, hence exhibiting viscoelastic behaviour. By using the cryogenic method, we are now able to 3D print super soft viscoelastic complex structures with a stiffness of $O(1)$ Pa to a precision of $O(100)$ μm , which is an unprecedented advancement in the capabilities of printing soft hydrogels. It is widely known that the histology of real soft tissues, in particular brain, is complex, which makes it difficult to test due to its sensitivity to environmental testing conditions and post-mortem time, among other factors. For both strain rates, the stiffness response of the 3D printed composite hydrogel is within the range of real brain results reported by other authors[210,211,346,347]. Furthermore, in comparison to real brain, the printed CH offers a greater repeatability and efficiency.

Another factor which may contribute to the slight increase in maximum stress at 30% strain for the 3D printed material compared to the cast moulded samples is the difference in freezing rate between two different methods. For the 3D printed samples, the freezing rate also decreases slightly as the layer height increases. However, it has been shown in literature that the freezing rate used when fabricating other PVA composite cryogels does not affect the mechanical properties of the hydrogel as greatly as the thawing rate, which is where the majority of the physical cross-linking bonds are formed [344,348–351]. Additionally, evidence that supports this is shown in Figure 73 (C)-(F) where a consistent average pore size across all layers is not greatly affected by the slight changes in freezing rate.

A further study on how the thawing rate affects the stiffness of the CH was shown in Figure 72. When 3D printing individual 10 mm diameter cylindrical samples, the surface area to volume ratio of a single sample is significantly greater than the samples obtained by biopsy punch from cast-moulding into a 40 mm diameter petri-dish. This suggests that the physical bonds between layers requires more time to form and strengthen than in a single layer. Moreover, given enough time, chains are able to form across the condensation between layers formed during the 3D printing process.

Structures of greater stiffness that can mimic other human soft tissues can also be achieved using the CH as ink. CH offers stiffness tunability by varying the composition of the constituent hydrogels. However, the viscosity of the ink increases when the concentration increases. The viscosity is a critical factor for this pneumatic extrusion based printing method. In future, additive silk particles, which have been used to greatly strengthen PVA without hugely affecting the solution viscosity, should be explored to create stiffer structures.

From the SEM images, the homogenous microstructure of the 3D printed solid structures is well matched with the scans obtained by Forte et al. of cast-moulded CH in terms of pore size and morphology. This indicates that physical cross-linking in the composite material does not occur during the freezing stage since the layers undergo the phase change at different times but instead crosslink during the thawing stage, which is consistent with our knowledge of cryogel physical bonding kinetics.

Additionally, images taken of the region across consecutive layers exhibit a clear crosslinking hydrogel network with similar porosity and morphology to the rest of the microstructure at 100 μm scale. This suggests that the hydrogel is able to bond across layers even though the fault line is clearly distinguishable at a wider view of 500 μm . Due to minor inaccuracies in the deposition of the hydrogel ink, micro bubbles of around 100 μm in size are formed, which have been captured in the SEM images. The size of these defects compared to a layer height of 1 mm indicate that the bubbles should not affect the response of the structure on the macroscopic scale, and indeed, the stress-strain curves from the compression tests show that these faults have little effect on the value of stress obtained at 30% strain compared to cast-moulded samples. This, however, might affect the resistance to damage of the printed material, which will be investigated in future studies. Interestingly, the hydrogel network exhibits diffusive behaviour in an apparent effort to mend those tears by crosslinking across the gap. Where the voids are small enough, the hydrogel can be seen to be able to cross-link and branch across the void Figure 73 (H). This is a very interesting observation as it suggests that the hydrogel is able to diffuse through the ice as it thaws whilst forming the network structure. This may suggest a self-healing mechanism as it allows the material to amend the errors created by an inaccurate print to some degree.

In summary, a printing technique focusing on producing soft structures using a composite hydrogel as ink has been developed. This has largely been unachievable thus far as applying a traditional layer-by-layer approach of additive manufacturing to such a soft hydrogel would result in the unstable collapse of the structure from its own weight. The development of this 3D printing method stabilises each printed layer immediately by utilising the phase change of the hydrogel solution to a solid frozen structure via cryogenic processes. Furthermore, the synergy of this technique with a composite hydrogel printing ink that requires a freeze-thaw cycle to crosslink is an intelligent and time-effective solution. The addition of a water-ice support material has allowed complex hollow structures to be built so that the resulting 3D scaffold can be used to investigate a wide range of tissue engineering studies in the future, owing to the biocompatibility of the composite hydrogel which has been shown in the previous chapter.

7. Conclusions

7.1. Summary and Conclusions

This thesis has aimed to improve the tissue mimicking abilities of hydrogels by providing an in depth material characterisation of a range of mechanical properties in comparison to real biological tissues. Firstly, the mechanical properties of three different biological tissues were characterised. The results from the biological tests of porcine brain, lung and liver, showed a good agreement with literature values where available. Furthermore, the effect of the internal structures on the directionality of the tissues at the macroscale was investigated. The stress-strain curve obtained at 0.01 s^{-1} strain rate was characterised using a first order Mooney-Rivlin hyperelastic model. The relaxation response was characterised using a 3rd order Prony series model. The characterisation of the biological tissues provided the ground truth for the determination of a mechanically accurate tissue mimicking material. This material would need to be able to mimic the independent mechanical characteristics already established for biological tissues.

A composite hydrogel formulation constituting of PVA and phytagel was chosen to be investigated as the blend of two different hydrogels provided a greater degree of flexibility and tunability over its mechanical properties. In order to achieve this, material characterisation of a range of properties was firstly carried out. The mechanical behaviours of biological soft tissues depend greatly on environmental conditions, such as strain rate, temperature, hydration and post-mortem time[8,60,228,347]. In order to determine the most accurate tissue mimicking materials that match three different mechanical properties of three different biological soft tissues, the hydrogel specimens were tested using the exact same testing conditions as the biological tissue samples.

3D plots mapping each mechanical characteristic with respect to the PVA and PHY concentration were created from the systematic testing of a range of different hydrogel compositions mentioned above. The behaviour of the hydrogel at high strain rates was also mapped in order to suggest a tissue mimicking composition that can be used as a tissue phantom for destructive impact tests that are unethical to perform *in vivo*. The 3D maps were used to reveal a selection of matching compositions. Where multiple characteristics were required to be mimicked simultaneously, the best tissue mimicking composition was determined, produced and evaluated by direct comparison with the biological tissue results.

One of the main areas of improvement for the tissue mimicking ability of the composite hydrogel is its homogeneity, compared to the anisotropy of biological tissues evidenced at the beginning of this work in reference to their internal structures. To tackle this, the final aim of this thesis was to develop a novel fabrication method that can create hydrogel samples with improved geometrical and structural mimicking abilities for the application of mechanically accurate 3D tissue engineering scaffolds.

The main contributions of this thesis are structured by topic.

Biological Tissues

The brain and lung shared a similar stiffness of around 500 Pa, whereas the stiffness of the liver was an order of magnitude higher at around 10 kPa. Combining this with the results from the insertion tests, the brain was found to be soft and easily punctured as the insertion forces are very low. Comparatively, the liver was much stiffer and had much higher insertion forces. Yet the results from the lung indicated that although it was as soft as brain, it experienced insertion forces that were closer to liver, suggesting that these mechanical properties are independent of each other, posing a greater challenge to tissue mimicking materials.

The brain does not show any mechanical anisotropy at the sample size tested in this work. Conversely, the compression testing results for lung and liver showed a significant difference between samples taken in axial and transverse directions relative to their respective internal structures, signifying a clear mechanical inhomogeneity. The lung and liver needle insertion results suggest a similar trend but are less conclusive as large insertion force peaks are observed in both axial and transverse directions. It is therefore suggested that the factor resulting the insertion force peaks is the encounter between the needle and any internal structure regardless of its position relative to the main bronchi or hepatic vein. Hence, a large insertion peak is likely due to the needle penetrating smaller internal structures that branch out perpendicularly from the main structure.

The stress-strain response of brain and liver were found to be more non-linear than lung, which was almost linear elastic. The hyperelastic parameters for brain and lung model their biological counterparts well and can be readily used to define computational models. The relaxation response of the liver was shown to be the greatest, followed by brain and lastly, lung. It is suggested that this is may be due to the impermeable connective tissue that encapsulates each lobule of the liver microstructure, which creates a larger degree of fluid pressurisation than exhibited by the other tissues tested in this work.

Composite Hydrogel

The systematic testing of PVA and PHY revealed the effect of each constituent hydrogel on the overall material behaviour.

- Increasing PVA and PHY resulted in a higher value of stress at 30% strain, with PVA having more influence on the increase of insertion and friction forces.
- PHY was found to decrease the composite hydrogel's ductility and increase its hyperelasticity and viscoelasticity,
- Low concentrations of PVA also resulted in a higher viscoelastic response.

The hyperelastic and viscoelastic parameters reported in this work can be used readily to define computational models as the materials models were well matched to the hydrogel test results.

Soft Tissue Phantom

Firstly, tissue mimicking compositions for surgical applications were evaluated. A comparison between the real tissue and CH compositions show a good match in terms of stiffness and insertion forces. In particular, the CH_Brain and CH_Lung show a very similar insertion force profile to the biological tissue as they are able to replicate the multiple fractures that occur during the entire insertion period. Although the CH_Liver is not able to show this characteristic as well, the stiffness, average insertion force and friction force are well matched, resulting in CH_Liver being the most accurate liver mimicking artificial material to date.

The use of the CH_Brain as a CED phantom has elucidated the effect of therapeutic solution viscosity on its diffusive behaviour through the hydrogel microstructure. This is a major step forward in the area of biomaterials towards the use of artificial tissue phantoms for surgical planning and training. This material offers surgeons an accurate tactile experience with invaluable and unrestricted opportunities to practice operations due to the ease of procurement, as there are no ethical issues, with the hopes of increasing surgery success rate.

Furthermore, this work also suggests a composition that is able to mimic the mechanical behaviour of brain at impact rates related to traumatic injury. This opens up the application of the material to be used as a traumatic brain injury phantom to elucidate how the impact wave loading patterns cause primary and secondary brain damage. Coupled with the cell viability results that show excellent biocompatibility, the tissue phantom can be used to explore an ever increasing range of biomedical and tissue engineering applications.

3D Printing

A 3D printing technique focused on producing brain mimicking tissue scaffolds using a composite hydrogel as ink was developed. First of all, the efforts were focused on fabricating complex structures for a wide range of tissue engineering and mechanobiological applications that are extremely difficult to achieve using a cast-moulding method because of their super soft mechanical characteristics and hollow geometries. This was accomplished by incorporating a cryogenic procedure. The use of a freeze-plate allowed the CH ink to instantly solidify upon contact with the plate. Each layer was built on previous solid layers and a stable 3D structure was created. This technique integrates the freeze-thaw process required for the formation of physical hydrogen bonds between the –OH groups of the PVA and phytagel and is therefore an elegant and time-reducing method, owing to the instantaneous freezing step. The crucial factor in achieving successful prints is to ensure the evenness of each printed layer, which should be a key area for development in the future.

Upon thaw, the material properties of the CH were very closely matched to that of the traditional cast-mould fabrication method, and well within the standard deviation of the samples tested. Moreover, SEM scans revealed that the microstructure of the printed CH was well matched to previous findings of cast-moulded CH in terms of pore size and morphology, further validating this method. The physical cross-linked network was exhibited across different printed layers and formation of bonds across boundary defects, such as voids, suggests a self-healing mechanism during the thawing stage.

The use of the CH as an ink in cryogenic 3D printing opens the doors to many applications that have yet to be explored due to the inability to fabricate super soft materials that mimic the stiffness of brain, lung and other human tissues characterised by a complex geometrical structure. This work has shown that the cryogenic technique used for the fabrication by 3D printing of the CH provides the capability to manufacture synthetic materials of complex shape and good mechanical properties and microstructure.

7.2. Future Work

This work could be improved by undertaking the following suggestions, sorted by topic:

Biological Tissue Characterisation

Although brain anisotropy was not elucidated in this work, future work involving mechanical testing of white and grey matter on the microscale will be able to quantify any difference between the axons and the supporting matrix, and hence be able to evaluate directionality and anisotropy in the tissue that may affect CED drug diffusion.

To improve the mechanical characterisation of liver, higher order models should be applied to simulate the stress-stretch curve of liver as the first order model is not sufficiently non-linear. Different models could also be explored, involving the exploration of a model based on the geometrical structure of its lobular microanatomy. The work on biological tissue characterisation could be extended in future by investigating the strain rate dependency of these different tissues so that they can be mimicked for applications occurring at higher strain rates, for example traumatic blast injury. Furthermore, the effect of the cylindrical sample's shape variation on its measured stress-strain behaviour should be analysed through FE modelling.

Hydrogel Development

Whilst this work focused on mimicking the biological tissues on the softer side of the spectrum, the CH has the potential to mimic stiffer tissues, namely cartilage. The testing matrix should be expanded to include higher concentrations of PVA and PHY formulation that mimic cartilage and further studies surrounding the frictional interactions between CH and common joint replacement materials to evaluate its potential for cartilage replacement and regeneration.

The accuracy of the CH_Brain could be improved by functionalising the hydrogel chains. Coupled with an injected solution that reacts with the functionalised groups on the material, the chemical interactions present in the real scenario could be mimicked. The biological accuracy could also be improved by seeding tumour cells inside the hydrogel and subjecting them to the local perfusion of anticancer drugs in a 3D *in vitro* environment. In fact, a tissue phantom where individual diffusive parameters can be investigated by controlling other parameters, will actually enable a deeper understanding of the effect of each property on the overall diffusive behaviour. Therefore, this tissue phantom offers great potential as a controllable drug diffusion model.

3D Bioprinting

The next step will be to add another extrusion head onto the 3D printer to allow two different hydrogel compositions to be printed interchangeably. This would allow samples with induced directionality to be created and the inclusion of complex internal structures could be 3D printed.

To create larger 3D structures with the future aspiration of 3D printing entire organs, the layer height must be kept even. This could be achieved by:

- Reducing condensation by sealing the printing chamber and pumping through dry inert gas.
- Finer control of printing ink flow rate by using a higher precision stepper motor developing the moter control code to include flow reversal.
- Insulating the chamber to increase overall print height.

Cell Studies

Cells are known to react to the mechanical cues of the substrate they are seeded on, including whether the environment is 2 or 3D. The following fascinating studies will be carried out in future work:

- The consequences and manifestation of primary and secondary damage to neuronal cells seeded on the CH composition that mimics brain at traumatic injury loads.
- The damage and resulting mechanisms that occur when cells seeded inside the CH_Brain phantom are strained and destroyed during the surgical needle insertion process.
- The ability of mechanical cues to guide the proliferation, migration and differentiation of stem cells towards a specific lineage.
- The efficacy of anti-tumour drugs on cancer cells in a mechanically accurate 3D culture.

Overall, the excellent biocompatibility of the CH with a collagen coating will allow the hydrogel structures to be used in various mechanobiology and, potentially, regenerative medicine studies of soft tissue-like CH substrates, including the exploration of mechanical cues on cell behaviour. In addition, the CH may also be used as a mechanically accurate tissue phantom for surgical training and also for more in-depth and destructive tests that are unethical to perform in vivo, such as traumatic brain injury experiments.

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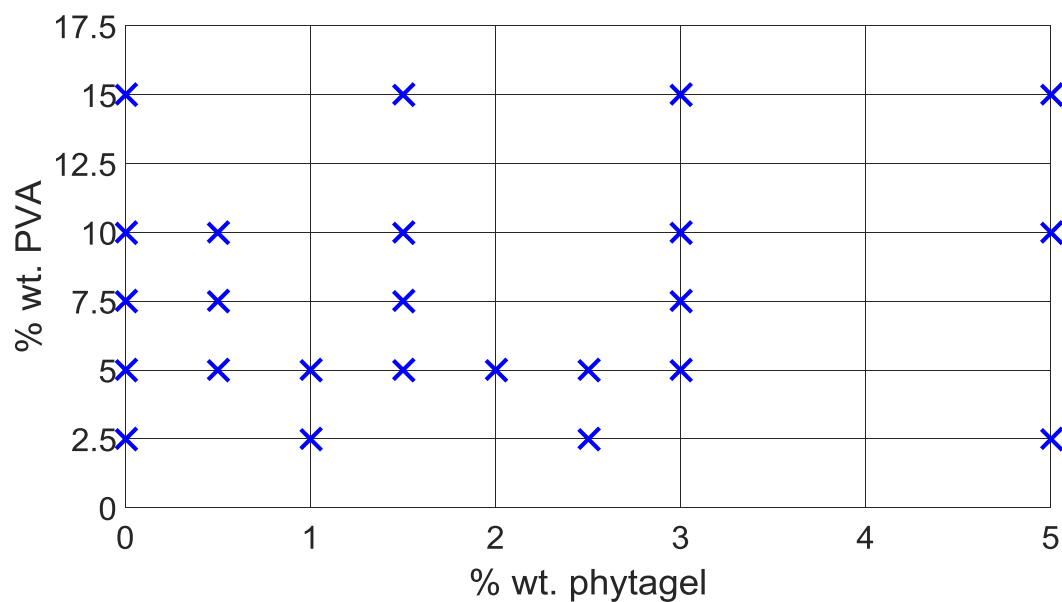
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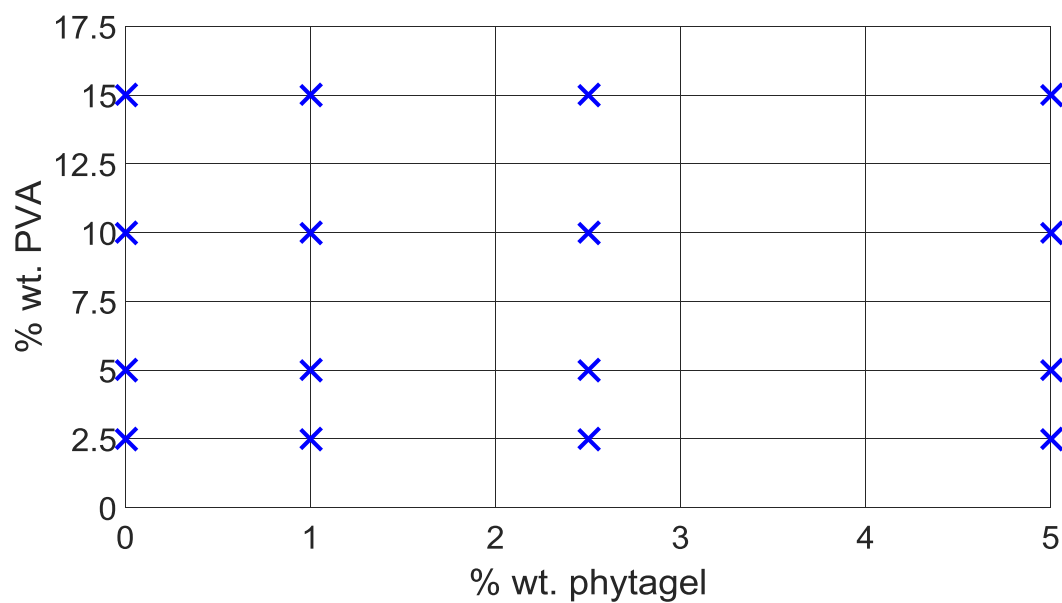
Appendices

Appendix A

[A]



[B]



Individual hydrogel composition testing matrix for (A) compression-relaxation tests and (B) needle insertion and high strain rate tensile tests.

Appendix B

Stress at 30% strain hydrogel results.

PVA wt%	PHY wt%	1 s^{-1}	0.0001 s^{-1}	PVA wt%	PHY wt%	0.01 s^{-1}	SD
5	0	111.5704	30.9171	2.5	1	242.9	694
5	0.5	253.2695	125.6686	2.5	2.5	4381.2	12518
5	1	574.8572	156.9428	2.5	5	24566	70189
5	1.5	1669	450.5378	5	0	86.0251	246
5	2	5519.4	1134.7	5	1.5	714.84	2042
5	2.5	6127.4	1848.2	5	3	6764.3	19327
5	3	7729.8	4846.8	7.5	0	214.2198	612
7.5	0	263.3549	149.8718	7.5	1.5	1075.9	3074
7.5	0.5	453.4953	160.3479	7.5	3	8516.1	24332
7.5	1.5	2871.3	717.1454	10	0	475.2296	1358
7.5	3	10936	6288.6	10	1.5	2976.2	8503
10	0	503.7167	427.1437	10	3	14000	40000
10	0.5	635.5528	433.2876	10	5	27610	78886
10	1.5	3770.3	2359.3	15	0	1418.3	4052
10	3	16480	10341	15	1.5	8557.4	24450
10	5	35423	17997	15	3	28081	80231
15	0	1597.7	1160.7	15	5	81415.1	232615
15	1.5	9108.3	6057.3				
15	3	34451	21430				
15	5	92521	64628				

Logarithmic equations describing strain rate dependency of hydrogels over 0.0001 , 0.01 and 1 s^{-1} strain rates.

● 5% PVA 0% PHY	$y = 8.7568 \ln(x) + 116.5$ $R^2 = 0.9571$
● 5% PVA 1.5% PHY	$y = 132.29 \ln(x) + 1554$ $R^2 = 0.9035$
● 5% PVA 3% PHY	$y = 313.02 \ln(x) + 7888.5$ $R^2 = 0.9649$

● 5% PVA 1.5% PHY	$y = 132.29 \ln(x) + 1554$ $R^2 = 0.9035$
● 10% PVA 1.5% PHY	$y = 153.2 \ln(x) + 3740.8$ $R^2 = 0.9948$
● 15% PVA 1.5% PHY	$y = 331.26 \ln(x) + 9550$ $R^2 = 0.799$

First order Mooney-Rivlin hyperelastic fitting parameters for hydrogel.

PVA %wt	PHY %wt	C1	C2	R ²
2.5	1	-44.55	-57.99	0.9982
2.5	2.5	3378	-4037	0.9988
2.5	5	2.13E+04	-2.43E+04	0.9998
5	0	-12.59	-22.44	0.9997
5	0.5	38.13	-110.1	0.9997
5	1	87.43	-162.8	0.9977
5	1.5	171.6	-376.1	0.9963
5	2	950	-1480	0.9971
5	2.5	1569	-2349	0.9974
5	3	5249	-6088	0.9962
7.5	0	-28.66	-58.83	0.9999
7.5	0.5	-12.89	-79.1	0.9993
7.5	1.5	175.9	-509.5	0.9971
7.5	3	6230	-7380	0.9961
10	0	-109.1	-97.55	0.9994
10	0.5	-48.89	-150.2	0.9993
10	1.5	2070	-2517	0.9994
10	3	12340	-13570	0.996
10	5	30950	-31760	0.9993
15	0	-42.46	-501.1	0.9999
15	1.5	4835	-6501	0.9986
15	3	22620	-26090	0.9986
15	5	77460	-85710	0.9986

3rd order Prony series viscoelastic fitting parameters for hydrogel.

1 s-1 strain rate			% wt. PVA											
			5.0			7.5			10.0			15.0		
		Properties	1	2	3	1	2	3	1	2	3	1	2	3
% wt. PHY	0.0	τ	0.68	11	168	0.60	6	194	0.67	7	184	0.6	3	217
		k	42	30	114	69	48	139	96	77	123	230	121	254
		η	28	345	19087	41	269	26936	64	548	22592	138	362	55240
	0.5	τ	0.58	9	176	0.48	7	174	0.43	7	198			
		k	108	74	228	220	105	136	207	110	114			
		η	63	678	40218	106	689	23622	89	737	22480			
	1.0	τ	0.38	5	142									
		k	180	137	233									
		η	68	751	33025									
	1.5	τ	0.28	11	188	0.42	11	182	0.35	11	177	0.35	9	186
		k	843	479	570	1334	620	894	1650	762	1095	3308	1415	2646
		η	236	5269	107160	564	6788	162512	578	8233	193641	1158	12577	491489
	2.0	τ	0.26	11	172									
		k	3700	1750	2052									
		η	962	18375	353864									
	2.5	τ	0.22	10	182									
		k	3290	1606	2747									
		η	724	16134	498805									
	3.0	τ	0.21	9	180	0.22	8	181	0.35	9	182	0.29	9	183
		k	3830	2600	3504	4200	2900	4653	7594	3516	6786	18661	7288	14783
		η	804	23400	630927	924	22040	842456	2658	32861	1234033	5412	65252	2706080
	5.0	τ							0.34	9	181	0.28	10	181
		k							18387	9718	20881	52942	24833	54433
		η							6252	89383	3775785	14824	236059	9829378

Relaxation Ratio

RELAXATION RATIO			% wt. PVA											
			5.0			7.5			10.0			15.0		
COMPRESSION SPEED (mm/s):			8.3	0.083	0.00083	8.3	0.083	0.00083	8.3	0.083	0.00083	8.3	0.083	0.00083
% wt. PHY	0.0		2.5803	2.8633	1.4451	1.6175	1.2101	1.1449	1.3104	1.1676	1.0266	1.1579	1.0866	1.0395
	0.5		2.3545	1.4710	1.2735	1.5643	1.2612	1.0598	1.3369	1.1682	1.0776			
	1.0		1.6335	1.6458	1.2107									
	1.5		1.6690	1.6410	1.2284	1.5428	1.2351	1.0657	1.4819	1.2812	1.0861	1.4020	1.2446	1.0984
	2.0		1.9547	1.4864	1.1620									
	2.5		1.8305	1.4784	1.1369									
	3.0		1.8875	1.5320	1.1961	1.6419	1.4319	1.1552	1.6249	1.4017	1.1601	1.7202	1.4766	1.2263
	5.0								1.9514	1.5850	1.2315	2.0274	1.7094	1.3265

Appendix C

PVA wt%	PHY wt%	Average Insertion Force /N	SD	Fracture Depth / mm	Friction Force / N	Friction Coeff
2.5	1	0.0752	0.0106	22.7	0.0709	0.162614679
2.5	2.5	0.1281	0.0156	20.9	0.0938	0.025439408
2.5	5	0.3422	0.0174	18.9	0.2277	0.01460739
5	0	0.1573	0.021	36.4	0.3128	0.757042254
5	1	0.2761	0.0538	27.3	0.3093	0.416184971
5	2.5	0.2442	0.0349	20.0	0.2442	0.04252462
5	5	0.3605	0.1163	17.3	0.2938	0.056283542
10	0	0.5409	0.0778	32.4	0.1135	0.1347981
10	1	0.6366	0.0807	26.2	0.3492	0.208602151
10	2.5	0.8402	0.1414	18.5	0.7125	0.086975098
10	5	1.2666	0.2258	16.7	0.8022	0.060370259
15	0	1.7522	0.2469	30.0	0.6944	0.335329341
15	1	1.8165	0.0893	24.7	0.7543	0.112716677
15	2.5	2.7223	0.1637	18.0	1.4048	0.074296594