

Smart Biomaterials: Embedding Microbubbles into Folded Protein Hydrogels



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Declaration

The candidate confirms that the work submitted is their own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

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Abstract

Hierarchical structures in nature demonstrate a diverse range of functions, that if replicated could be utilised in materials for biomedical applications. Designing biomaterials from the ‘bottom-up’ approach allows for a full understanding of complex materials, with several levels of hierarchy. Proteins are naturally functional and can be mechanically resistant, providing interesting properties to biomaterials where proteins are the building blocks. Taking inspiration from nature, the project reported here utilised folded proteins creating folded protein hydrogels. The focus of this study was to focus on using folded protein hydrogels in biomedical applications. Control over properties such as the hydrogel porosity, stiffness and diffusion of molecules in hydrogels can be challenging, however they are important for the success in a biomedical setting. Ultrasound responsive microbubbles are clinically used as ultrasound contrast agents, with recent focus on utilising them for triggered drug release. Microbubbles can be loaded with drugs, and burst with ultrasound, releasing the loaded drugs into the surrounding environment. This composite system aimed to address challenges in creating a complex drug release profile and porosity of folded bovine serum albumin (BSA) protein hydrogels, phospholipid microbubbles were embedded into the BSA hydrogels (BSA:microbubble hydrogels).

To explore the potential of BSA and BSA:microbubble hydrogels in different biomedical applications, this thesis explored produced BSA microgels with microfluidics to produce BSA microgels of 100 μm in diameter. Cell viability studies were conducted on BSA hydrogels with pancreatic ductal adenocarcinoma (PDAC) cultures, finding that rinsing the components that did not cross-link in the BSA hydrogel were required for maintaining cell viability. Finally, the lifetime of microbubbles embedded in the BSA:microbubble hydrogels was investigated. The lifetime of the microbubbles was longer for the microbubbles in the BSA:microbubble hydrogels compared to microbubbles stored in a solution of buffer.

A cross-length scale study was demonstrated in this thesis, aiming to connect nanoscale protein stability with circular dichroism, to the mesoscale protein network with small-angle neutron scattering (SANS) and the bulk mechanical properties of the protein

hydrogel with rheology. The impact of the presence of microbubbles in folded protein hydrogels across each length scale provided a wealth of information across the composite system. Rheological characterisation showed that the stiffness of BSA:microbubble hydrogels was reduced at higher concentrations of microbubbles (10^9 MB/mL). Investigations with SANS showed that the presence of the microbubbles in the BSA hydrogels induced a decrease in the number of protein clusters in the hydrogel network. Reducing agent dithiothreitol (DTT) induces the unfolding of BSA molecules by breaking down the disulphide bonds. A reduction in stiffness of the bulk BSA hydrogel was also observed with the addition of DTT post-formation of the BSA hydrogel network. SANS studies showed that the number of protein clusters was reduced by the addition of DTT. Adding DTT to BSA:microbubble hydrogels resulted in a further reduction of number of protein clusters in the hydrogel network. This level of detail in understanding BSA:microbubble hydrogels could provide the ability to tune the BSA hydrogel properties for biomedical applications and predict the behaviour of new folded proteins.

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Publications

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List of abbreviations

Experimental Technique

MB	Microbubbles
US	Ultrasound
AFM	Atomic Force Microscopy
SFMS	Single Molecule Force Spectroscopy
MI	Mechanical Index
BSA:MB	Bovine Serum Albumin hydrogel with microbubbles
UV	Ultraviolet
CD	Circular dichroism
SAXS	Small-angle X-ray scattering
SANS	Small-angle neutron scattering

Model/Properties

η	viscosity
G	Shear modulus
G*	Complex shear modulus
G'	Storage modulus
G''	Loss modulus
F	Shear force
λ	wavelength of radiation

mg	milligram
g	gram
kg	kilogram
mL	millilitre
μ l	microlitre
l	litre
M	Molar
MW	Molecular Weight
kPa	kilopascal
Pa	pascal
pN	piconewton
nN	nanonewton
Å	Ångstroms
nm	nanometre
μ m	micrometre
mm	millimetre
cm	centimetre
m	metre
s	seconds
min	minutes
RCF	Relative centrifugal force

Databases

PDB	Protein data bank
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Biological molecules

BSA	Bovine serum albumin
MBP	Maltose binding protein

DNA

Deoxyribonucleic acid

Important Chemicals

bFGF	basic fibroblast growth factor
EC	ethyl lactate
NaPB	Sodium phosphate buffer
Ru(II)bpy_3^{2+}	Tris-bipyridylruthenium(II)
NaPS	Sodium persulfate
DPPE	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DSPE-PEG-2000	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [amino (polyethylene glycol)-2000]
IPA	Isopropanol
PDMS	polydimethylsiloxane

Constants

k_b	Boltzmann's constant
h	Plank's constant

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Chapter 1

Introduction

1.1 Hierarchical Structures

In nature it is common for small and simple building blocks to be used to create structures of a higher order, with increasing complexity. These structures can then be arranged to form even higher order structures, such that for some systems, the different levels of order span over multiple length scales. This type of material is defined as a hierarchical system, which describes how items are arranged either ‘above, below or at the same level as one another in different systems’ [1]. Without hierarchical systems, nature would be confined to form anything more complex than the chemical elements such as carbon, nitrogen and oxygen. Structural hierarchy in nature allows for more complex and functional materials, that would not be possible with the individual building blocks. A principle of some hierarchical systems, is that if a change occurs at one of the levels then this can impact the higher levels. An example of hierarchical behaviour is observed in pine cones, where when wet pine cones dry out their seed scales bend downwards. This results in the pine cone opening out in dry weather (Figure 1.1A) [2]. The cause of the change in pine cone structure crosses multi-length scales. The seed scales are composed of two layers of cellulose microfibrils which are aligned in different orientations. The cellulose microfibrils are composed of extensor cells that can change in length and volume depending on the water in the local environment. It is these extensor cells that control the outcome of the

pinecone. If the conditions are humid, wet conditions the extensor cells swell, if it is dry the extensor cells shrink. When the extensor cells lose liquid they shrink, and go from a turgid state to a flaccid state (Figure 1.1B). This results in the seed scales bending down and opening out, which allow the pine cones to release their airborne seeds.

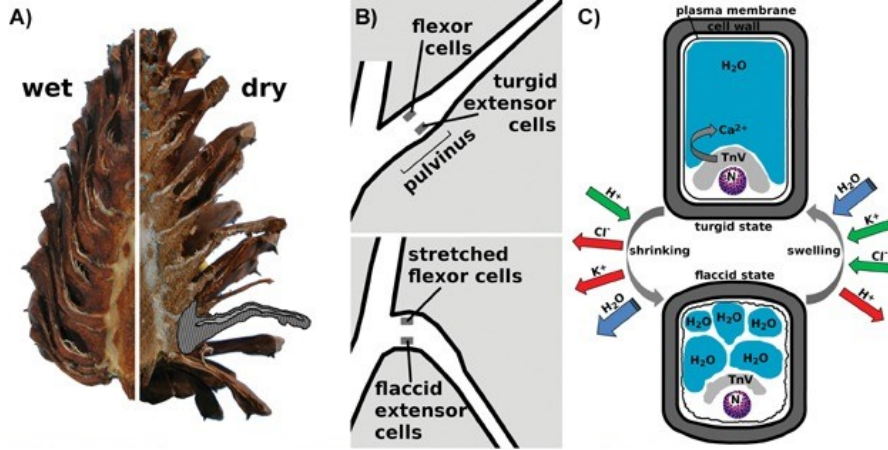


Figure 1.1: A) The pine cone is an example of a function that is performed by a hierarchical structure, that has seed scales that close when wet and open when dry. B) Top: The seed scales bend upwards due to extensor cells being in a turgid state. Bottom: The seed scales bend downwards due to extensor cells being in a flaccid state. C) the turgid state of extensor cells is caused by the cells swelling in wet humid conditions, and the cells become flaccid in drier conditions due to the osmotic movement of water. Adapted from with permission from Wiley Materials [2].

By taking inspiration from nature, new complex, hierarchical materials can be formed, that have functional building blocks that respond to changes in the environment. Small changes to many building blocks can create affect the macroscale properties. This thesis aims to explore how functional, hierarchical materials that span from the nanoscale through to the macroscale can be controlled and utilised for biomedical applications.

1.1.1 Hierarchical Living Systems

Living systems naturally form hierarchical structures with multiple levels of organisation from the molecular level through to the body as a whole. The arrangement of proteins and cells control functions in our body vital for life. Interesting examples of hierarchi-

cal systems include titin, tubulin, actin and fibrinogen, with more details of exemplar hierarchical systems described in this section [3–6].

1.1.1.1 Microtubules

In cells the cytoskeleton provides structural support to define the shape of the cell, as well having a role in the function of the cell [7]. Microtubules are a type of biopolymer in the cytoskeleton, that play a vital role in defining the cell shape, providing a mechanical support for mitosis and cell movement [8]. The building block in microtubules is the globular protein tubulin, a heterodimer with subunits α -tubulin and β -tubulin. The tubulin dimers self-assemble into linear strands known as protofilaments. The protofilaments laterally associate to form a hollow rod structure of 25 nm in diameter, providing the microtubule with a high bending stiffness [9]. The mechanical stiffness of the microtubules is phenomenal with a Young’s modulus of 1.4 GPa, 100 times stiffer than other components of the cytoskeleton [10]. The stiffness, resilience to shear and twisting forces, are key to the intracellular movement of organelles and the shape as the cell through cell migration, allowing the microtubules to be flexible and bend while being stiff enough to provide mechanical support [11]. The importance of the hierarchy in microtubules is evident in these mechanical properties, as a tubulin or protofilament would not provide the bending stiffness and functions alone.

Each level of hierarchy in microtubules plays a part in the function and properties of the overall microtubules. For example, microtubules are responsible for cell migration. The tubulin dimers at the ends of the microtubule are able to polymerise in the presence of guanosine-5'-triphosphate (GTP), hydrolysing to guanosine diphosphate (GDP), leaving the GDP-tubulin susceptible to depolymerisation. The polymerisation and depolymerisation of tubulin is a dynamic process, leading to the growth and shrinkage of the microtubule [13]. The arrangement of the heterodimers in the protofilament contributes to the movement of motor proteins across microtubules. As an α -tubulin always binds to a β -tubulin, and vice versa, this results in an α -tubulin end to the microtubule that is more negative and β -tubulin end which is more positive. This arrangement creates a

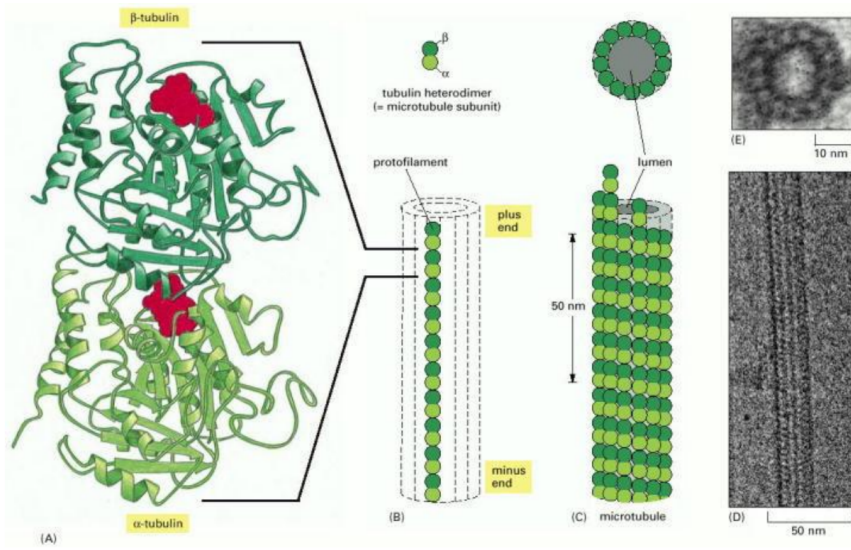


Figure 1.2: Microtubules are hierarchical biopolymers composed of A) α -tubulin and β -tubulin, that form a B) tubulin heterodimer. The tubulin heterodimer is the building block for the protofilaments that associate into C) microtubules. D) and E) show the structure imaged with transmission electron microscopy. Reproduced from with permission Oxford University Press [12].

polarity, essential for motor movement along the microtubule [14]. This process allows the cells to migrate in response to the environment. The properties of microtubules are a source of inspiration for functional biomaterials, however it remains a challenge reproduce the complex, functional properties in synthetic materials based on microtubules [15].

1.1.1.2 Collagen

Collagen is a structural, fibrous protein which makes up for 30% of the protein in the body, and is a major component of load bearing tissues such as bone, skin, tendons, and cartilage [16, 17]. Collagen is a fascinating example of a hierarchical structural in nature, with multiple levels of organisation as shown in Figure 1.3. Collagen fibers provide connective tissues with the ability to withstand tension [18]. The building block of the collagen fiber is an amino acid sequence at ≈ 2 nm long. At the molecular length, collagen is formed of 3 helical polypeptide chains known as tropocollagen (TC) which are ≈ 300 nm in length [19, 20]. TC has a rope like structure where the individual strands of

collagen molecules are twisted together, and held together by hydrogen bonds between collagen molecules. TC has a Young's modulus of 7 GPa and a fracture strength of 11.2 GPa [17]. Single triple helices are unstable at 37°C, as it is energetically favourable to form a random coil confirmation [21]. Instead, TC arranges into a staggered structure to form a 3D crystal lattice, the collagen fibril with lengths of 10 μm . The staggered structure, stabilised by non-covalent intermolecular bonds, between the TC molecules gives collagen fibrils strength and rigidity, with a Young's modulus of 5 GPa and fracture strength of 0.5 GPa. [22, 23]. The weak intermolecular bonds also allowing for slipping between the TC molecules, allowing collagen to withstand a lot of energy by dissipating the energy through slippage.

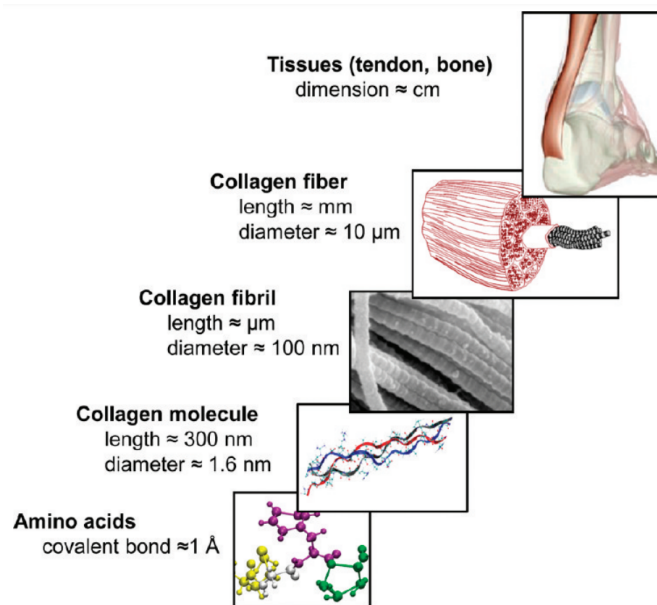


Figure 1.3: Collagen is a hierarchical structure that is vital for tissues such as tendons and bone. Three peptide chains are twisted together to form the collagen molecules. Collagen molecules are arranged into a 3D lattice known as the collagen fibril which can withstand high stress. Several collagen fibrils bundle together into the collagen fiber, and give tendons and bone their unique mechanical properties. Reused with permission from Gautieri *et al.* (2011) [24], copyright (2022) American Chemical Society.

Collagen fibrils arrange to form fibres that have a diameter of 1 mm, these fibres arrange either randomly or in an orientated fashion to form different tissues [25]. The collagen fibril is present in both articular cartilage and tendons, however both have different mechanical properties. Articular cartilage can withstand high load in joints, while tendons are more capable of absorbing large quantities of elastic energy, reducing

the chance of injury in a high impact scenario [26]. These difference are down to the arrangement of the collagen fibers in the different tissues, showing that the same building block can produce tissues with distinct structural and mechanical properties.

1.1.2 Structural Hierarchy in Proteins

Proteins are the most abundant biological macromolecule. Being present in every living cell, proteins provide essential functions that occur on the cellular level through to the body level [27]. Proteins are responsible for chemical reactions and regulation in the body, allow for transportation through cell membranes, as well as providing mechanical resilience, structure and shape for cells. The level of protein organisation spans multiple length scales, meaning proteins are hierarchical. The building block of proteins is the amino acid, which is an organic molecule with a central carbon atom covalently bonded to a carboxylic group ($-\text{COOH}$), an amino group ($-\text{NH}_2$) and a side chain (represented as R), where the side chain is unique to each amino acid [28]. Figure 1.4 shows the

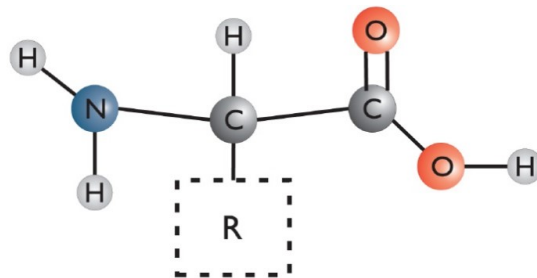


Figure 1.4: Top: Schematic of a general amino acid structure, the subunit of all proteins. The amino acid has a central carbon atom, covalently bonded to an amino group ((NH_2)), a carboxyl group (COOH), and a R group. The R group is a side chain that characterises the type of amino acid, and its chemical properties. Note that R-groups tyrosine and cysteine are particularly relevant to this thesis. Reused from Hughes *et al.* (2016) [29] with permission from IOP publishing.

characteristic structure of an amino acid and R groups of the 20 standard amino acids. The different functional R groups of the amino acid determine the hydrophobicity and charge of the amino acid. The R groups can be classed by the chemical properties of their side chains, as shown in Figure 1.5.

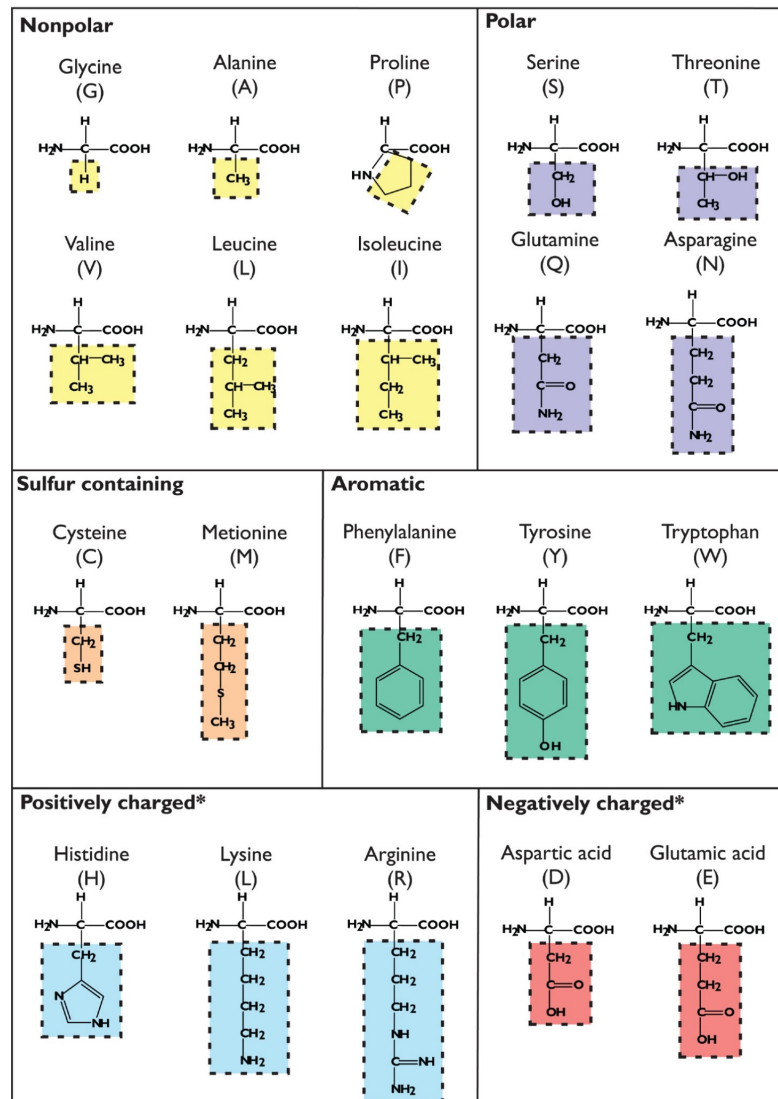


Figure 1.5: Top: Schematic of a general amino acid structure, the subunit of all proteins. Reused from Hughes *et al.* (2016) [29] with permission from IOP publishing.

Amino acids covalently link via peptide bonds, to give the first level of hierarchy in proteins, the polypeptide chain. The order of the amino acids in the polypeptide chain is referred to as the protein sequence. The amino acid sequence of a polypeptide is controlled by the encoded DNA. This level of hierarchy is known as the primary structure of proteins, and shown in Figure 1.6. The different amino acids control how the polypeptide chain folds. The secondary structure is defined as the local folded structure of the polypeptide chain in space. This level of structure occurs due to the

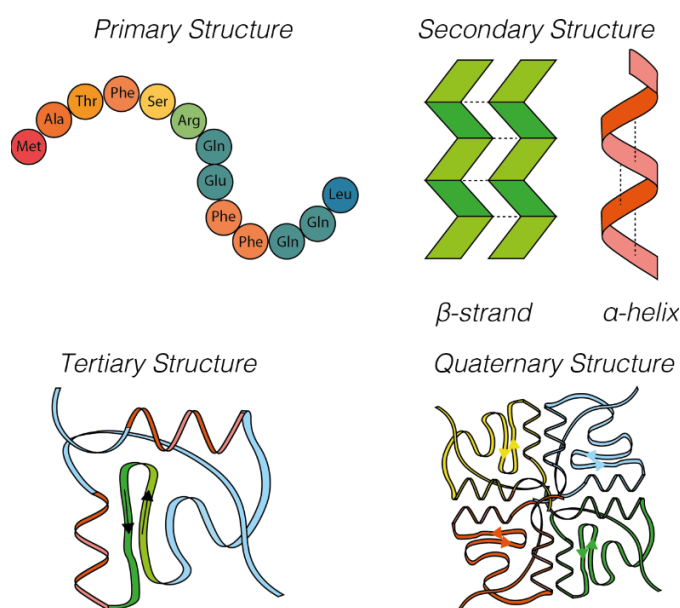


Figure 1.6: Top left: Proteins have amino acid building blocks link with peptide bonding to make up the primary structure. Top right: Interactions between backbone polypeptide chain form structures such as the α -helix and β -sheet classed as the secondary structure. Bottom left: The tertiary structure is the 3D folded structure. Bottom right: Some proteins have an additional level of hierarchy, the quaternary structure, where multiple polypeptide chains interact. Reused with permission from [30] under a CC BY 4.0 licence.

interactions between the backbone of the polypeptide chain, where the backbone is the polypeptide without the R groups. Hydrogen bonds form between amide groups to create the secondary structure, therefore the amino acids in the primary sequence dictate the secondary structure of the protein. The secondary structures formed are classified as α -helices, β -sheets (β -strands that align in parallel or anti-parallel), β -turns and random coils (Figure 1.6). Different structures occur depending on where the intramolecular non-covalent interactions occur. The 3D structure that the protein forms due to interactions between the R groups is the tertiary structure, and is classed as all α , all β or α/β structure. The interactions results in a 3D folded structure, known as the protein fold, which dictates the biological function of the protein. The different arrangements polypeptide chains occupy due to these interactions results in a variety of proteins in all living things. These inter-polypeptide chain interactions include many non-covalent bonding such as dipole-dipole interactions, hydrogen bonding, ionic bonding, and London dispersion forces. Hydrophobic interactions are important to the tertiary structure, where amino acid residues with hydrophobic (water fearing)

side chains are arranged into clusters inside the protein, while the hydrophilic (water loving) residues are arranged on the outside, and able to interact with water. Disulphide bonds can form a covalent bond between two sulfur containing side chains of the cysteine amino acid [31]. These are strong bonds that can be thought of as ‘nano-staple’ between sections of the peptide chain. A further level of protein hierarchy is present in some proteins, referred to as the quaternary structure. This is defined as when multiple polypeptide chains interact, commonly via weak interactions such as hydrogen bonding and London dispersion forces, rather than a singular chain. When a polypeptide has its intramolecular interactions broken, and becomes unfolded, the structure is assumed to be in a random coil formation. In the situation where the protein is no longer folded, the protein may be unable to perform its normal functions [32].

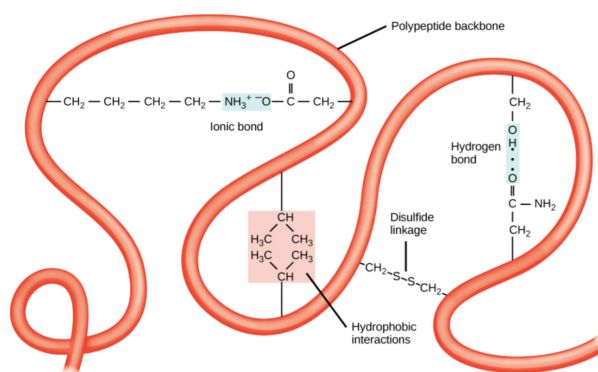


Figure 1.7: The tertiary structure of proteins is held together by different interactions between the polypeptide chain. Breaking these bonds, the protein becomes unfolded, with the possibility of losing its functionality. Reused from OpenStax with permission from Creative Commons Attribution 4.0 International License [33].

1.1.3 Protein Stability

Most proteins must remain folded, in its native state, to perform biological functions. The previous section described different interactions that hold the protein in its folded conformation. The folded state is therefore important to maintain, however changes to the environment of the protein can cause the protein to unfold. The stability of the protein to remain in its folded state can be quantified by the change in free energy of protein unfolding.

The folding of a polypeptide involves bond formations and changes to the structure, resulting in energy changes occur in the system. The transition between folded and unfolded states is a thermodynamic process. The free energy of a state is a measure of how favourable it is to be in that state, or in other words, how much energy available in the state to do work. Free energy change is defined as the work done on a system for the state to transition to another state, factoring in for the energy lost as heat to the surroundings [28]. In the case of a constant pressure (isobaric) and temperature (isothermal), the change in Gibbs free energy, ΔG , of the transition to state is:

$$\Delta G = \Delta H - T\Delta S \quad (1.1)$$

where ΔS is the entropy change and ΔH is the enthalpy change is the heat absorbed in a reaction that occurs at constant pressure, defined by Equation 1.2:

$$\Delta H = \Delta E - P\Delta V \quad (1.2)$$

where ΔE is the change in internal energy of the system and $P\Delta V$ is the work done at constant pressure. The entropy of a system is a measure of the states available:

$$S = k_B \ln \Omega \quad (1.3)$$

where Ω is the amount of states accessible for a single particle, and k_B is Boltzmann's constant. For a high entropy system, there are many available states each with its own associated energy, while a lower entropy system has less available states. A protein in its native folded state has an associated energy barrier to overcome in order to unfold [34]. The free energy change between folded and unfolded states of proteins can be quantified with ΔG_u and used to determine if transitioning to a state is favourable or unfavourable. For the protein to remain in its folded state, Equation 1.1 demonstrates that there is a balance between the entropy and enthalpy of a system. For a spontaneous process to occur, ΔG must be negative. If a transition between states has a positive ΔG , then energy is required for the transition to occur. When a protein is in a folded state, it can unfold and in the case for some proteins, refold. For spontaneous folding to occur, the

change in free energy to transition from a unfolded to folded state must have a negative ($\Delta G_{U-F} < 0$). If the change in free energy to fold a protein is positive ($\Delta G_{U-F} > 0$), then additional energy is required to fold the protein. Figure 1.8A depicts a two state energy landscape, showing a single energy barrier required to access each state, where the protein is either in a folded or unfolded state. Amino acids can rotate about the peptide bond, giving polypeptides routes to different possible conformations [34]. The number of conformations of a folded protein is less, as the movement is restricted. The entropy of the system therefore decreases when a protein folds to a a more ordered state with less possible conformations to change. When unfolding, proteins often have multiple intermediate unfolded state. The energy landscape of a protein can therefore be complex, with many pathways of folding [35]. Figure 1.8B shows a funnel-like free energy landscape for multiple possible pathways of a protein to unfold, with multiple local minima and one global minima. The top of the funnel represents all the possible unfolded conformations, where the entropy is maximum, and the bottom of the funnel is the folded state with the least entropy. The intermediate states can be complex, where both hindering and facilitating towards protein folding in the intermediate states can occur.

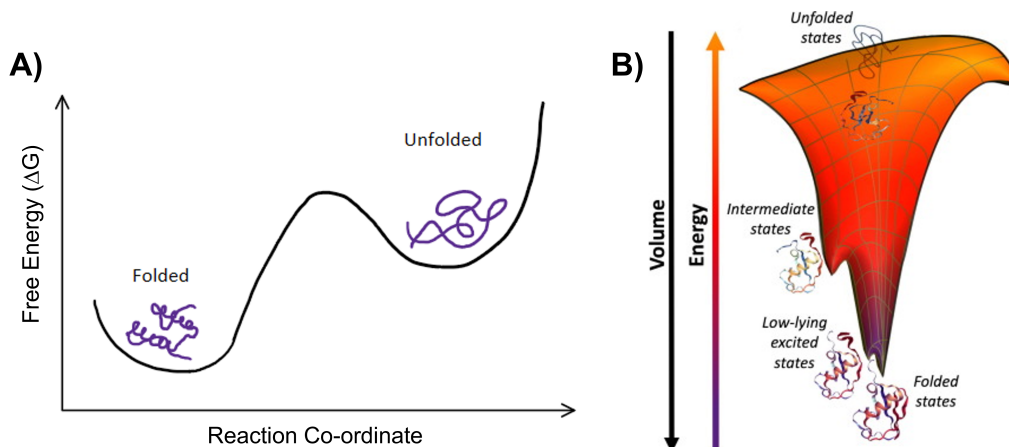


Figure 1.8: A) The energy of landscape of the folding and unfolding of a protein, where the troughs represent stable conformations. There is energy barrier is associated with going from the folded state (left) to the folded state (right). The rates of unfolding and folding are determined by curve of the energy landscape. B) An energy landscape funnel of a protein where the unfolded state is represented as the top of the funnel, with multiple stable intermediate conformations before reaching the final folded protein conformation. Reused from Roche *et al.* (2017) [36] with permission Elsevier.

1.1.4 Mechanical Stability of Proteins

For some proteins the unfolding process is essential to achieve their function, or is part of a mechanical process. An example of a protein that partially unfolds to achieve its function is in mitochondria as it moves through the membrane (Figure 1.9A) [37, 38]. The power stroke model predicts that the intramitochondrial, matrix protein (chaperone mtHsp70) undergoes a conformational change, controlled by ATP hydrolysis. This conformational change pulls the peptide chain through the membrane channel, driving unfolding of folded domain on the mitochondria surface [39]. Further examples of proteins that use mechanics in their function are shown in Figure 1.9.

In biological systems some proteins are able to withstand relatively high forces, often the function in the body requires them to be mechanically stable. Some cells are mechanosensors, meaning that when a force is exerted the cell has a response. Titin kinase, part of the muscle protein titin, reacts to mechanical force playing a role in defining

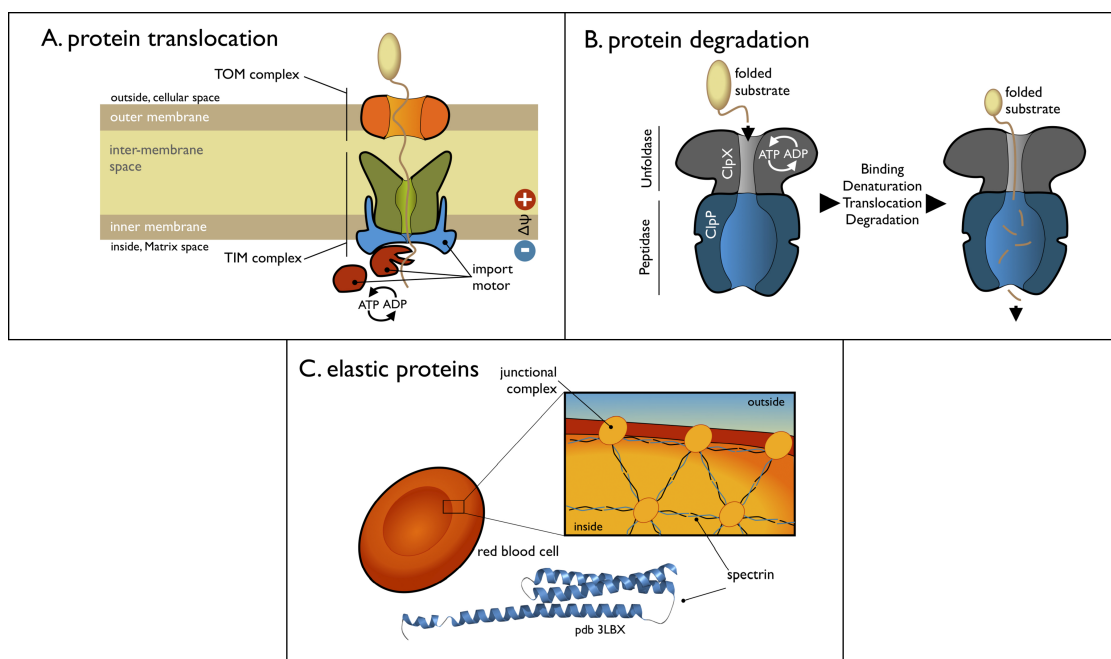


Figure 1.9: Proteins have fascinating functions on the body, where mechanical forces come into play. A) Protein translocation through the membrane into mitochondria involves the protein partially unfolding. The power stroke model predicts that ATP hydrolysis activates the matrix chaperone mtHsp70 to pull the unfolded protein through the membrane. B) In chaperone protein Clp, ATP hydrolysis provides energy to create a mechanical force. This mechanical force can remove proteins that are either misfolded or no longer required by force the protein to unfold, and pull the peptide chain through. C) Spectrin is an example of a protein that is resilient to mechanical force. Spectrin is found in the cytoskeleton of red blood cells, giving red blue cells their shape and mechanical properties. Adapted from [40] with permission from the Royal Society of Chemistry.

the response of the muscle to force [41]. The mechanical resilience and malleability of proteins to remain intact and folded, or have the ability to unfold and refold, can be determined with the use of single molecule force spectroscopy (SMFS) techniques. SMFS techniques are used to investigate the mechanical stability of proteins, atomic force microscopy (AFM), optical tweezers and magnetic tweezers can be used. These techniques allow for the application of low forces onto the protein (10-100 pN), which allows the protein to be probed over small length scales. These techniques can determine the forces required to unfold a protein and give information on the energy landscape of unfolding/folding. Using SMFS techniques, proteins have been observed to withstand high mechanical forces even to > 100 pN [40]. The structure of the protein can often be related to the mechanical force that the protein can withstand. α -helical structures are

typically found to be mechanically weak [40, 42–44]. Relating this to their secondary structure, α -helices has no hydrogen bonding between the polypeptide chains, allowing them to be unfolded with the application of only low pulling forces [45]. β -sheets are able to withstand higher forces and mixed α/β proteins fall in between. The mechanical resistance of β -sheets is due to hydrogen bonding between the β -strands giving the protein a high resistance to force than hydrophobic interactions between helices in solely α -helical structures.

For proteins with similar structures, different mechanical stability properties are found. For example, protein I27 domain from titin has a β -sheet structure and is relatively mechanically resistant, while green fluorescent protein has a β -sheet barrel is much less resistant to mechanical forces [46, 47]. The secondary structure is therefore not the only factor influencing mechanical stability. Brockwell *et al.* (2003) investigated protein E2lip3 with AFM, immobilising two different parts of the protein allowed the protein to be pulled in two different ways [48]. In one pulling direction, E2lip3 was pulled such that the protein was ‘unzipped’ where each hydrogen bond was broken one by one, with a much lower mechanical force required to unfold E2lip3 (Figure 1.10A). In a second pulling direction, β -strands were sheared, and a relatively high mechanical force was required to break the hydrogen bonds and unfold E2lip3 (Figure 1.10B). This

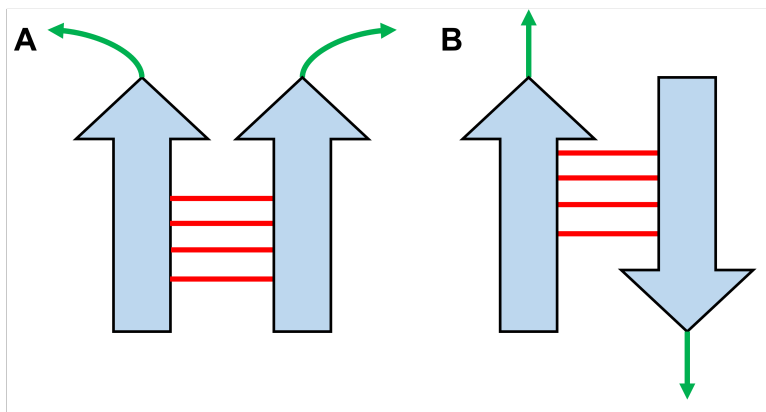


Figure 1.10: Schematic of two different pulling profiles of proteins. Protein being A) unzipped and B) sheared under pulling force, causing hydrogen bonds between β -strands to break.

study demonstrated that the direction of pulling the protein changed how the protein

responded to mechanical force, due to different ‘mechanical clamps’ characteristic to the protein. Mechanical clamps refer to the region of a protein that experiences the greatest amount of force, or the load-bearing region. Further mechanical clamps have been identified, Figure 1.11 shows that type of mechanical clamp is related to the mechanical stability of the protein. AFM-SFMS, along with Monte Carlo simulations, has revealed

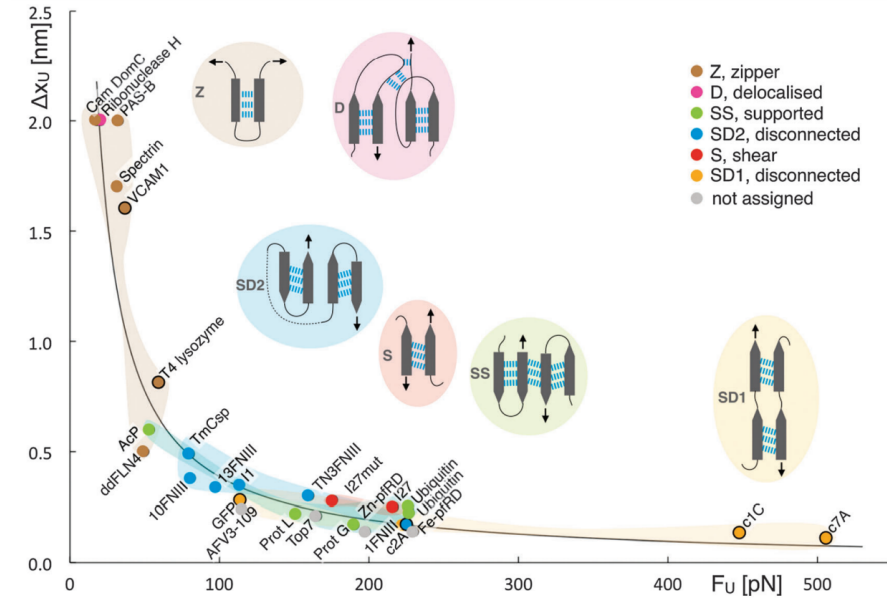


Figure 1.11: The relationship between the pulling distance from the folded to unfolded transition (ΔX_u) and the force required to unfold the protein (F_u) for several proteins, with different mechanical clamp motifs (top right). Pulling speeds were 600 nm s^{-1} . Reused from Hoffman *et al.* (2013) [49] with permission PCCP Owner Societies.

the energy landscape of many proteins with stable intermediate structures that were previously unknown without these techniques [45, 50]. The mechanical resistance of proteins is not only dependant on the amino acid sequence, the structure they form but also how the protein is unfolded in terms of rate and direction of force, demonstrating how each level of hierarchy is important for proteins.

This section highlights the complexity of proteins introducing how in nature proteins respond to changes in the environment. The relationship between how proteins cope with mechanical force is dependant their structure and intra-molecular bonds. Hierarchy in nature is apparent, and science can take inspiration from these complex, multiple level systems to create exciting, new biomaterials than can respond to changes in their

environment or be tuned to a specific purpose. SFMS techniques have been able to reveal information about the mechanical properties of individual proteins, showing that proteins can offer inherent functionality and mechanical resilience that can be utilised in novel biomaterials.

1.1.5 Hierarchical Systems: Building Blocks to Bulk Properties

These examples describe only a fraction of how diverse and complex nature's hierarchical systems are, structure and function to living beings. Recreating the hierarchical behaviour of natural systems into synthetic materials, with the level of complexity and functionality demonstrated in nature, is challenging. Hierarchical materials in biomedical application could offer materials that are suitable for use *in vivo* in terms of structural support and mechanical behaviour, while also having being responsive to changes in the environment. Often materials are designed with a purpose in mind, referred to as the 'top-down' approach. Nature does the opposite, where the building blocks have been optimised to achieve the next level of complexity through the evolutionary process. This is referred to as 'bottom-up', an inspirational approach to apply to understanding biological systems and engineering new materials.

1.2 Synthetic Hierarchical Biomaterials

A hydrogel is a 3D structured network of cross-linked, hydrophilic building blocks that by definition is swollen with at least 10% water [51]. The combination of large water content and structure from the network results in a swollen, porous material with a network that provides mechanical support to the hydrogel. Examples of building blocks used in hydrogels include polymers, peptide chains and DNA, and can be classed as synthetic or natural [52, 53]. The cross-links between the building blocks can be either physical or chemical bonds. Chemically cross-linked hydrogels have covalently bonded building blocks, while physical bonds involve non-covalent cross-links such as entanglements, hydrogen bonds or electrostatic interactions that often can have reversible properties [54].

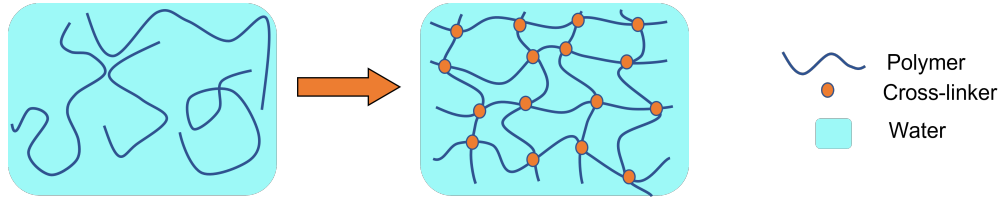


Figure 1.12: Schematic of cross-linked polymers swollen with water forming a hydrogel network. Left: Pre-gelation. Right: Post-gelation

Hydrogels exhibit hierarchical behaviour, with the building blocks able to form porous and structured networks. The type of building block, the type of cross-link and the volume fraction can influence the hydrogel, leading to a variety of different macroscale properties, such as network porosity or the mechanical stiffness of the bulk hydrogel [55, 56]. When exploring hydrogels, there are multiple different properties to understand when considering what applications would be suitable. Due to high levels of water content, hydrogels can have similar mechanical properties to tissues and the extracellular matrix, making hydrogels an ideal candidate for biomaterials [57]. The complexity and tunability provides an exciting diversity of hydrogel based biomaterials, that has the potential to be tailored to specific biomedical applications[58].

1.2.1 Hydrogels for Drug Delivery

Conventional drug delivery methods frequently resort to repeated or high dosages for successful treatment [59]. This can lead to a lower efficacy of the treatment, a resistance to the drug, and toxic side effects [60, 61]. Challenges with the efficacy of orally administered drugs, the most common method of drug delivery, include short circulation times and lack of targeting to the region of interest (area which requires treatment) [62]. Figure 1.13 demonstrates the difference between the desired controlled release rate for maximum efficacy, compared with the conventional release of drugs with the administration of multiple doses [63]. The conventional drug release rate has sharp burst increases of drug level associated with each dose. This can result in an overall drug concentration greater than desired, side effects or a lower efficacy. The desired release rate is a constant

exposure of drugs once the dose has been administered, for the time required to fulfil the treatment of the patient [64]. Further issues with conventional drug delivery methods include how the body affects the drug, for example drug denaturation and aggregation in the body can occur on short time scales, reducing the lifetime of the drug [65]. The focus of current drug delivery research has explored different methods encapsulating the drug in a vessel, and investigating how drug release can be controlled [66].

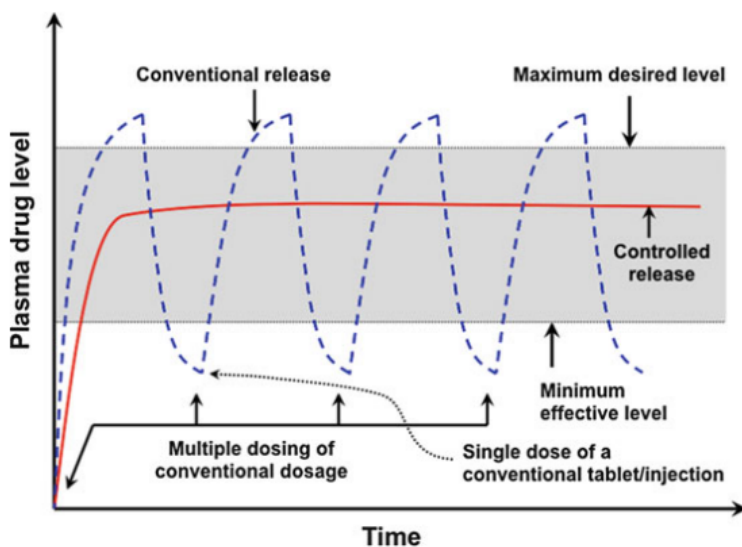


Figure 1.13: Comparison of conventional drug treatment involving multiple doses of drug (blue curve) to an ideal controlled drug release treatment (red curve). The greyed area is the desirable plasma drug level, above which the drug could be toxic and below which the drug is ineffective for treatment [63].

Hydrogels offer an alternate method of drug delivery, where hydrophilic drugs can be easily loaded into the hydrogel network due to the high water content of hydrogels. The hydrogel network offers protection from proteins in the body reaching the encapsulated drug, therefore the rate degradation of the encapsulated drug could be decreased when in a hydrogel [67]. Drugs encapsulated within hydrogels are released by a range of methods: diffusion, degradation of the network, polymer-drug interactions, controlled swelling or deformation of the pores [68]. Hydrogels have successfully demonstrated the ability to release drugs at a slower rate compared to free drugs, increasing the time period of drug uptake in the region of interest. Furthermore, the use of ‘smart’ hydrogels has attracted research for stimuli responsive drug release [69]. Smart hydrogels, when exposed to

a stimulus such as a change in the environment or application of mechanical force, can respond with a change to hydrogel. Stimuli that can induce changes to hydrogels include temperature, pH, chemical, enzyme, light, and magnetic field [52, 70–73]. Smart hydrogels for drug delivery applications have demonstrated the capability to induce a controlled release in drug flow out of the hydrogel in response to a stimulus [74–76]. The

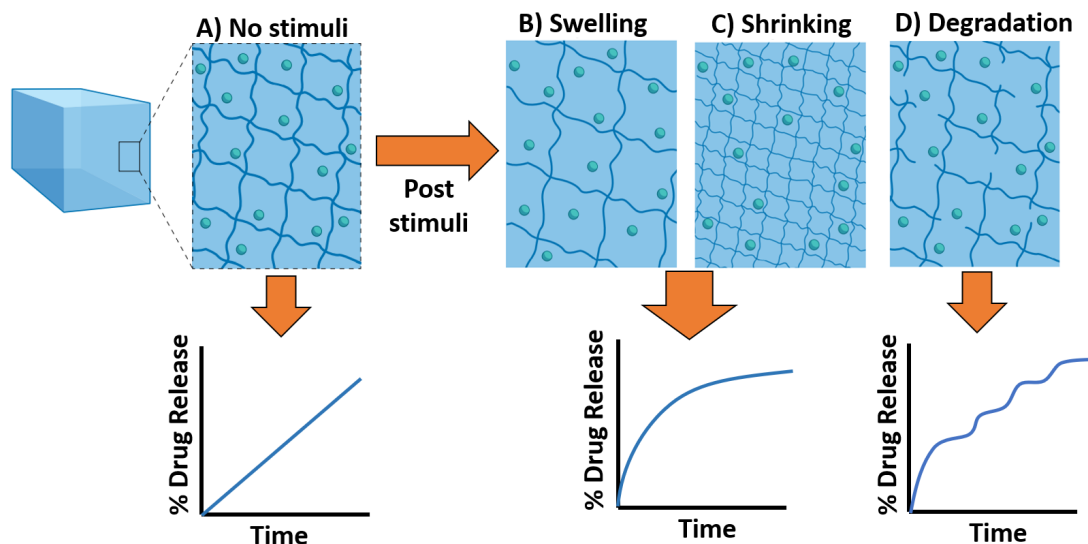


Figure 1.14: Schematic displaying examples of how different stimuli can affect the hydrogel network (top) and how this correlates to different drug release profiles (bottom) Adapted from Allen *et al.* (2022) [77] with permission from Springer Nature.

pore-to-drug size ratio can be changed by different stimuli to vary the speed of drug release, for example the steric hindrance of the network on large drug molecules slows down the drug movement, while small drug molecules move through larger pores faster [78]. Figure 1.14 shows different drug release methods of diffusion, network degradation, swelling and shrinking. Diffusion is the simplest method of drug release, where the drug moves through the hydrogel network until it exits the hydrogel. A common response to a stimuli for hydrogels is a change in the degree of swelling (Figure 1.14B). This increases the pore size of the network causing an increase in the diffusion of encapsulated drugs from the hydrogel. If the hydrogel is stimulated to shrink, the force of the network contracting can squeeze out encapsulated drugs. An example study of implanting and removing a nanocellulose-hyaluronic acid hydrogel from mice demonstrated the ability to shrink in size and change in porosity after 7 days *in vivo* [79]. Network degradation can be a result of the cross-link or polymer building block breaking down, allowing drugs to

diffuse through holes in the network created from degradation. The different drug release mechanisms result in distinct drug release profiles from the hydrogel. Hydrogel network degradation can be accelerated with the presence of enzymes or occur naturally over time [71]. Hydrogels offer an exciting method of drug delivery, that have the potential to improve the control over drug release when implanted *in vivo*.

In addition to the responsive properties hydrogels, they can be easily be moulded into different sizes and shapes to suit the application as demonstrated in Figure 1.15. Macroscopic sized hydrogels are suitable as patches that can be placed on the surface of the skin or implanted in surgery [80–83]. The mechanical properties of the hydrogel patches are vital for their success. For example, in the application of wound-healing, a hydrogel patch applied to an area that undergoes frequent stress, the hydrogel needs to remain intact until after the treatment is complete, otherwise this will leave the wound exposed to infection [84]. The location of hydrogel requires consideration of the stiffness, elasticity, brittleness and compressibility. Hydrogels have similar mechanical properties to some tissues. Furthermore hydrogels can be tuned, making them a suitable material for patches [85]. Hydrogels can be produced in smaller sizes such as microgels ($>1\ \mu\text{m}$) or nanogels ($>1\ \mu\text{m}$) to open up options of oral or inhalation administration methods [86–88]. Shear thinning hydrogels, which undergo shear stress, have a lower viscosity. This useful property of shear thinning hydrogels allows hydrogels to pass through a needle when undergoing the shear stress of injection [68]. The adaptability and breadth of the macroscopic and mechanical properties in hydrogels makes hydrogels an exciting, diverse tool for drug delivery.

1.2.2 Hydrogels for Cell Culture and Tissue Engineering

Cell culture is the practice of using cells from a natural source and growing them in an unnatural setting. Once the cells have proliferated (increased in number), the cell culture can be used as a model of the *in vivo* environment to experiment with the cells for example in the response of the cells to novel drugs. Cells are complex, and the *in vivo* extracellular matrix (ECM) properties influences biochemical and physical cues

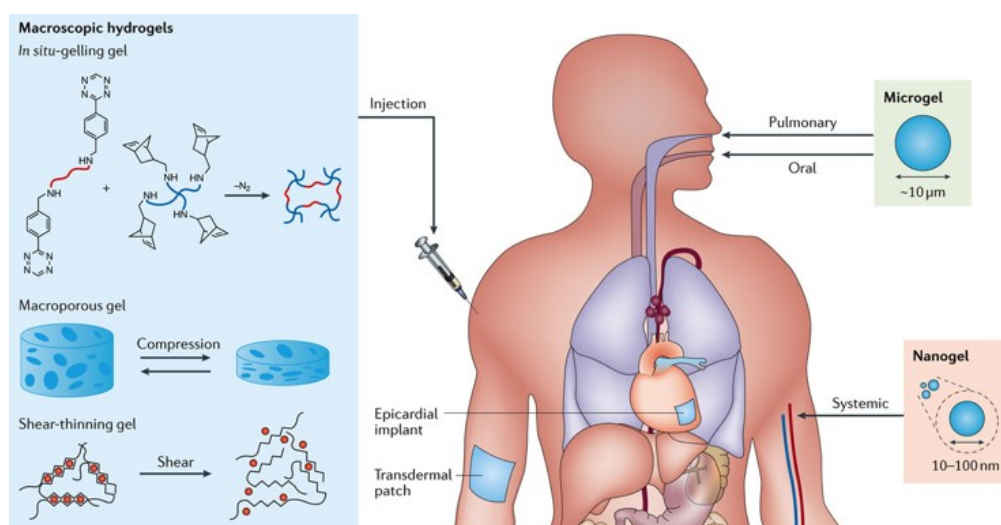


Figure 1.15: Different delivery routes of drugs from hydrogels for biomedical applications require different macroscopic properties. Left) Shear thinning or *in situ* gelling hydrogels are suitable for administration via injection. Middle) Hydrogel implants and patches are required to remain intact with repeated exertion. The size of implants and patches limits them to be used on the surface of the skin or inserted during surgery. Right) Microgels and nanogels are much smaller in size than patches, and can therefore be administered via injection, or ingested orally. Reproduced from Li *et al.* [89] with permission from Springer Nature.

that control cell behaviour. For example, stiffness of the ECM affects cell shape and migration [90]. Traditional cell culture practice uses 2D cell culture on plastic or glass substrates, offering a cost effective and efficient way to grow cells [91]. However glass and plastic substrates lack the mechanical, structural and compositional cues to influence cell function and it is generally accepted that these not to represent the physiological conditions [92]. Cell culture on stiff plastic and glass substrates result in abnormal cell behaviour such as flattened, more spread out cells, abnormal cell division and a different response to drugs than in an *in vivo* environment. Hydrogels provide an attractive alternative material for culturing cells, as the stiffness, rigidity and structure of a hydrogel can mimic aspects of the natural tissue [93–95]. Cells require a stiff substrate to adhere to, hydrogels provide the stiffness that cells would experience in the ECM, in addition to having porosity that allows for cell-cell communication [96, 97]. Figure 1.16 shows the how different the morphologies of cells can be when comparing cells cultured on increasing stiffnesses of hydrogel compared to the morphology on glass. The success of culturing cells onto hydrogels in 2D cell culture, lead to 3D cell culture in hydrogels to

repair or improve tissue function. Tissue engineering requires cells, a scaffold to support the cells and the simulation of growth in the patient [98]. Hydrogels have demonstrated the ability to act as a scaffold in supporting cells for tissue engineering, mimicking soft tissues in the body [99–101].

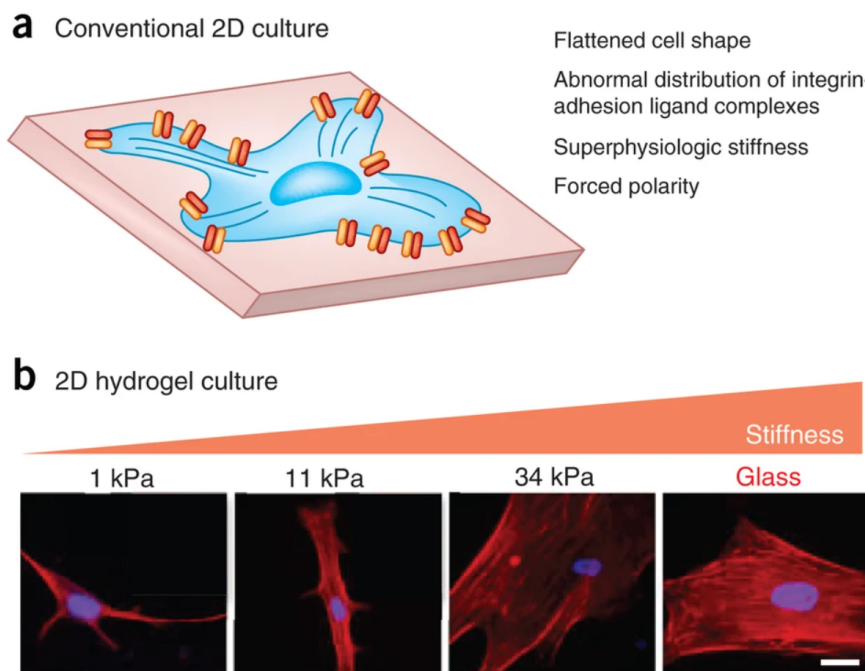


Figure 1.16: Cells are dependant on the stiffness of the substrate upon which they are cultured. A) In 2D cell culture on a hard substrate, such as glass or plastic, there are limitations associated with the method that affect the cell behaviour. B) Confocal microscopy images of cells cultured onto substrates of increasing stiffness. The morphology of cells differ with the substrate. Adapted from Caliri *et al.* (2016) [91] with permission from Springer Nature.

1.2.3 Biomolecular Hydrogels

Proteins are inherently biocompatible and biodegradable, in addition some proteins have mechanical resilience and functionality encoded into the protein, making them a unique component for biomedical applications. Proteins are purified from natural sources and can be engineered to have interesting properties [102]. Proteins are used as the building blocks of cross-linked hydrogel networks, where protein hydrogels have been used for a wide range of applications including tissue engineering [103–105], drug delivery [106, 107] and cell mechanobiology [108]. Peptides can be used as the building blocks for hydro-

gels, demonstrating a range of tunability and control over the behaviour of the hydrogel properties. Elsayy *et al.* (2022) demonstrated that the release of chemotherapeutic drug doxorubicin from peptide hydrogels can be tuned by changing the interactions between doxorubicin and the peptide [109]. Gao *et al.* (2017) compared different peptides as the building block for peptide hydrogels, where the peptides all had the same length, but had different interactions between peptide fibers due their differing amino acid sequences [110]. The resultant network structure, characterised with small angle neutron scattering (SANS), showed that the interaction mechanisms between peptide fibers not only changed the structure of the the network, but the bulk rheological properties. By switching one of the amino acids in the peptide sequence, the peptide hydrogels went from a network of fibers with strong interactions between them and a stiff bulk hydrogel ($G' = 1300$ Pa), to a hydrogel with a network of entangled fibers , no association between them and a weaker hydrogel ($G' = 13$ Pa).

Deoxyribonucleic acid (DNA) is a programmable, biological molecule that is attracting attention as a building block for creating smart and functional hydrogels [111]. Nishikawa *et al.* (2011) showed an exciting potential of DNA hydrogels in the sustained release of chemotherapeutic drugs through the network degradation resulting in significant reduction in the rate of tumour growth in mice [112].

Due to each level of hierarchy having a different organisation, the characterisation of each length scale requires different methods in order to build an overall picture of the system [113]. Figure 1.17 shows that on the molecular level, techniques like nuclear magnetic resonance (NMR) can reveal information on the molecular interactions. Proteins can form clusters or fibers that are the basis for network formation, these can be investigated with small-angle X-ray scattering (SAXS) and SANS to find their structure. The fibers or clusters interact, forming a network and require different techniques to understand the topology of the network. Electron microscopy or ultra-small angle neutron scattering (USANS) could be suitable depending on the size of the network. Finally, bulk properties on the macroscale can be characterised with rheology to understand the viscoelastic properties. Few studies however go into such detail of each length scale, likely due to the range of techniques and length of study required to achieve this. For

example study of L-cysteine gels by Menger *et al.* (2000) found a sudden decrease in the stiffness of the gel with increasing temperature suggesting that a sudden phase change is occurring [114]. Differential scanning calorimetry showed that this was in fact not the case, but the gel had a continuous gel-to-sol conversion where the sudden decrease in the stiffness actually suggests that the gel instantly becomes unable to withstand the shear force. This study illustrated how important cross length scale studies are for complex hydrogels, and that characterising only the bulk properties may elude to the wrong conclusion.

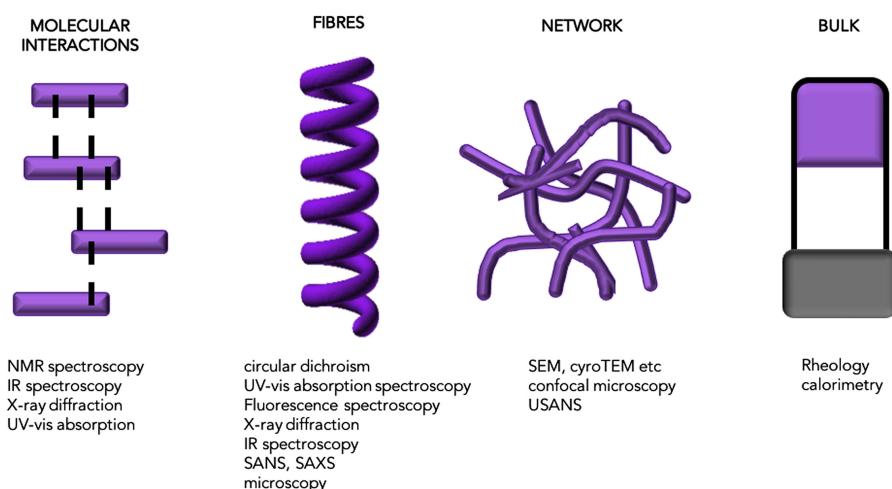


Figure 1.17: Techniques that can be used to characterise each length scale of peptide hydrogels. By characterising each length scale, a full understanding of the peptide hydrogel can be achieved. Reproduced from Draper and Adams (2017) [113] with permission under Creative Commons CC-BY.

1.2.4 Folded Protein Hydrogels

Folded proteins are vital for the vast majority of functions in biological processes, with some proteins even defining mechanical properties that span across numerous length scales, as described in Section 1.1.2. Though many studies have investigated protein hydrogels, fewer have retained the functionality of the protein fold to create a smart biomaterial. Using nature as an inspiration, protein engineering can create new constructs to create mechanically robust and functional protein hydrogels. Utilising the knowledge and characterisation of proteins, hydrogels can be designed with a ‘bottom-up’ approach

to create predictable, well understood materials that overcome challenges hydrogels face in applications. To understand and predict the properties of folded protein hydrogels, information about the individual folded proteins is vital. Folded proteins form hydrogel networks by chemical cross-linking folded, globular proteins, providing the ability to utilise the unique and exciting functions of proteins [115–118]. This section highlights some examples of folded protein hydrogels that have been carefully designed. A pioneer-

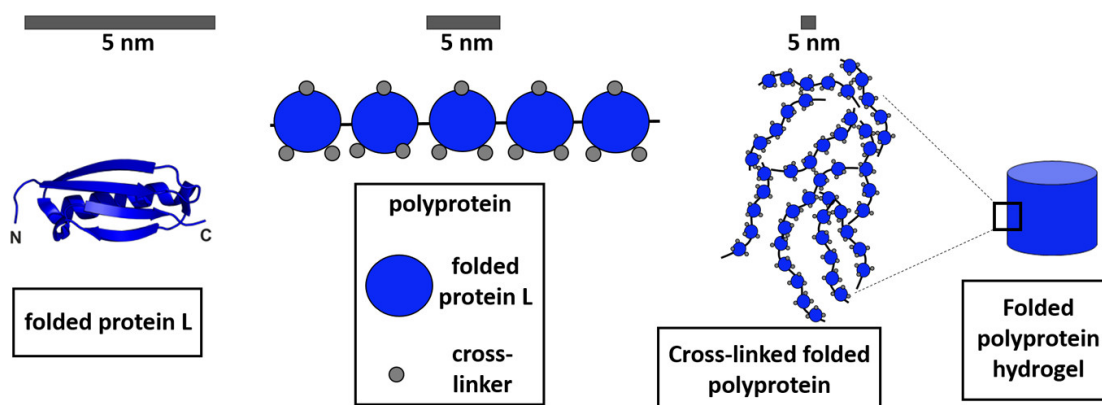


Figure 1.18: Folded proteins form hydrogels that have cross length-scale hierarchy. Proteins can be engineered into polyproteins, that cross-link to form a folded polyprotein hydrogels. Reproduced from da Silva (2017) [119] with permission under Creative Commons CC-BY.

ing example of using a well characterised, functional, biocompatible building block for creating folded protein hydrogels was inspired from giant muscle protein titin. Titin was pulled with AFM (see Section 1.1.2), which revealed that the folded globular domains of titin unfolded one-by-one when a stretching force is applied [46]. This allows the protein to dissipate the energy through unfolding, rather than the protein breaking. Furthermore, the unfolded proteins were found to refold when the stretching force was removed. Overall, titin has exciting mechanical resistant properties that could be beneficial in biomaterials. Taking inspiration from titin, Lv *et al.* (2010) engineered a polyprotein from protein GB1 (PDB: 1GB1), the streptococcal B1 immunoglobulin-binding domain of protein G, selected due to having similar mechanical properties to the folded titin immunoglobulin domains, and resilin, a random coil like protein to mimic the unstructured part of titin (see Figure 1.19A) [120, 121]. Several polyproteins were compared with different sequences of GB1 and resilin were stretched with single molecules AFM, showing

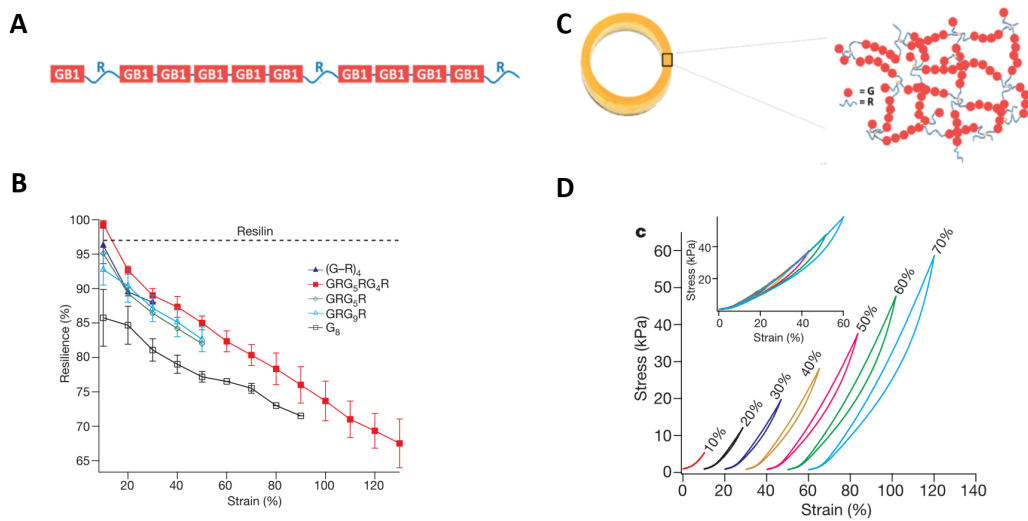


Figure 1.19: A) Rationally engineered polyprotein construct consisting of folded protein GB1 (G) and random coiled like protein resilin (R) to mimic giant muscle protein titin. B) Mechanical resilience of different polyprotein constructs of GB1 and resilin to being pulled with AFM. C) The polyprotein in GRG5RG4R was selected due to displaying high mechanical resilience in (C) and consequently was chemically cross-linked to create a folded protein hydrogel. D) Tensile stress measurements of polyprotein GRG5RG4R hydrogel of increasing strain. NEED Permission

that the arrangement of protein domains in the polyprotein controlled the mechanical properties (Figure 1.19B). The polyprotein that demonstrated the highest resilience (GRG5RG4R) was selected to be photo-chemically cross-linked to produce a folded protein hydrogel. The mechanical properties of polyprotein GRG5RG4R hydrogel showed at low strain, the hydrogel was highly elastic, while at high strain the hydrogel could reach a remarkably high stress (>25 kPa) acting as a shock-absorbing hydrogel (Figure 1.19D).

The approach of selectively using known load-bearing molecules has lead to engineering several new protein hydrogels with predictable mechanical properties. Wu *et al.* (2018) designed several protein hydrogels to cross-link (see Figure 1.20 for examples) with a functionalised 4-armed polyethylene glycol (PEG), demonstrating that mechanical properties of the resultant hydrogel could be predicted from the type of cross-linker and protein used [122]. The first scenario (Gel 1 in Table 1.1) investigated a cross-linker (tax-interacting protein 1 (TIP-1), PDB: 3DJ1), which fused to Kir peptide on

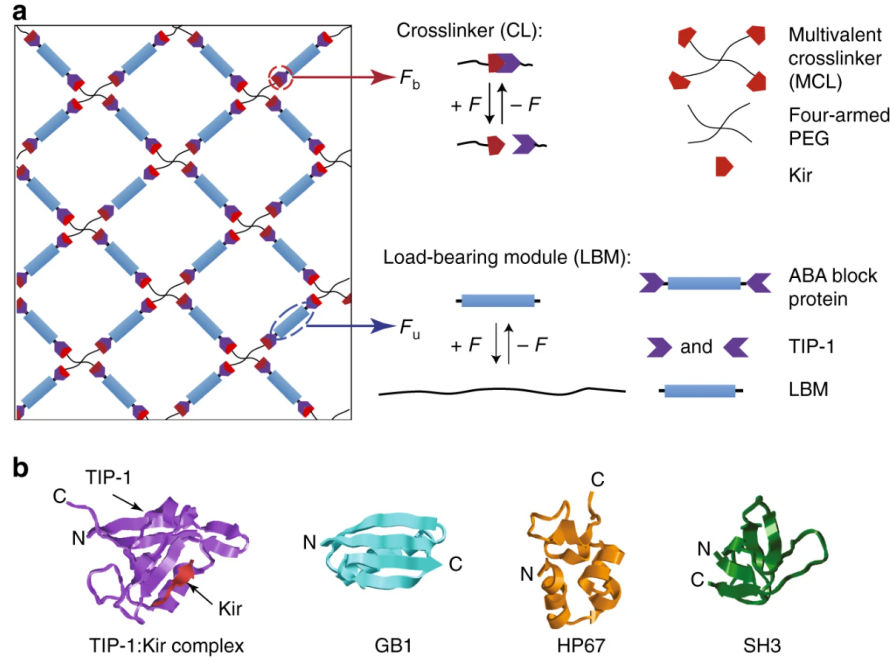


Figure 1.20: A) Schematic of protein hydrogel network design composed of cross-linkers between load-bearing molecules. The application of force to the cross-linker will break the cross-linker. Force applied to load-bearing protein will unfold the protein. B) Examples of proteins investigated for protein hydrogels: TIP-1:Kir, PDB: 3DJ1, GB1 (PDB: 3GB1), HP67 (PDB: 2RJY), and SH3 (PDB: 1PRL). Figure reused from Wu *et al.* (2018) [122] with permission from CC BY 4.0.

the functionalised 4-armed PEG molecule) that had a rupturing force (F_{break}) that was weaker than the force required to unfold (F_u) the load-bearing protein, GB1 (PDB: 3DJ1). The second scenario (Gel 2 in Table 1.1) investigated load-bearing protein SH3 (PDB: 1PRL), which required a lower force to unfold than the force required to rupture the TIP-1 cross-linker: Gel 1 showed that when the unfolding force of the protein is

Table 1.1: Properties of different proteins and the predicted properties of the protein hydrogel behaviour as a result of cross-linking the proteins. Data taken from Wu *et al.* (2018) [122].

Hydrogel	Protein	F_U	Forces	hydrogel properties
Gel 1	GB1	180 pN	$F_U > F_{break}$	rigid, brittle
Gel 2	SH3	25 pN	$F_U < F_{break}$	Rigid, extensible, tough

greater than the force required to rupture the protein ($F_U > F_{break}$), the cross-link will break before the protein is unfolded. This leads to mechanical properties in the protein hydrogel showing a lack of ability to be stretched over a long distance. Conversely in

Gel 2, when the unfolding force of the protein is weaker than the force required to rupture the protein ($F_U < F_{break}$), the protein unfolded before the cross-link broke. This allowed the protein hydrogel to extend over longer distances and was less brittle than Gel 1. This example demonstrated that not only does the protein influence hydrogel behaviour, but the mechanical strength of the cross-linker is also important. Predicting the bulk mechanical properties of folded protein hydrogels from the nanoscale protein is an important example of ‘bottom-up’ material design.

The previous studies mentioned here link the nanoscale and macroscale properties of folded properties, without giving insight to the in between mesoscale properties, i.e. the structure of the network formed by the proteins. A unique approach taken by de Silva *et al.*[119] combined the single molecule AFM force spectroscopy of unfolding different proteins, structural data from small angle neutron scattering (SANS), and viscoelastic properties from rheology experiments with the aim to find links between nanoscale and macroscopic properties. de Silva *et al* investigated two different pentameric polyproteins (I27)₅ and (pL)₅ and compared an (I27)₅ protein hydrogel, (pL)₅ protein hydrogel and a 1:1 blend of (I27)₅/(pL)₅ of the two proteins. The stiffness of the individual (I27)₅

Table 1.2: Properties of different proteins and the predicted properties of the protein hydrogel behaviour as a result of cross-linking the proteins. ξ is the correlation length of fractal-like clusters of proteins. Data taken from de Silva *et al.* (2017) [119].

Polyprotein	Folded G' (kPa)	Unfolded G' (kPa)	ξ (Å)
(I27) ₅	27 ± 6	2.7 ± 0.5	200
(pL) ₅	26 ± 4	0.051 ± 0.009	172
(I27) ₅ /(pL) ₅ 1:1 blend	7.6 ± 1.4	1.0 ± 0.2	174

and (pL)₅ protein hydrogels exhibited similar mechanical properties, while the blend ((I27)₅/(pL)₅) produced a significantly weaker hydrogel. To understand the origin of the difference in stiffness, SANS was used to characterise the structure, predicting that all the protein hydrogels observed had fractal-like clusters of proteins connected with chains of proteins in between the clusters. The correlation length (ξ) of the clusters of proteins in the protein hydrogel network can be conceptualised as the size of the largest clusters. Comparing ξ revealed that the (I27)₅/(pL)₅ blend and (pL)₅ hydrogels were similar, and significantly smaller than that of the (I27)₅ protein structure. This result

suggested that the (pL)₅ forms more compact, smaller clusters of proteins than the (I27)₅. This demonstrates that the choice of protein can control the mesoscale structure even when the bulk properties are similar, in addition to being able to tune the stiffness of the bulk hydrogel taking two different proteins and mixing them together. Further studies have demonstrated that changes to the protein or the cross-linking procedure can result in vast changes to the resultant mesoscale and mechanical properties, allowing for tuneable stiffness in protein hydrogels .

Monomeric BSA (PDB: 3V03) protein hydrogels have demonstrated tuneable properties through different hydrogel preparation methods and by manipulating the BSA protein. Aufderhorst-Roberts *et al.* (2020) showed for photo-initiated cross-linking, the stiffness of BSA hydrogels can be increased by elevating the rate of chemical cross-linking with increased power of the LED used for photo-initiation [123]. The rate of cross-linking allowed control over the network formation, where fast reaction rates had diffusion limited aggregation of the protein, while in the aggregation of protein with slow reaction rates, the reaction rate limited aggregation. Khoury *et al.* (2020) demonstrated that BSA hydrogels that adsorb to divalent cations such as Ni²⁺ and Cu²⁺ were able to increase the stiffness (17-fold) of the BSA hydrogels [115]. These BSA hydrogels were used to control a change in shape of the bulk hydrogel by the addition of divalent cations.

Using unfolded proteins to assemble a protein hydrogel network has previously been demonstrated to change the mechanical stiffness of the bulk protein hydrogel. At 60°C BSA begins to denature and as the BSA monomer unfolds, it re-assembles into amyloidic fibers. Khanna *et al.* (2020) demonstrated a novel gelation method whereby protein aggregation was initiated through short burst of low temperatures (65°C), and gelate the BSA without any external cross-links [124]. These BSA hydrogels were able to reversibly transition from sol to gel states, however at the cost of further unfolding the BSA. In the initial sol state the BSA structure was composed of 61% α -helices, after heating to transition to the gel state 39% of the secondary structure was % α -helical, and reversing to the sol state only 8% of the structure remained % α -helical. The time for gelation and transition time from sol to gel was tuned by the concentration of BSA and the addition salt at different concentrations. A study by Haas *et al.* (2022) showed when

preparing BSA hydrogels with ethanol, increasing the concentration of ethanol causes an increase in the stiffness of the final hydrogel [125]. Changes to the nanoscale protein were confirmed with attenuated total reflectance-infrared spectroscopy (ATR-IR), where with BSA hydrogel preparation with low concentration of ethanol had a secondary structure with a lower ratio of α -helix: β -sheet than when the BSA hydrogel was prepared at a higher concentration of ethanol. This confirmed that changes to the secondary structure of the protein were influencing the behaviour of the bulk, rheological properties.

The level of control with folded protein hydrogels on each level scale makes them a fascinating material to work with. Tuning how the hydrogel is prepared, the building block, or the cross-links between the building blocks (Figure 1.21), can result in multi-length scale changes from the mesoscale structure to the viscoelastic properties of the bulk. Changes made to the protein, the cross-linking mechanism or environment in gelation can all tune protein hydrogel behaviour. Armed with this information, the future of patient tailored hydrogel drug delivery with the production of hydrogels with chosen strength, functionality and drug release profile could be utilised for improved therapeutic treatment. This provides the opportunity for tuning the properties of folded protein hydrogels for different biomedical applications such as cell culture, where the stiffness of the hydrogel is important for providing cells an environment that mimics their natural tissues. The range of mechanical properties exhibited by tissues in the body ranges from 1 kPa up to 1 GPa, demonstrating the diverse needs for biomedical hydrogels in tissue replacement and drug delivery patches. Figure 1.22 demonstrates that even single folded protein hydrogels can be tuned to have stiffness in the range of 1-50 kPa, with the potential of tailoring the mechanical stiffness to the application. It is desirable to have predictive control of hydrogel mechanics to replicate any given tissue stiffness, or tuning the structure of hydrogel to tailor the drug release profile to the application.

Folded proteins hydrogels have demonstrated the ability to respond to external stimuli such as chemical modulation and light exposure [138], have programmable shape changes [139], are responsive to the hydrogel solute environment [140], as well as the ability to control and preserve mechanical properties [121–123, 141]. Using a functional

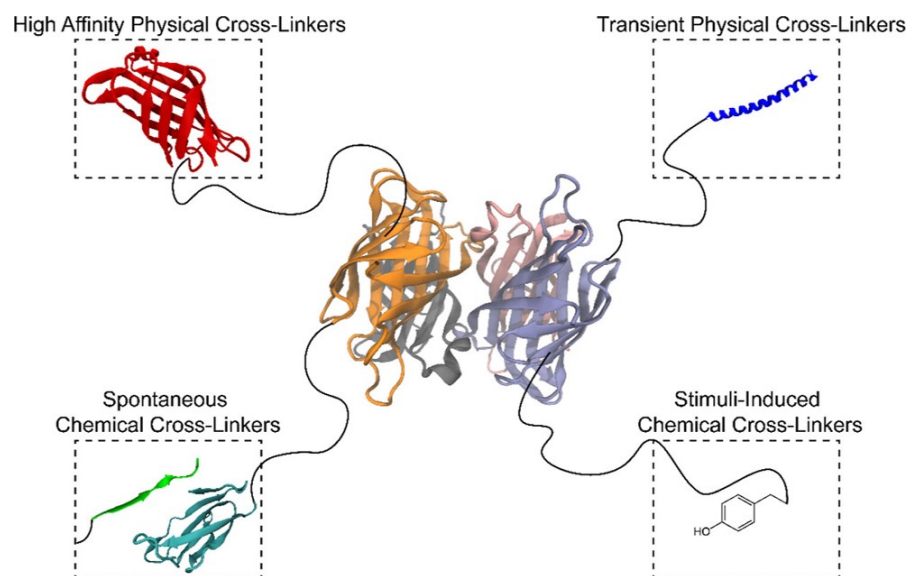


Figure 1.21: Comparison of different ways to cross-link protein hydrogels with both physical and chemical cross-links streptavidin homotetramer (centre). Top left: streptavidin monomer physical cross-linker. Top right: coiled-coil protein monomer physical cross-linker (PDB: 1MZ9). Bottom left: SpyTag-SpyCatcher spontaneously formed chemical cross-linker (Spy in cyan and green, PDB: 4MLI). Bottom right: dityrosine photo-initiated chemical cross-linker. Figure reused from Knoff *et al.* (2022) with permission from [?]. Copyright 2022 American Chemical Society.

protein building block that has inherent thermodynamic stability, in addition to chemical or mechanical functionality can be advantageous over a polymer or peptide hydrogel. The functions that proteins can perform is vast, but these are yet to be fully utilised for functionalised hydrogels that could lead to new applications in bio-medics[121, 142]. Huerta-López *et al.* (2021) highlights that although folded protein hydrogels have a wide variety in tunable properties, for applications in drug delivery further research is required to have more complex drug release profiles for controlling drug release from folded protein hydrogels [108]. Typically, drug release from protein hydrogels relies on diffusion of the drug out of the hydrogel leading to short times for drug release and the lack of control to tune the kinetics of drug release to suit different applications. Incorporating a triggered release mechanism would allow for the release of drugs at the desired time point, increasing the window for efficacy and potentially decreasing the side effects of the drug.

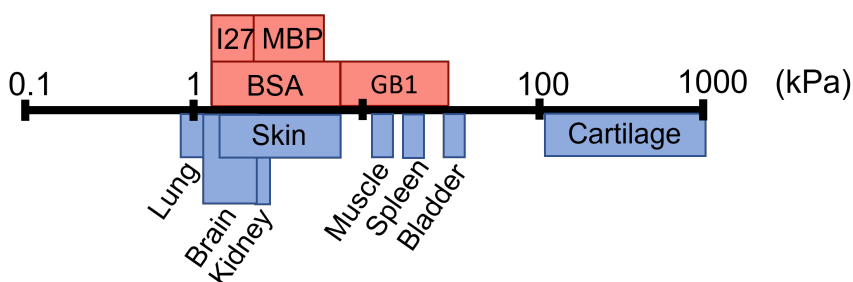


Figure 1.22: Shear modulus (G') of different biological tissues (blue) and the G' of folded protein hydrogels (red) including bovine serum albumin (BSA), maltose binding protein (MBP), guanine nucleotide-binding protein (GB1), and interleukin-27 (I27), where the data is collated from multiple sources. [115, 119, 126–137]

1.3 Microbubbles

The following section introduces the background and relevant information to microbubbles. This includes their ultrasound responsive properties utilised in microbubble ultrasound contrast agents, and the development of MB for improved stability and the application of microbubbles for drug delivery.

1.3.1 Introduction to microbubbles

A microbubble (MB) is defined as gaseous core with a diameter of 1-10 μm [143]. The gaseous core is typically stabilised by a surfactant, polymer or protein shell, to improve the lifetime of the MB. Due to MBs being highly compressible, they undergo volumetric oscillations when ultrasound is applied to them [144]. MBs gained initial interest as an ultrasound (US) contrast agent to monitor the flow of blood; there is a significant acoustic impedance mismatch between the MB gas core and surrounding liquid medium, in this case blood allowing MBs to be highly visible with US [145]. This caused the MBs to scatter more US than the red blood cells, and therefore blood flow could be monitored by tracking the movement of the MBs in the blood with US [146]. The echoscattering (echogenicity) is also distinguishable from tissues, therefore increasing the US signal intensity and reducing signal-to-noise ratio in imaging in some tissues [147, 148]. The use of US is an attractive method of drug release in the clinic, due to being a

non-ionizing technique [149–151]. MBs are clinically available, and are routinely used for imaging the left ventricle of the heart, and used in the diagnosis of some cancers [152]. Clinically accepted MBs are less than 3 μm in diameter, as larger MBs could cause embolisms in the vasculature system [147, 153, 154]. The development of MBs over the last few decades has seen improvements in the lifetime of storage and circulation *in vivo* [155–157], the ability to target MBs to the region of interest [158, 159], and echogenicity of MBs under US [160, 161]. The choice of shell is also important in terms of the MB acoustic response. MBs are often stabilised by a lipid shell, as lipids self-assemble into a monolayer and allow for a more flexible shell, that can withstand higher strain without rupturing [162]. The stability of MBs is also important for their success in clinic. MB lifetime can be improved with inclusion of stabilising molecules into the MB shell. PEGylated lipids have shown to increase stability of lipid shelled MBs with long circulation times *in vivo*, reducing the removal rate of the drug encapsulated in MBs [163]. This is a result of PEGylated lipids cause steric hinderance between the MB and *in vivo* biomacromolecules, preventing biomacromolecules from interacting with the MB shell. Microbubbles can be produced either by mechanical agitation, where shell components are saturated with gas and violently shaken, or with microfluidics [164]. In the microfluidic method, typically a flow-focusing device forces gas through a small nozzle, where the stream of gas is 'pinched' by incoming streams of liquid and the changes in pressure resulting in MB formation [165].

1.3.2 Microbubbles for drug delivery

MBs are clinically approved as ultrasound contrast agents [145, 150, 166, 167], and more recently phospholipid MBs have been investigated for drug delivery therapeutics [147, 168, 169]. Phospholipid shelled MBs can be adapted for loading with drugs via multiple routes, as demonstrated in Figure 1.23 [170]. Hydrophobic drugs can be loaded into the lipid shell by mixing the drug with the lipids before the MBs are produced, where the drug molecules associate with the lipid tail. The drug loading capacity is limited by the MB shell thickness [171]. To improve drug-loading, fat soluble hydrophobic drugs can be incorporated into the inside of the MB shell by dissolving drugs into an oil layer

[172, 173]. Electrostatic interactions can be used to load hydrophilic drugs onto the surface of the MB. This can be achieved by including lipids within the shell that have positively charged head groups, allowing for negatively charged drugs such as plasmid DNA to be loaded to the MB surface [174, 175]. Alternatively, drugs can be loaded into nanoparticles by linking the shell to the nanoparticle [176]. Biotinylated lipids and avadin can be used to conjugate liposomes (aqueous core stabilised by a lipid bilayer). By incorporating the biotinylated lipids into the lipids shell of the MB and liposomes, they both can link to avidin, allowing for 10^5 liposomes to be loaded to the MB surface [177, 178].

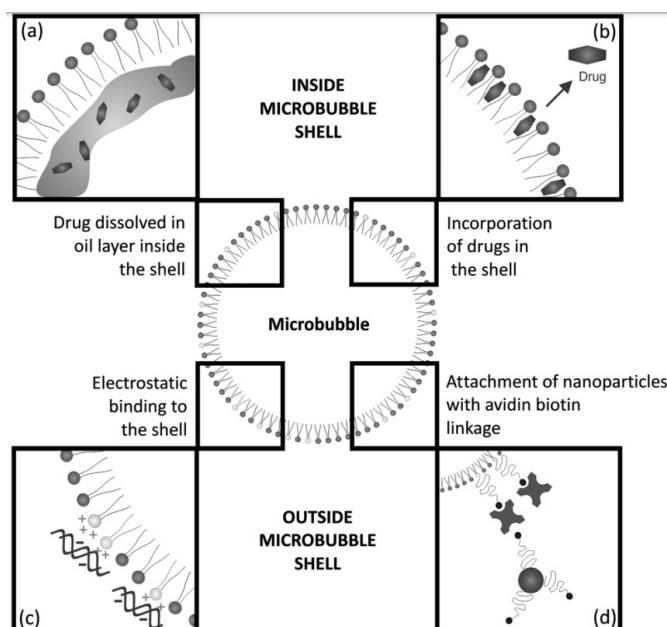


Figure 1.23: Phospholipid microbubbles (MB) can be loaded with drugs either in an oil layer coating the inside of the MB, into the inside of the lipid shell, with electrostatic interactions on the outside of the shell, or in nanoparticles that are attached to the outside of the shell. Reproduced from Lentacker *et al.* (2009), with permission from the RSC [170].

When exposed to US, MBs undergo volumetric oscillations known as stable cavitation. If the US pressure is high enough, the MB will expand to at least twice its original size and violently collapse, referred to as inertial cavitation. This is described in more detail in Section 1.3.5. Drug release from drug-loaded MBs can be triggered by the application of US induced MB destruction. The ability to target MB drug release to the region of interest can allow for improved treatment, as demonstrated in Figure 1.24. An

US transducer is applied to the body to instigate MB drug release local to the region of interest. The US beam can be focused to a small region of interest [179]. This allows the time and location at which US is applied to be controlled, releasing of drugs from only those in the path of the US beam [180–182]. Chemical targeting can be achieved by including a targeting moiety in the lipid shell, which attaches to the region of interest [181, 183]. The exposure of drugs to healthy cells is reduced and therefore the negative side effects that come with exposure to the drug is also reduced, as demonstrated in Figure 1.24 [60, 158]. Targeting is particularly important in the case of chemotherapeutic drugs which have severe side effects, and can reduce the quality of life of the patient [184]. Due to the volumetric oscillations of MBs when exposed to US, MBs have also demonstrated the ability to exert a shear stress on cell membranes. The acoustic response of MBs to US includes microstreaming from stable cavitation, and shockwaves from inertial cavitation where by causing a shear stress on cell membranes the cells become more permeable [185, 186]. This mechanism is known as sonoporation and can enhance the drug uptake of cells from their increase in permeability [187, 188]. Binding drugs that degrade quickly in the bloodstream to MBs, such as nucleic acids and proteins, has the additional advantage that the rate of degradation from the MB protecting the drug could be reduced [189]. Additionally, as MBs can be imaged with ultrasound, imaging and drug delivery can be combined, which can be advantageous to accurately find the region of interest for triggering drug release. Bai *et al.* 2019 demonstrated MBs that were able to target to tumours, determine the difference between tumours and healthy tissues, and show increased uptake of chemotherapeutic drugs in the cells [190].

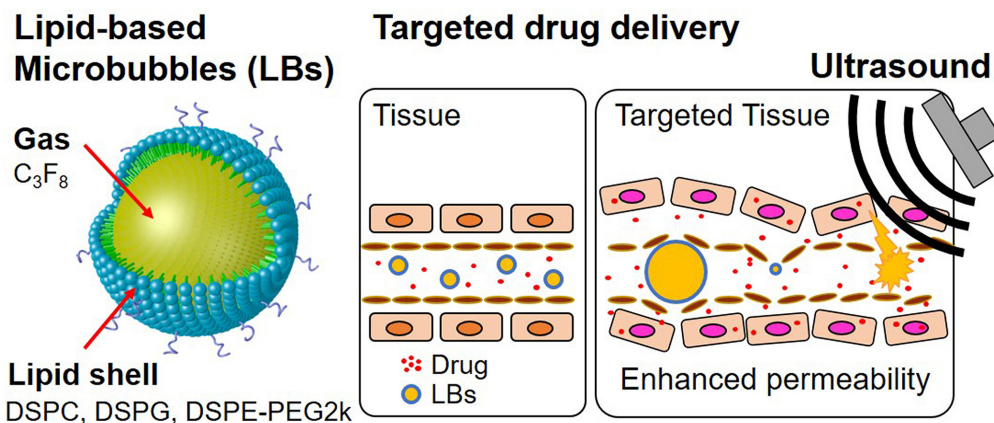


Figure 1.24: Microbubbles (MBs) can be used for drug delivery. Left: A typical microbubble composition with a perfluoropropane (C₃F₈) gas core, stabilised by a phospholipid shell. Right: MBs can be targeted to specific cells and tumours, alternatively the location of drug release can be selected with the application of ultrasound local to the region of interest. Reproduced from Omata *et al.* (2020) [191] with permission from Elsevier.

1.3.3 Microbubbles in Hydrogels

The previous section described how MBs are an excellent tool as an ultrasound contrast reagent and in controlled drug delivery. Their ultrasound responsive properties are more recently being applied to MBs embedded within soft materials such as hydrogels [192]. By bringing together hydrogels and microbubbles into one composite system, the attractive qualities of the individual components can be taken advantage of in a more complex system. Embedding MBs within hydrogels offers a solution to some of the challenges faced by microbubbles and hydrogels biomedical applications. Introducing hydrophobic drugs into hydrogels can be challenging due to the high water content [193]. Alternatively, hydrophobic drugs could be incorporated into hydrogels by including MBs loaded with hydrophobic drugs. Smart hydrogels have offered an array of methods for drug release from changes to environment *in vivo*, as described in Section 1.2.1. If drug release from hydrogels is desired at a defined time point, and there is no *in vivo* change to trigger drug release, having acoustically response hydrogels offers a solution for triggering drug release without the need for internal cues [194]. Furthermore, hydrogels can also be used as a model of MBs in tissues *in vivo*. The oscillations of MBs in a liquid environment can be more violent than in viscoelastic solids such as soft tissues, Imaging of MBs in

hydrogels can help determine the appropriate US properties to maximise the oscillations of MBs in viscoelastic solids, optimising image quality. The reader is directed to other studies on this topic [195, 195, 196].

Mechanically stiff hydrogels are often required for biomedical applications involving stiff tissues, however increasing the porosity can compromise the stiffness of the hydrogel. Stiffness is an important parameter in biomedical applications of hydrogels [197, 198], therefore pore formation by a method which does cause large decreases in the hydrogel stiffness is desirable. Gao and Cho (2022) determined for hydrogels with a low polymer volume fractions, the bulk modulus (κ , quantifying the hydrogel stiffness) can be related to permeability (P , how easily fluid can flow through the hydrogel network) by $\kappa^{-9/8} \propto P$ [199]. In the scenario of wanting to tune the stiffness of a hydrogel, for example increasing the cross-linking density to increase the stiffness, there is a trade off with the reducing solute permeability through the hydrogel network. MBs offer an alternative way of creating porous networks, that exhibits only minor change the mechanical stiffness of the hydrogel [200, 201]. Pores are commonly introduced in hydrogels via freeze drying, however this can also compromise mechanical stiffness of the final hydrogel [202]. Using MBs to create pores in hydrogels is preferential for pore formation that does not involve a drastic decrease in temperature. Pores can be introduced into a hydrogel by embedding MBs within the gel and bursting the MBs with ultrasound after gelation. Furthermore, the size and monodispersity of MBs can be controlled, leading to uniform pores of a desired size in materials upon destruction of the MBs [203–205]. Increasing the porosity of hydrogels increases the diffusion of entrapped molecules, leading to a larger release of the entrapped molecules from the hydrogel. The porosity of hydrogels is a key component in biomedical applications, affecting both both drug delivery and tissue engineering. By increasing the porosity of hydrogels the release of entrapped drug molecules from the hydrogel could increased for drug delivery, or the release of nutrients for improving cell growth.

1.3.3.1 Ultrasound Stimulated Drug Release from Hydrogels

Different methods of acoustically stimulated drug release from hydrogels has been explored in the literature [194]. Acoustic droplets are liquid droplets that can be vaporised under the application of US. The vaporisation occurs due the liquid being superheated due to the changes in pressure of an US wave. Studies using acoustic droplets in hydrogels for drug delivery have been explored for controlled drug release, and some of the techniques could be applied to MBs within hydrogels. The controlled burst release from the vapourisation of acoustic droplets within hydrogels has been explored by changing the acoustic pressure of the ultrasound. Moncion *et al.* (2018) [206] produced two samples of acoustic droplets the first with a perfluorohexane core, and the second with a perfluoropentane core. Due to the two different compositions used in the core, the application of a low acoustic pressure of 2 MPa primarily vaporised the acoustic droplets with a lower boiling point (perfluoropentane) and a second acoustic pressure of 8 MPa vaporised both acoustic droplets with perfluoropentane and perfluorheptane cores. Both sets of acoustic droplets contained fluorescent dextran in the core, and two acoustic droplets samples were mixed together to monitor the release profile of the dextran from the blended solution (Figure 1.25A). This achieved a controllable release profile of two different dextrans, triggered by changing the acoustic pressure ultrasound waves (Figure 1.25D), which slowed down the release of dextran from the hydrogel when compared to dextran free in the hydrogel. This control has the potential for co-delivery of two different therapeutic payloads from fibrin hydrogels, in a controlled manner, however high acoustic pressures were required to vapourise the droplets.

Epstein-Barash *et al.* (2010) loaded liposomes with phenazine methosulfate drug and encapsulated the liposomes in an injectable hydrogel [200]. The drug release from the hydrogel was compared for different scenarios including free drug in the hydrogel, drug loaded liposomes in the hydrogel, and drug loaded liposomes in the hydrogel with the addition of US application. The drug loaded liposomes prolonged the time over which drug was released from the hydrogel, and the further addition of US application saw to the hydrogel with drug loaded liposomes increased the total drug released from the

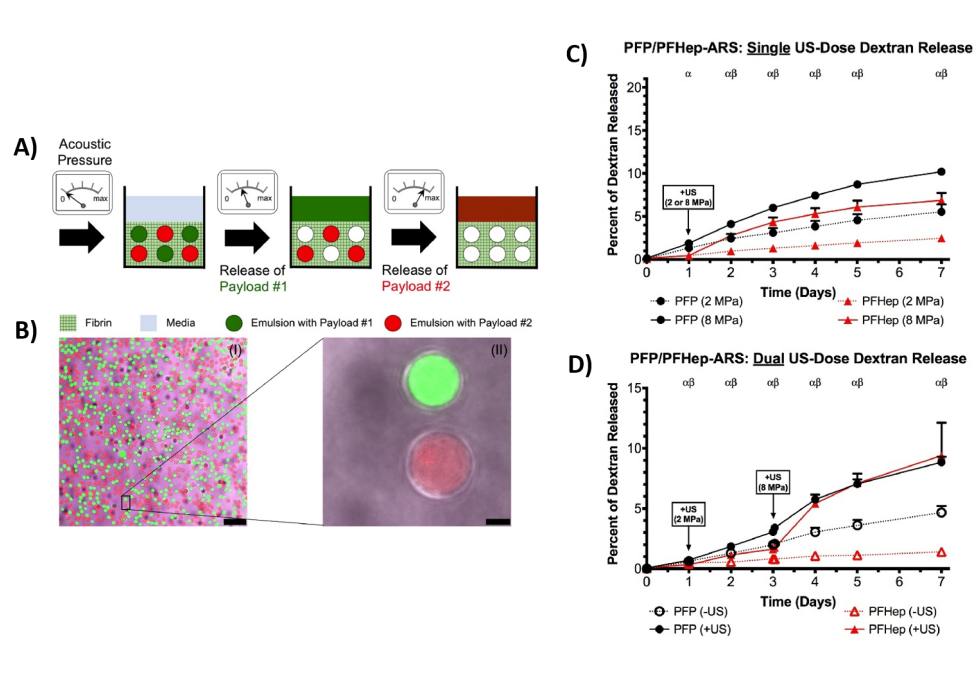


Figure 1.25: A) Schematic US triggered release of two different fluorescently labelled dextrans (Alexa Fluor 488 and Alexa Fluor 594-labelled dextrans) at a lower and higher acoustic pressure from MBs into hydrogel scaffold and B) confocal images of the different fluorescent dextrans from the acoustic droplets. C) A mix of perfluoropentane (PFP) and perfluoroheptane (PFHep) droplets were vapourised in the hydrogel (referred to in this study the acoustic responsive scaffold, or ARS) on day 1 and the release of fluorescent dextrans monitored from the two emulsions over time. D) PFP and PFHep droplets encapsulated in the hydrogel with a lower US pressure on day 1 (2 MPa) followed by higher US pressure on day 3, with the release of fluorescent dextran from the vapourised droplets over 7 days. Reproduced from [206] with permission from Elsevier.

hydrogel. Lin *et al.* (2016) aimed to address one of the challenges of treating bladder cancer, where drugs are rapidly removed from the bladder due to renal clearance [207]. To prevent a hydrogel from blocking the urethra, or irritating the bladder, Lin *et al.* produced a hydrogel with large MBs (20 - 400 μm) encapsulated such that the hydrogel floated in urine. Three scenarios were compared: free Rhodamine (RhB), RhB in a non-floating hydrogel, and the floating hydrogel were compared in the bladder. The release of RhB in the bladder was extended over a longer period of time from the floating hydrogel, with an higher total release of RhB from the floating hydrogel, compared to the non-floating hydrogel. This result is likely to do with the higher porosity in the floating hydrogel increasing RhB diffusion. The non-floating hydrogel bound to the inside of the bladder wall, resulting in irritation of the bladder and was quickly removed from

the bladder. The floating hydrogel remained in the bladder for a longer period of time, demonstrating less bladder irritation.

These studies show the potential of using a composite MB-hydrogel system for controlled drug delivery, however to the best of the author’s knowledge only a handful of studies have demonstrated controlled drug delivery from MBs encapsulated with hydrogels. In drug delivery, increasing the porosity of a hydrogel also allows for increased diffusion of entrapped drug for treatment. By bursting drug-loaded MBs in hydrogels, the hydrogel becomes more porous at the same time the drug is released into the hydrogel network. Acoustic droplet vapourisation offers several examples of successfully controlling drug release from hydrogels, however high acoustic pressures were required which could cause unsafe levels of heating when considering translation to the clinic. Using MBs for ultrasound triggered drug release from hydrogels has the potential to create a drug release profile, that can be controlled by the application of US with safe acoustic pressures, in a wider range of treatments than are currently reported.

1.3.3.2 Hydrogels with Microbubbles Embedded as Tissue Engineering Scaffolds

Embedding MBs within hydrogel scaffolds is beneficial for tissue engineering, firstly being able to trigger the release of nutrients for cells or growth factors and secondly by bursting the MBs, a more porous hydrogel is produced for increased cell-to-cell communication [208–211]. Hydrogels can be used for culturing cells on top of the hydrogel for 2D cell culture, or cultured within the hydrogel for 3D cell culture. The importance of mechanical properties is highlighted when using hydrogel scaffolding to culture cells, where cells have been shown to respond differently to a range of mechanical properties of hydrogels [212]. Implantable hydrogels are chosen to deliver growth factors over conventional injections of solutions of growth factors for maximal efficacy, and to further control the release of basic fibroblast growth factor (bFGF) from the fibrinogen hydrogel scaffolds. Dong et. al. demonstrated a burst release of bFGF from MBs encompassed within hydrogel scaffolds enhance endothelial network formation and stimulating vessel

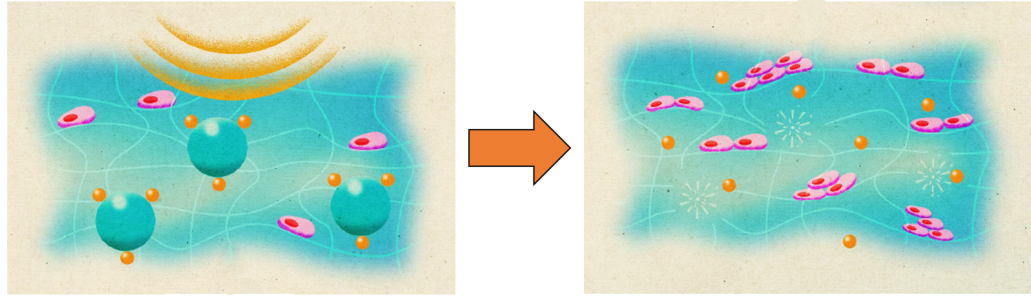


Figure 1.26: Schematic of Left) MBs loaded with nutrients (orange) in a hydrogel used as a tissue scaffold for cell (pink) growth. Right) After US application, the encapsulated nutrients for the cells are released, increasing the proliferation of cells [213].

growth[214]. Melino *et al.* (2016) investigated enzyme coated MBs with air cores were used in PEG-fibrinogen hydrogels, to stimulate the production of hydrogen sulfide (H_2S) in the hydrogel [201]. H_2S is a gastrotransmitter (a gaseous signalling molecule, that acts as a neurotransmitter), therefore a hydrogel that slowly releases H_2S is an ideal model for understanding how H_2S affects cell proliferation. The MB-hydrogel was tested as a 3D scaffold for the growth of cells of human Sca-1^{pos} cardiac progenitor cells (hCPCs), and found an increase in cell proliferation correlated with the production of H_2S . Melino *et al.* not only addressed the importance of biological cues for cell growth, but also the mechanical stiffness observing a minor decrease in the stiffness of MB loaded hydrogels. The selection of gas in the MB core has been considered for improving cell proliferation in hydrogels. Lui *et al.* (2022) used oxygen MBs embedded within an alginate hydrogel to supply oxygen to cells for 3D cell culture and increase the porosity of the hydrogel upon ultrasonically bursting the MBs [215]. This resulted in a higher proliferation of cells with the alginate hydrogel pores as well as an increased rate of wound healing in type I diabetes rat models, where lack of oxygen is often a problem. Cartilage tissue has a poor recovery after a trauma, motivating Wu *et al.* (2018) [216] to explore gelatin hydrogel scaffolds for cartilage tissue engineering . Different sized MBs were embedded during gelation and burst to create gelatin with different, controlled pore sizes and then used to culture human adipose-derived stem cells (hSACs). The hSACs cells behaviour showed

that larger pore sizes enhanced the chondrogenic differentiation, emphasising that a large pore size in hydrogel scaffolds for tissue engineering is vital for cell culture, where cell-to-cell communication and access to nutrient is improved with a porous, permeable hydrogel.

1.3.4 Composition and stability of MBs

The initial discovery of MBs were air bubbles without any shell, used to improve the imaging contrast of left ventricle of the heart [217]. Since then, the lifetime of MBs for biomedical applications has been increased by using a shell to encapsulate the gas core, and selecting gases that have higher molecular weights than air[218–220]. This section will discuss the background to MB stability and common MB compositions chosen to enhance the lifetime of MBs.

The stability of MBs is often characterised by the change in the size of the population of MBs over time. How the size of a MB changes over time is related to the internal gas pressure, the external pressure of the medium surrounding the MB and from surface tension of the liquid shell. The Laplace pressure describes the pressure difference between the internal and external MB pressures:

$$\Delta P = P_b - P_a = \frac{2\sigma}{r} \quad (1.4)$$

Where P_b is the internal pressure within the bubble, P_a is the hydrostatic pressure external to the bubble, σ is the surface tension and r is the radius of the bubble. Gas movement out of the MB changes the pressure within the MB, where the pressure a MB experiences is a balance between internal gas pressure and external pressure from surface tension of the liquid shell. If small changes in the size of MB occur, the system is not thermodynamically stable, and the Laplace pressure states that the size of the MBs will drastically increase or decrease, this phenomenon is known as the ‘Laplace bubble catastrophe’. MB stability is dependant on the rate that of gas dissolution from the MB into the surrounding medium, which is taken into consideration with the Epstein-Plesset

model:

$$-\frac{dR}{dt} = H \frac{1 - f + \frac{2\gamma}{P_a R}}{1 + \frac{4\gamma}{3P_a R}} \left[\frac{D_w}{R} + \left(\frac{D_w}{\pi t} \right)^{1/2} \right] \quad (1.5)$$

where R is the radius of the bubble, P_a is the ambient pressure, γ is the surface tension of bubble, f is a ratio of the concentration of the gas initially dissolved in the system to the concentration of gas at saturation, D_w is the diffusivity of gas in the surrounding medium, and H is the Ostwald coefficient:

$$H = \frac{C_a}{C_g} \quad (1.6)$$

where c_a is the gas concentration in the aqueous phase, and c_g is the gas concentration in the gas phase.

The presence of a shell around the gas core provides a barrier that the gas has to diffuse through, therefore decreasing the rate of gas dissolution and increasing stability of the MB. A modification to the Epstein-Plesset model, developed by Borden and Longo introduced a term (R_{shell}) which represents the resistance to mass transfer through a lipid monolayer. This accounts for the reduces rate of gas dissolution from a lipid stabilised MB [221].

$$-\frac{dR}{dt} = \frac{H}{\frac{R}{D_w} + R_{shell}} \left(\frac{1 + \frac{2\gamma}{P_a R} - f}{1 + \frac{4\gamma}{3P_a R}} \right) \quad (1.7)$$

The composition of the MB shell therefore plays a vital role in defining the surface tension and rate of gas diffusion out of the MB, therefore controlling the stability of the MB. This was demonstrated by Borden *et al.* (2002) for lipid MBs, where a MB shell composed of 1,2-diacyl-sn-glycerol-3-phosphatidylcholine lipids (DPPC) with 16 carbons in the acyl chain, was compared to the same lipid with a longer acyl chain length of 22 carbons (Di-22-PC). MB shells composed of Di-22-PC had higher gas permeability and longer lifetimes over MB shells composed of DPPC. MBs are often stabilised by a lipid shell, as lipids self-assemble into a monolayer and allow for a more flexible shell, that can withstand higher strain without rupturing.

Another route to MB instability is through the buoyancy of the MBs, whereby the MBs rise to the surface of the liquid. MBs at the liquid-gas interface become unstable

and rupture due to the lack of gas-liquid interface that was previously supporting the MB. The dynamic viscosity of the medium surrounding the MB (μ) and density of the core are crucial to the velocity at which the MBs rise (U):

$$U = \frac{gd^2\Delta\rho}{18\mu} \quad (1.8)$$

where g is the acceleration due to gravity, d is the MB diameter, $\Delta\rho$ is density difference between the MB core and the medium surrounding the MB. This is determined by Stokes drag force ($F_d = 6\pi\mu rU$) and buoyancy force ($F_b = \frac{4}{3}\Delta\rho\pi r^3$), where the MB is assumed to rigid and spherical.

The previous instabilities described only take into account a singular MB, when in reality a MB will be in a sample surrounded by many other MBs. When two or more MB meet, they can merge, referred to as coalescence. In addition, the population of the MBs also influences the MB stability. Ostwald ripening is a mechanism that describes that as small particles shrink, large particles grow from the displaced material of the smaller particles. Considering MBs, as small MBs shrink, the fluid local to the MBs becomes supersaturated with gas, allowing the larger MBs to increase in size [222]. This behaviour is based on the thermodynamic instability of the MBs, where larger particles being energetically more favourable than smaller MBs. Ostwald ripening is dependant on the surface tension of the MB, as the stability of the molecules at the liquid-gas interface varies with the MB size. Kelvins equation 1.9 states the chemical potential between the MB surface and core ($\Delta\mu$) is inversely related to the size of the MB:

$$\Delta\mu = \frac{2\gamma\Omega}{r} \quad (1.9)$$

where Ω is the molar volume of the MB and the chemical potential (μ) is defined as:

$$\mu = kT \ln\left(\frac{P}{P_{sat}}\right) \quad (1.10)$$

where P is vapour pressure and the P_{sat} is the saturated vapour pressure. The gas dissolution from small MBs to the surrounding liquid upon MB shrinking, which is then

transferred to larger MBs as larger MBs are more energetically favourable. The Gibbs-Thomson equation defines a critical radius (R_c), where MBs smaller than R_c will shrink and MBs greater than R_c will grow:

$$\frac{C}{C_{eq}} = \exp\left(\frac{R_c}{r}\right) \quad (1.11)$$

$$R_c = \frac{2\gamma\Omega}{k_B T} \quad (1.12)$$

where C is the solute concentration, and C_{eq} is the equilibrium concentration.

1.3.5 Microbubbles and Ultrasound

Sound waves propagating through soft materials behave as longitudinal waves, where the particle movement is parallel to the direction as the wave. The sound wave gives energy to particles in its path, causing the particles to oscillate locally and transferring energy to neighbouring particles. The oscillation of particles results in regions in the sound wave of high pressure where the particles are more densely packed known as compression, or low pressure regions where the particles are more spread out, known as rarefaction, depicted in Figure 1.27. The wavelength of a sound wave (λ) is therefore determined by the distance between two regions of compression. The speed of a sound wave (c) is described by Equation 1.13, where f is the sound wave frequency.

$$c = \lambda f \quad (1.13)$$

Ultrasound is defined as sound waves with a frequency above 20 kHz, generated and detected by a transducer, where a piezoelectric crystal vibrates under an oscillating electric field. When applying an US pulse with an oscillating pressure, MBs due to their compressible gas core, MBs to undergo volumetric oscillations, such that the MBs expand and contract in the rarefaction and compression regions of the ultrasound wave, demonstrated in Figure 1.27. At low acoustic pressures, MB oscillation is linear and symmetrical. Energy not used in volumetric oscillations is radiated as acoustic emission. Higher acoustic pressures result in non-linear and non-symmetrical oscillations

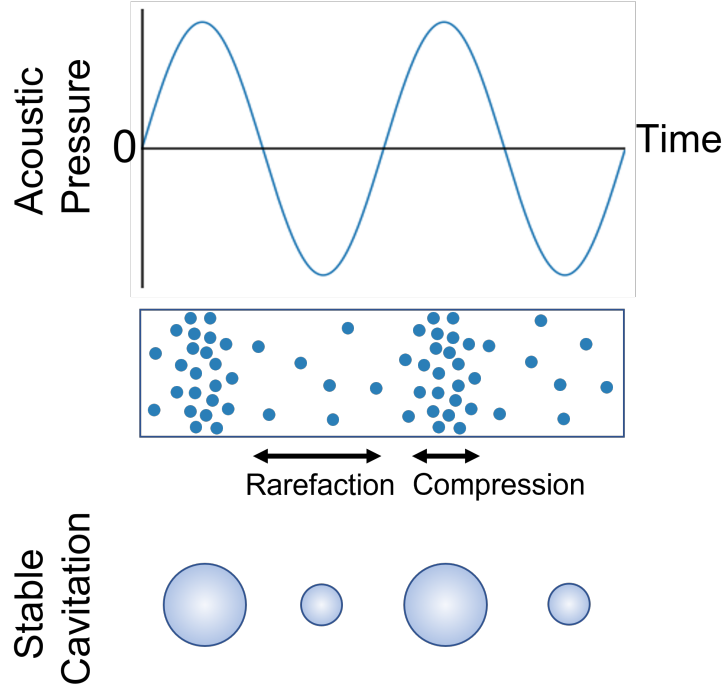


Figure 1.27: Ultrasound waves cause oscillations of particles, with areas rarefaction (lower pressure) and compression (higher pressure). When MBs are exposed to ultrasound, the small changes in acoustic pressure cause the MB undergo stable cavitation, where the MB volume expands and contracts.

and harmonic, sub-harmonic and ultra-harmonic emissions. When the acoustic pressure is increased further, eventually the pressure from the surrounding fluid is far greater than the internal pressure of the MB, and the MB violently collapse, and results in broadband emission. This response is known as inertial cavitation. Rayleigh-Plesset equation is used to model the oscillation of a bubble in an incompressible fluid. This model has been further developed by a number of groups for describing the oscillations of MBs with shells in a liquid. The Rayleigh-Plesset equation models the volumetric oscillations of a MB:

$$R \frac{d^2 R}{dt^2} + \frac{3}{2} \left(\frac{dR}{dt} \right)^2 + \frac{4\nu_L}{R} \frac{dR}{dt} + \frac{2\gamma}{\rho_L R} \frac{\Delta P(t)}{\rho_L} = 0 \quad (1.14)$$

$$\Delta P(t) = P_\infty(t) - P_B(t) \quad (1.15)$$

where R is the radius of the MB, ν_L is the kinematic viscosity of the surrounding medium, γ is the surface tension, $P_\infty(t)$ is the pressure external to the MB, at a point which is infinitely far away, and $P_B(t)$ is the internal pressure experienced by the MB. Further models have since been developed to modify the Rayleigh-Plesset equation to add terms for related to the presence of a lipid shell, which creates a viscous damping effect on the oscillations, as well as having a frictional term associated with the movement of lipids through a fluid medium [144, 223]. The relationship between the resonant frequency of a MB with a lipid monolayer $f_{resonance}$ was considered in the de Jong model [144]:

$$f_{resonance}^2 = f_{free}^2 + \frac{S_{shell}}{4\pi^2 m} \quad (1.16)$$

$$f_{free}^2 = \frac{1}{2\pi R_0} + \left(\frac{3\gamma P_0}{\rho} \right)^{\frac{1}{2}} \quad (1.17)$$

where f_{free} is the resonant frequency of a MB with no lipid shell, S_{shell} is the stiffness of the shell, and m is the mass of the MB. The MB shell composition is important for determining the rigidity of the of the MB, and therefore the acoustic properties. For example, a more flexible shell results in a MB has a lower resonant frequency. The stiffness of the MB shell can be increased by either using lipids with long acyl chains, increasing the number of interactions between acyl chains, or packing of lipids determined by the size ratio between the head group and the acyl chain. Lipids therefore are an attractive component for MB shells due to a vast array of different lipids which allows for choice in the head and chain groups, and therefore control over the properties of the MBs [224], over polymers or proteins that can create stiffer and less compressible MBs.

1.4 Project aims and objectives

This chapter discussed how folded protein hydrogels have developed from the knowledge of mechanically stable, and functional proteins. Folded protein hydrogels can be rationally designed by choosing the protein, cross-linker and gelation method to open up a variety of tuneable hydrogels for biomedical applications [119, 123, 136, 225]. The stiff-

ness of hydrogels in both drug delivery and tissue engineering the stiffness of hydrogels is vital for the success. When considering the application of folded protein hydrogels, limited studies have investigated drug release. A major current challenge for protein hydrogel systems is the ability to control the triggered release of drug molecules from these hydrogels. Typically, drug release from protein hydrogels relies on diffusion of the drug out of the hydrogel. This has limitations of short timescales for drug release and the lack of control to tune the kinetics of drug release to suit different applications. Incorporating a triggered release mechanism would allow for the release of drugs at the desired time point, increasing the window for efficacy and potentially decreasing the side effects of the drug. In the event of wanting to make a more porous hydrogel for tissue engineering, the stiffness of hydrogels is often compromised.

This project aims to create a composite system composed of phospholipid microbubbles encapsulated in folded protein hydrogels for biomedical applications. Drug-loaded MBs, embedded within a hydrogel, provide a potential opportunity to impede the diffusion of drug molecules in the hydrogel and prolong the release profile. The time of drug release from the MBs into the surrounding hydrogel network can be controlled with the application of US which instigates the destruction of MBs. To date, MBs have not been embedded in folded protein hydrogels. Such a multi-composite system provides an opportunity to exploit the bursting of MBs to modulate the hydrogel porosity and small molecule or loaded drug diffusion, which could be controlled by the concentration of MBs.

This aim of this thesis to answer the following questions:

- 1) How is the protein hydrogel affected by the encapsulation of phospholipid MBs within the folded protein hydrogel? Does protein remain folded when surrounded by phospholipids and MBs? Do interactions between the MBs and the protein result in changes to the rate of the protein network formation or changes to the structural properties protein network formed, such as the number or size of fractal-like clusters of proteins? How are the bulk mechanical properties different, for example the stiffness of the protein hydrogel, or the strain required to break the hydrogel?

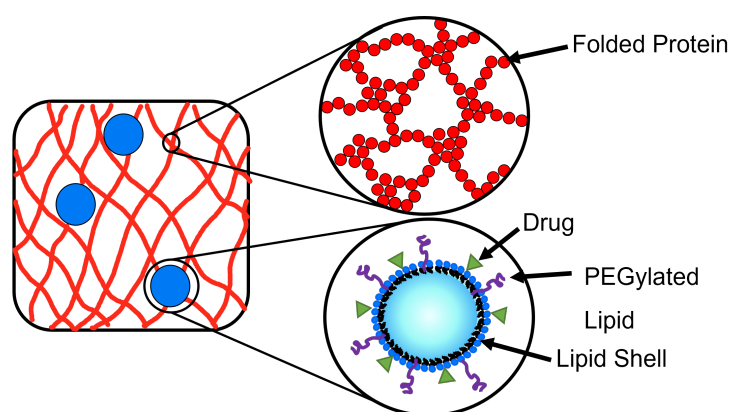


Figure 1.28: Schematic depicting microbubbles (blue spheres) encompassed within a folded protein hydrogel network (red lines), where the hydrogel network is composed of cross-linked folded proteins (red spheres). The inset of the MB shows drug molecules (green triangle) attached to the lipid shell, where the lipid shell stabilises the gas core.

2) Considering the biomedical applications of folded protein hydrogels, what happens to the protein, the structure of the protein network and the bulk mechanical properties of the protein hydrogel with encapsulated MBs if the unfolding of the proteins is triggered?

3) In what areas of biomedical applications could folded protein hydrogels with encapsulated MBs have the potential to succeed in? How is the stability of the MB affected by encapsulation in protein hydrogels? Could the size of the hydrogels be scaled down for entry to the body via an oral route? Are the components required to initiate the cross-linking of the proteins toxic?

Chapter 2

Materials and methods

This section outlines the experimental techniques used in this thesis, along with the relevant background and theory to the experimental techniques. A list of materials and suppliers used in this thesis is detailed in Appendix A.

2.1 Buffers

For the microbubbles (MBs) and protein hydrogels, 25 mM sodium phosphate buffer (NaPB) at a pH of 7.4 was prepared from sodium phosphate monobasic monohydrate and sodium phosphate dibasic dihydrate in ultrapure, Milli-Q water (resistivity of 18.2 M Ω). For improved stability of MBs, 1% (v/v) glycerol was added to the NaPB [203]. For neutralising acid, a 0.1 M Na₂CO₃-NaHCO₃ buffer was prepared at a pH of 9.9 from sodium bicarbonate and sodium carbonate anhydrous in Milli-Q water. Buffers were filtered through a 0.22 μ m filter for sterilisation.

2.2 Microbubble Production

2.2.1 Lipid Preparation

All lipids were dissolved in a 1:1 mixture of methanol and chloroform. Lipids 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [amino (polyethylene glycol)-2000] (DSPE-PEG-2000) were mixed in 95:5 mol% ratio, giving a final lipid concentration of 2 mgmL⁻¹. For fluorescence microscopy, 0.1 mol% of fluorescent lipids (DOPE-Atto 647n) was added to the mixture of DPPC and DSPE-PEG-2000 (94.9 and 5 mol%). To remove the solvent, the lipid mixture was dried under nitrogen for 1 hour and stored overnight under vacuum before resuspension. The dried lipid film was resuspended in NaPB and 1% (v/v) glycerol, involving vortexing for 1 min and stirring for 20 mins at 60°C. The resuspension of lipids was followed by tip sonicating the lipid solution for 30 mins to promote resuspension. The lipid solution was centrifuged at 17,000 g for 30 mins, in order to remove titanium deposited during tip sonicating. The supernatant was removed and pipetted into a fresh centrifuge tube to be used for producing MBs.

2.2.2 Production and Separation of Microbubbles

The lipid solution, stored in a centrifuge tube, was saturated with perfluorobutane (C₄F₁₀) gas for 2 mins at 300 mbar. The MBs were produced with mechanical agitation using CapMixTM (3M-ESPE) to shake the lipid solution at 71.7 s⁻¹. Differential buoyancy was used to separate MBs. Larger MBs are more buoyant than smaller MBs and can be separated by allowing the MBs to rise over time. Figure 2.1 shows the predicted rising distance for MBs using the Hadamard-Rybczynski equation [226]. Derived from the Navier-Stokes equation, the Hadamard-Rybczynski equation (Equation 2.1) considers buoyancy force and the opposing drag force on MB, while neglecting the surface tension and inertia forces.

$$W_b = \frac{2}{3} \frac{R^2 g (\rho_b - \rho_0)}{\mu_0} \frac{\mu_0 + \mu_b}{2\mu_0 + 3\mu_b} \quad (2.1)$$

where R is the radius of the MB, g is the gravitational acceleration (9.81 m s^{-1}), ρ_b is density of the MB, ρ_0 is the density of the surrounding medium, μ_b is the viscosity of the MB and μ_0 is the viscosity of the surrounding medium. Figure 2.1 shows a theoretical prediction based off Equation 2.1, where the following terms were used: $\rho_b = 11.2 \text{ kg m}^{-3}$ for a gas core of C_4F_{10} , $\rho_0 = 999.76 \text{ kg m}^{-3}$ for the NaPB-1% glycerol buffer, $\mu_b = 4.65 \times 10^{-4} \text{ Pa s}$ for the viscosity of C_4F_{10} gas, $\mu_0 = 9.1959 \times 10^{-4} \text{ Pa s}$ is the viscosity for the NaPB-1% glycerol buffer.

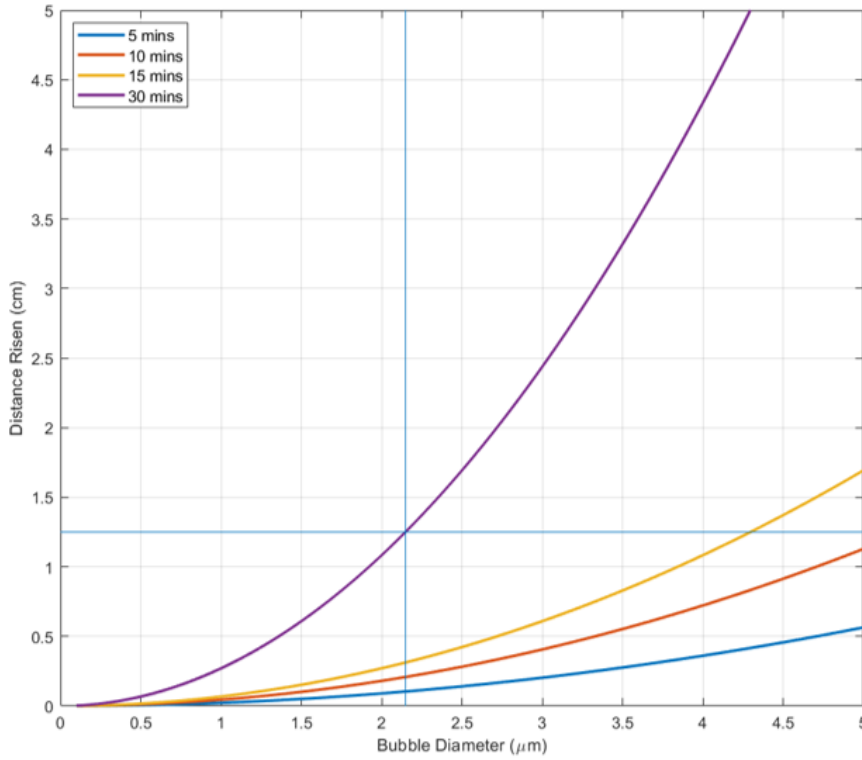


Figure 2.1: Predicted time for bubbles with a C_4F_{10} in 1% glycerol solution based on the Hadamard-Rybczynski equation[226]. Lines in light blue are guides for the eye at $x = 2.15 \mu\text{m}$ and $y = 1.25 \text{ cm}$. Adapted Matlab script from Dr. Damien Batchelor.

Figure 2.1 predicts that after 30 mins, MBs with a diameter of greater than $2.15 \mu\text{m}$ are predicted to rise by a distance of at least 1.25 cm , which is equivalent to the height of the centrifuge tube the MBs are stored in. For this reason the MBs are left for 30 mins for the larger, more buoyant MBs to float to the top half of the centrifuge tube. The smaller MBs were separated by pipetting the MBs from the lower half of the centrifuge tube, in order to reduce the population of MBs to those with diameters of less

than $2\mu\text{m}$.

2.3 Protein Hydrogel Preparation

Bovine serum albumin (BSA) hydrogels for this study are formed as a result of a photo-activated cross-linking reaction between protein residues.

2.3.1 Photo-initiated Cross-linking Reaction

The photo-initiated cross-linking reaction requires tris-(bipyridine)ruthenium(ii) ($\text{Ru}(\text{bpy})_3^{2+}$), a light harvesting molecule, in the presence of sodium persulfate (NaPS) and visible light to initiate the reaction ($\lambda_{\text{max}} = 452 \text{ nm}$). There are multiple suggested mechanisms of bond formation between proteins, described by Fancy *et al* 1999 [227]. One possible mechanism (Fig. 2.2) involves $\text{Ru}(\text{bpy})_3^{2+}$ oxidising to $\text{Ru}(\text{bpy})_3^{3+}$, prompting the donation of an electron to the persulfate ion. As a result, the oxygen-oxygen bond from the persulfate ion is suggested to cleave, producing a SO_4^{2-} and a free radical $\text{SO}_3^{\bullet}$. The $\text{Ru}(\text{bpy})_3^{3+}$ could then oxidize aromatic residues on the tyrosine while in the presence of H^+ ions, resulting in the formation of tyrosine free radicals. The free electron of the tyrosine is located on the carbon atom adjacent to the hydroxyl carbon in the benzene ring. This tyrosine free radical reacts with a neighbouring tyrosine, forming a carbon-carbon covalent bond between the proteins. Alternatively, the free radical could attack a cysteine side chain and form a heteroatom-arene linkage with the free radical tyrosine. In the presence of free cysteine it has been shown that both routes are likely to occur [227]. For BSA, a number of the cysteine residues of BSA are already bonded in disulphide bridges therefore the cross-links formed are likely to be purely dityrosine bonds. The mechanisms are yet to be determined experimentally, but the authors in Fancy *et al.* (1999) have predicted these potential routes [227].

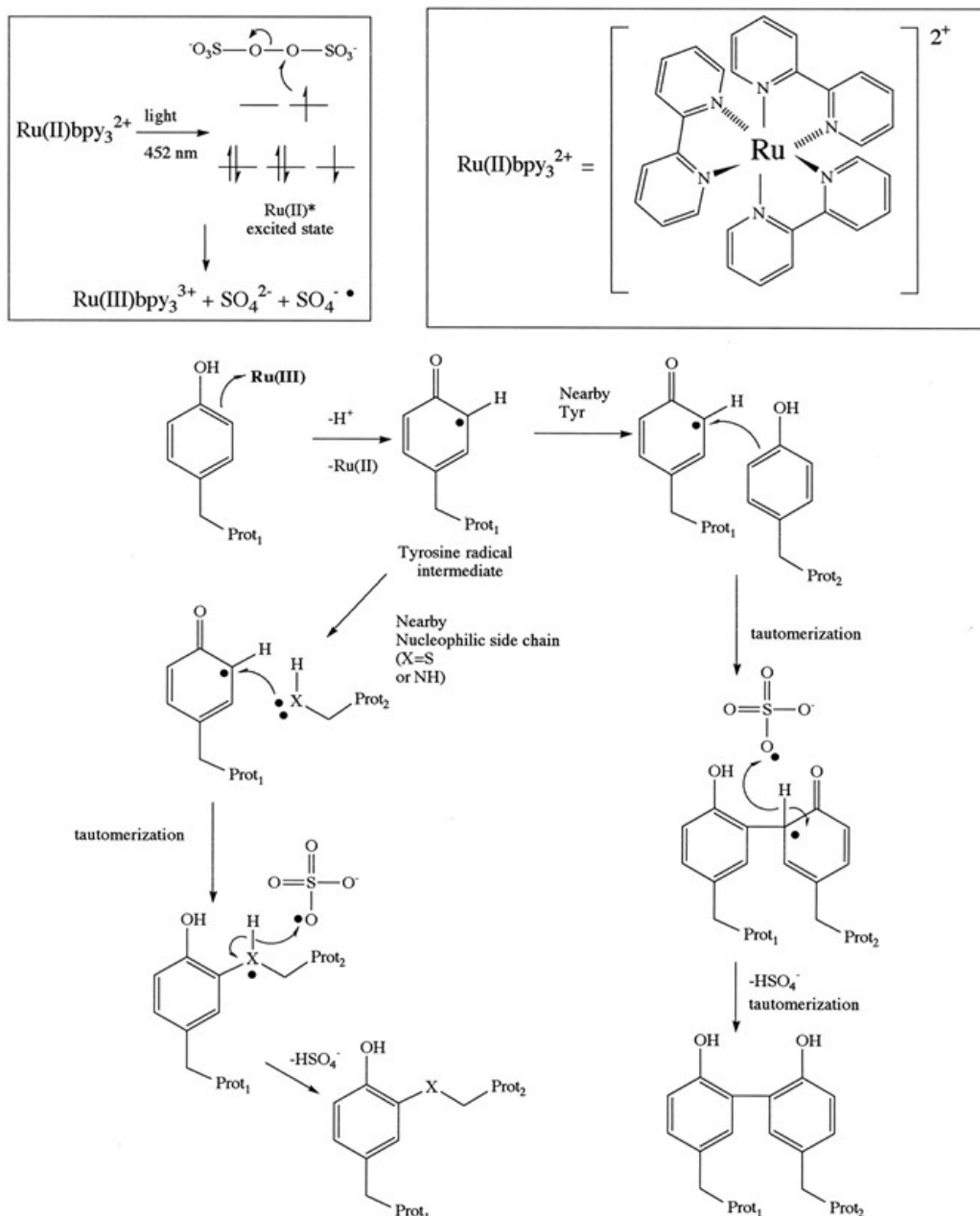


Figure 2.2: Top left: Ru(bpy)_3^{2+} in the presence of sulfate ions is excited by light ($\lambda = 452 \text{ nm}$), creating a free radical persulfate ion. Top right: chemical structure of Ru(bpy)_3^{2+} . Bottom: Potential mechanisms of the photo-initiated protein cross-linking reaction. In the first step of the mechanism, Ru(bpy)_3^{2+} oxidises the tyrosine residue, creating a tyrosine free radical. In one potential mechanism (left) involves a nucleophilic cysteine group nearby could attack the tyrosine radical, leading to production of a heteroatom-arene linkage site. In a second potential mechanism (right) a nearby tyrosine residue leads to coupling between the aromatic rings of two tyrosines. For the possible mechanisms, the removal of a hydrogen atom is required to produce a stable product. The sulfate radical produced from the excitation of Ru(bpy)_3^{2+} could assist in this hydrogen removal. These potential mechanisms are predictions, yet to be confirmed experimentally. Reproduced with permission from PNAS 1999[227].

2.3.2 Preparation of Reagent Stocks

The photo-chemical cross-linking reaction between BSA proteins requires reagents NaPS and $\text{Ru}(\text{bpy})_3^{2+}$. Initially 400 mM NaPS and 4 mM $\text{Ru}(\text{bpy})_3^{2+}$ are prepared separately in 1% (v/v) glycerol and NaPB, and each vortexed for 1 min for sufficient dissolving. The $\text{Ru}(\text{bpy})_3^{2+}$ is filtered through 0.22 μm pore to remove contaminants. To prepare the reagent stock NaPS, $\text{Ru}(\text{bpy})_3^{2+}$ and NaPB are mixed to concentrations of 150 mM NaPS and 0.3 mM $\text{Ru}(\text{bpy})_3^{2+}$. The reagent mix was aliquoted into 0.5 mL centrifuge tubes and stored at -80°C for up to a month before use.

2.3.3 Resuspension of BSA Protein

To resuspend BSA to a concentration of 200 mgmL^{-1} , the BSA powder was carefully wetted with buffer and the centrifuge tube upended until the BSA was fully dissolved. The BSA was centrifuged at 5000 RCF for 1 minute to pellet any BSA that had not been dissolved. The BSA supernatant was removed and pipetted into a fresh centrifuge tube for the preparation of the pre-gel solution. To determine the final concentration of the BSA supernatant, the BSA solution was diluted 300 times and the UV absorbance was taken at $\lambda = 280$ nm with Shimadzu UV-1800 spectrophotometer (Shimadzu UK Ltd, UK). Equation 2.2 denotes the Beer-Lambert Law used to calculate the concentration from the UV absorbance[228]:

$$A_{280} = c\varepsilon\Delta L \quad (2.2)$$

where A_{280} is the absorption of resuspension at $\lambda = 280$ nm, c is the concentration of the protein solution, ε is the extinction coefficient of BSA at $\lambda = 280$ nm, which for BSA is $43,824 \text{ M}^{-1}\text{cm}^{-1}$, and $\Delta L = 1$ cm is the path length of the sample [229].

2.3.4 Preparation of BSA Hydrogels

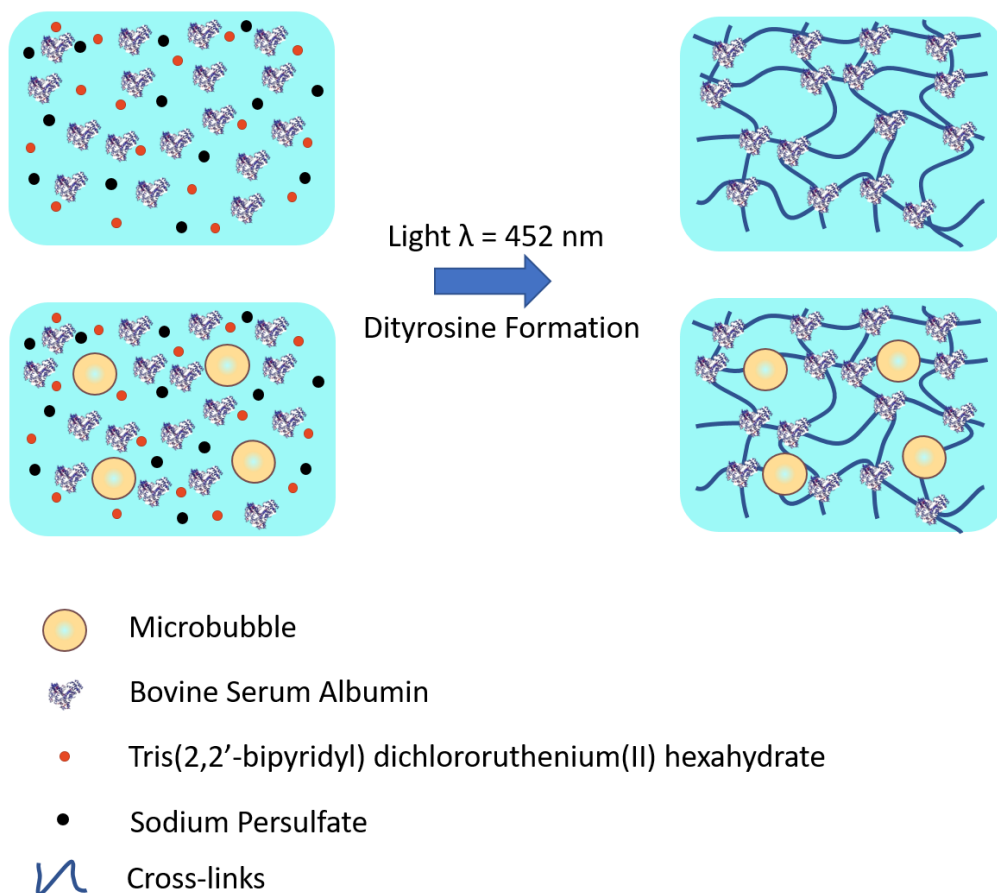


Figure 2.3: Schematic of Left) BSA protein pre-gel solution before photo-activation and Right) post BSA protein hydrogel formation after photo-activation, both in the absence and presence of MBs. Not to scale.

For the BSA hydrogels (without MBs), the reagent stock was diluted with buffer (NaPB and 1% (v/v) glycerol) and mixed with the resuspended BSA such that the final concentrations in the pre-gel solution are 100 mg/mL BSA, 50 mM NaPS, 0.1 mM $\text{Ru}(\text{bpy})_3^{2+}$. In the case of the preparation of BSA hydrogels encompassing MBs, (BSA:MB hydrogel) the MB solution at the desired concentration was used in place of buffer when mixing with the BSA, NaPS and $\text{Ru}(\text{bpy})_3^{2+}$ such that the concentrations in the final pre-gel solution with MBs is the same the pre-gel solution in the absence of MBs. To initiate gelation via excitation of $\text{Ru}(\text{bpy})_3^{2+}$, a LED lamp (Blue LED Emitter LZ4-00B208, LED ENGIN, USA) with $\lambda = 460 \text{ nm}$ illuminated the pre-gel solution for 5 mins. Dithiothre-

itol (DTT,) was added to the BSA hydrogel to induce unfolding of the BSA protein in the hydrogel, and to monitor the resultant hydrogel properties. DTT was introduced to the protein after the photo-initiation of chemical cross-linking. Once the BSA hydrogel is produced and soaked in buffer, 3 mM of DTT is added to to soak the BSA hydrogel in DTT.

2.4 Imaging Techniques

2.4.1 Bright Field Microscopy

For observing MBs in solution, the MBs were diluted from the stock concentration (between 0 and 100x) and imaged in a 50 μm high chamber, created by separating a coverslip and glass slide with a polyethylene terephthalate spacer (PTFE, Goodfellow Cambridge Limited). Optical images were taken of the MBs with the inverted microscope (Nikon 90i, Nikon, Japan), using a reflection bright-field microscopy set-up with 40x magnification objective [203]. This involves shining a white light onto the sample, and a camera to detect the reflected light. On average, 15-20 images were captured and analysed with a custom MATLAB (MathWorks, US) script written by Dr. Damien Batchelor using the Image Analysis Toolbox, to determine the size and concentration of the MBs [230].

2.4.2 Confocal Microscopy

Fluorescence based microscopy produces an image by detecting photon emission from fluorophores. Fluorophores enter an excited state by the absorption of a photon. When relaxing from the excited state to a lower energy state, a photon is emitted. The emitted photon has a wavelength equivalent to the energy difference of the excited and relaxed states. Energy in this transition is lost, therefore the emitted photon has a lower energy than the absorbed photon. Confocal microscopy differs from reflected bright field microscopy by using a pinhole, the size of which can be adjusted, to control the amount

of light emitted from the sample. The pinhole blocks out light emitted from different focal planes, reducing the background from planes other than the focal plane of interest (see Figure 2.4). This allows for high resolution fluorescent images, making confocal a desirable technique. Confocal fluorescence microscopy collects the light emitted from fluorophores to produce an image. Additionally, this allows for Z-stacks of the sample to be obtained, where multiple 2D images over a range of depths are ‘stacked’ together to create a 3D image. In this study, confocal laser scanning microscope (CLSM) system (SP8, Leica Microsystems GmbH, Wetzlar, Germany) was used to observe MBs, and fluorescent MBs, encapsulated in protein hydrogels, whilst simultaneously imaging with transmission mode, bright field microscopy.

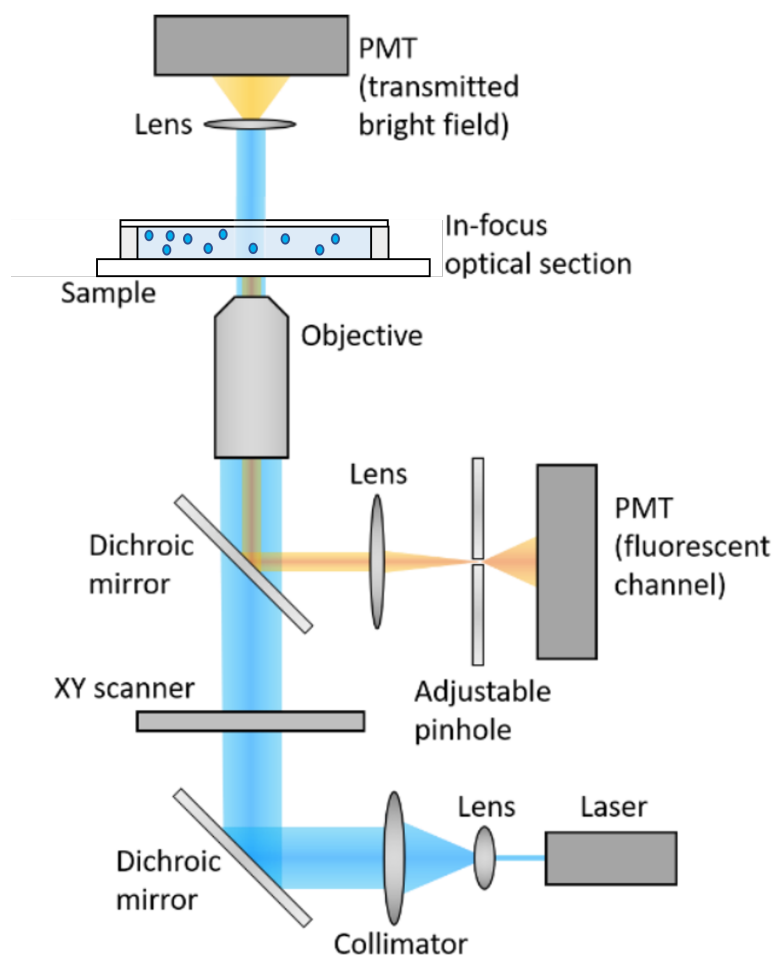


Figure 2.4: Schematic of confocal microscope set up. Reused with permission from [231] under a CC BY-NC-SA 2.5 licence.

Confocal microscopy was used to observe fluorescent MBs. Fluorescent lipids were used in MB preparation to visualise the MBs in the BSA hydrogel (see Section 2.2.1 for lipid preparation) and were excited using a 638 nm diode laser. Fluorescence emission from Atto-647n-DOPE lipid was collected from 590-768 nm. The confocal pinhole size was 1 A.U. and a 50x objective used.

The transmitted bright field microscopy on the CLSM system was used to monitor the stability of MBs in BSA hydrogels over 2 days. The CLSM system was chosen as an alternative to reflection, bright field microscopy, for higher resolution images due to the use of a laser (488 nm optically pumped semiconductor laser) and reduced background from the transmitted laser light being collected. Z-stacks of the BSA:MB hydrogels were comprised of 50 images taken in 1 μm steps. ImageJ was used to determine the concentration of MBs. The images were first binarised, then a particle tracking algorithm plug-in (MosaicSuite) was applied, which uses a circle finder algorithm, before determining if the detected particle appears over multiple image slices [232]. The cut off radius was set to 4 μm and the maximum displacement of the particle between frames was set to 1 μm .

2.5 Ultrasound Set-up and Application

Ultrasound (US) was used in this study for MB destruction, see Chapter 1 Section 1.3.5 for a background to US. A 2.25 MHz centre frequency, unfocused transducer (V323-SM, Olympus, Japan) with a single 6 mm element diameter was used to deliver the US signal. A burst of 22-cycle of sine waves was applied with a 1% duty cycle, with a pulse repetition frequency (PRF) of 1 kHz. The total duration of the sequence was 4 s. Peak negative pressure of the signal was 900 kPa, corresponding to a mechanical index of 0.6. The signal generated by a function generator (TG5011, Agilent Technologies, UK), was set-up via a MATLAB script written by Dr Damien Batchelor, and confirmed with an oscilloscope (WaveRunner LT342L, LeCroy Cooperation, USA). The signal was amplified from a peak-to-peak voltage (V_{pp}) of 0.4 to 178.7, with a + 53 dB RF power amplifier (A150, Electronics % Innovation, USA), required for MB destruction.

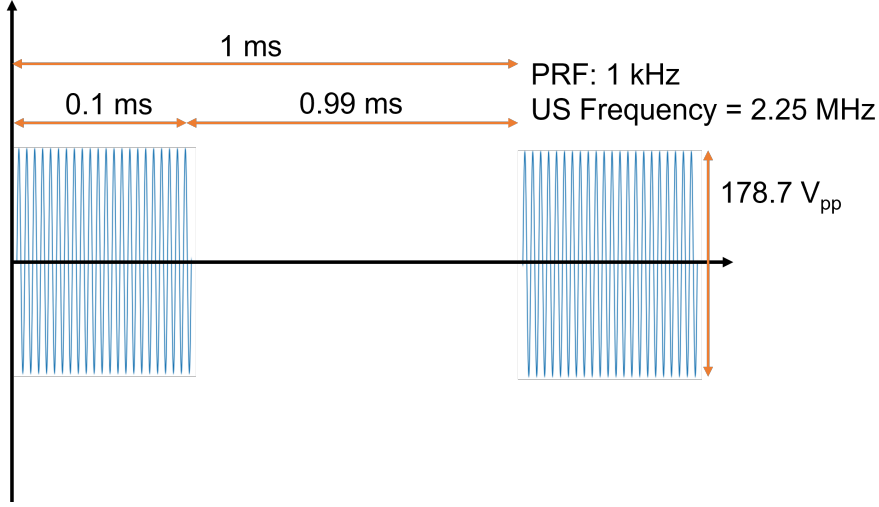


Figure 2.5: Schematic showing part of the 2.25 MHz ultrasound signal used for MB destruction. The US signal is composed of bursts of 22-cycle sine waves followed a period of time with no US with a pulse repetition frequency of 1 kHz. The total application of the US signal was 4 s.

For bursting MBs in the BSA hydrogel, the BSA:MB hydrogel was contained in between a glass slide and a coverslip, separated by 50 μm spacers. The transducer was coupled to the glass coverslip with US coupling gel and a gel pad (Aquaflex, Parker Laboratories, US). The gel pad thickness was at 2 cm to ensure that the US signal was greater than the near field distance, where the signal is more predictable and uniform. The near field distance (NFD) is the region of the US beam nearest to the transducer, where beam is more uniform (with maximums and minimums) and is the natural focal point for the transducer. The NFD is defined by the transducer is calculated with Equation 2.3:

$$NFD = \frac{D^2}{4\lambda} \quad (2.3)$$

where D is diameter of the transducer element, λ is the wavelength emitted by the transducer. The affect of the application of US to the MBs was monitored with optical, bright field microscopy as described in Section 2.4.1.

2.6 Characterisation of Proteins

Circular dichroism (CD) spectroscopy is a light absorbance spectroscopy technique that uses the differential absorption of circularly polarised light to differentiate between chiral molecules, providing information about their structure. Chiral molecules cannot be superimposed onto the mirror image of the molecule by any Euclidean transformation.

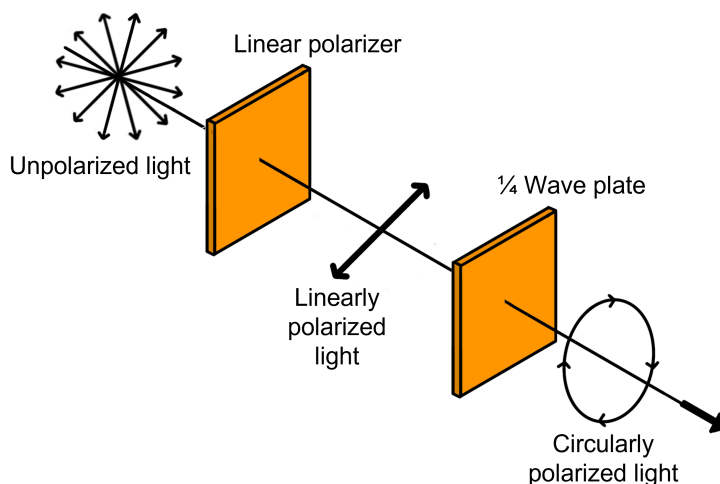


Figure 2.6: Schematic demonstrating the relevant axes in producing circularly polarised light. Linearly polarised light by passing through a quarter wave plate. Adapted from Cronin, 2018 [233].

To achieve circularly polarised light, unpolarised light is first passed through a linear polariser (See Fig. 2.6). The resultant light is linearly polarised, where the light oscillates in a single plane. To produce circularly polarised light, the linear polarised light is subjected to a birefringent quarter waveplate, where the waveplate axis must be 45° to the axis of polarisation. Circularly polarised light incident to a chiral molecule is absorbed by the chiral molecule, where the emitted light will be different to the incident light. The absorption between right (A_R) and left (A_L) circularly polarised light when interacting with chiral molecules differs, and CD spectroscopy uses the difference between the two absorbances (ΔA) to characterise the molecule:

$$\Delta A = A_L - A_R \quad (2.4)$$

To produce a CD spectra, the ellipticity, θ , of the light emitted from the sample is recorded as a function of wavelength.

$$\theta = \arctan(a/b) \quad (2.5)$$

where a and b are the minor and major axis of the elliptical light θ is also related to ΔA by $\theta = 32.98 \Delta \cdot A$.

Protein molecules are chiral and their distinct secondary structural features can be determined with the use of CD i.e. α -helices, β -sheets and β -turns (Figure 2.7). This allows for changes in their secondary structure to be identified with CD, such as unfolding of the protein and the thermal melts as a function of temperature [234, 235].

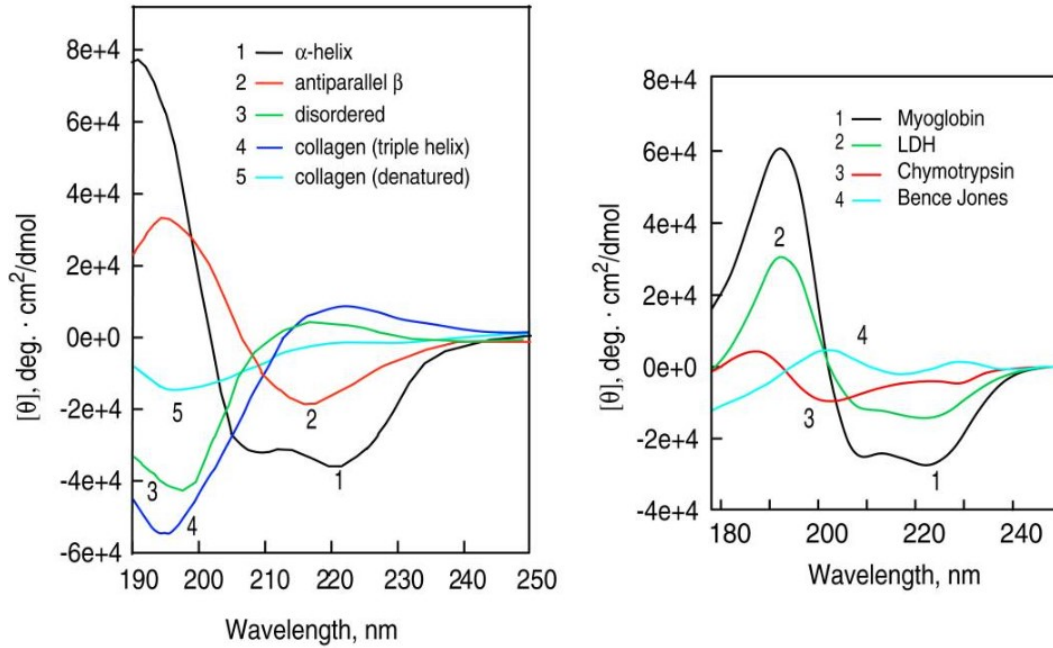


Figure 2.7: CD spectra for different secondary structures of proteins. Left) The CD spectra (where θ is the ellipticity of light emitted from the sample, see Equation 2.5) of poly-L-lysine controlled by pH: 1. (black line) shows pol-L-lysine in an α -helical confirmation, 2. (red line) in an anti-parallel β -sheet conformation and 3. (green line) in a extended disordered conformation. The CD spectra of collagen in the native triple helical state (4, blue line) and its denatured state (5, cyan line). Right) Examples of different proteins with a range of secondary structures: Reprinted by permission from Macmillan Publishers Ltd: Nature Protocols [236].

Distinctive features of proteins include peptide bonds, aromatic amino acids absorb electromagnetic radiation in the wavelength range 260-329 nm and disulphide bonds

absorb below 240 nm. The wavelength range of absorption for peptide bonds therefore makes the use of the ultraviolet (UV) light in CD spectroscopy to investigate the structural features proteins advantageous. Figure 2.7 shows the CD spectra of different proteins, with a variety of secondary structures [236]. The peaks in the CD spectra are used to characterise the secondary structure of the protein, with examples shown in Figure 2.7: α -helices are characterised by two negative peaks (~ 209 and 222 nm), β -sheets are characterised by one weak intensity, negative peak (~ 215 nm) and positive peak (~ 195 nm), and a disordered coil-coil structure is characterised by a strong intensity, negative peak (~ 195 nm).

CD was used to estimate the secondary structure of BSA before and after gelation and to quantify the amount of unfolding. The standard BSA hydrogel was compared to BSA:MB hydrogel. The CD experiments were performed with a Chirascan plus circular dichroism spectrometer (Applied PhotoPhysics). Due to using CD on high concentrations of protein (100 mg mL^{-1}), a cuvette with path length of $10 \text{ }\mu\text{m}$ was chosen for a short path length, in order to counteract the large amount of absorption of UV light. A range of UV wavelengths ($178 - 260 \text{ nm}$) was used to observe the CD spectra, in steps of 1 nm with a bandwidth of 5 nm for the maximum light.

2.7 Viscoelastic Characterisation

Rheology is the study of the deformation and flow of a material, and is applied to a wide range of materials. Rheology has been used to characterise a range of viscoelastic materials, such as gels, tissues, and foams, in order to understand their mechanical properties [98, 237, 238]. A viscoelastic material exhibits behaviour of both ideal viscous and ideal elastic materials simultaneously, however the time frame that the viscous or elastic behaviour dominates over can differ between viscoelastic materials. For example, the pitch drop experiment involves bitumen, a viscoelastic material with a high viscosity, slowly forming a drop. The time recorded for bitumen to drop can take up to 13 years, demonstrating that the viscous behaviour occurs very slowly while the elastic component dominates at shorter timescales [239]. Another example of a viscoelastic material is

cornstarch, where if a shear force is applied slowly, the viscous component dominates such that the cornstarch acts as a viscous liquid. However, when a shear force is rapidly applied to the surface of cornstarch, the elastic component dominates on short timescales, and the cornstarch solidifies [240].

2.7.1 Ideal Viscous Fluid

In an ideal viscous liquid undergoing a force, the molecules within the liquid flow over each other, creating internal friction. This friction results in a flow resistance, which is defined as the viscosity (η) of the fluid. The two plate model is used to define the rheological parameters and viscosity of a material [241]. For this model the sample is sandwiched between two parallel plates, where throughout the application of a shear force (F) the lower plate remains stationary. The applied shear force causes the upper plate to move parallel to the lower plate by distance (x) as shown in Figure 2.8.

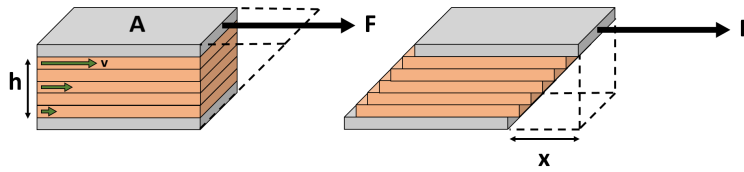


Figure 2.8: Two plate model for an ideal viscous liquid. A shear force (F) is applied to the top plate, causing deformation, where h is the distance between the top and bottom plates, A is the area of the plate, v is the velocity of the fluid, x is the displacement caused by the shear force.

$$\sigma = \frac{F}{A} \quad (2.6)$$

Equation 2.6 describes the resultant shear stress (σ) experienced by the the material due to the applied shear force, where A is area of the plate. The liquid is assumed to coat the area of both plates without slipping and under force exhibits laminar flow. The shear force is assumed to create a velocity gradient through the ideal viscous liquid, such that each small layer of the liquid has a smaller velocity near the lower plate and layers closer to the upper plate have a larger velocity. The shear rate ($\dot{\gamma}$) for a viscous liquid

is proportional to the velocity of rotation as a result of a shear force:

$$\dot{\gamma} = \frac{\delta\gamma}{\delta t} = \frac{v}{h} \quad (2.7)$$

where v is the velocity, h is the distance between the top and bottom plates. The viscosity (η) at constant temperature is defined by Newton's Law, describing the resistance of the flow as a result of an applied force, which arises from the friction between layers of liquid shown in Figure 2.8:

$$\eta = \frac{\sigma}{\dot{\gamma}} \quad (2.8)$$

A dashpot is a cylindrical damping device, consisting of a piston that moves through a fluid. A dashpot moving through an ideal viscous liquid with viscosity η , under loading of a constant force (Figure 2.9) will move continually through the liquid, when loading a constant force is applied proportional to the velocity of the piston. The unloading behaviour with the removal of the force results in the piston stopping and staying in this position. The energy of deformation is not stored, but used up to move the piston and heat loss to the surroundings, therefore with the removal of force there is no piston movement.

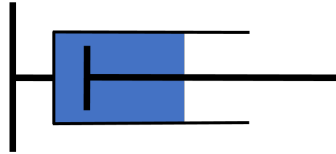


Figure 2.9: A dashpot moving through a ideal viscous liquid.

2.7.2 Ideal Elastic Solid

A perfectly elastic solid when stretched or compressed, force (F) applied to deform the solid is proportional to change in length (x) of the deformed solid. This relationship is known as Hooke's law ($F = kx$), where the proportionality constant (k) is the elastic modulus, characterising the stiffness of the elastic material [242]. In terms of the two plate model, where for a solid homogeneous deformation throughout the sample is assumed, the shear strain (γ), describes the amount of deformation the sample undergoes

with shear force applied:

$$\gamma = \frac{x}{h} \quad (2.9)$$

where x is the displacement due to the application of a force, and h is the height of the sample between two plates. For elastic solids at constant temperature, the ratio between the shear stress, σ , (Equation 2.6) and the shear strain, γ is constant. The proportionality constant is the shear modulus, G and defines the rigidity of the material.

$$\sigma = G\gamma \quad (2.10)$$

A spring model is used to describe the Hookean behaviour of ideally elastic materials [241]. Upon loading of a constant force, the spring immediately deforms either stretching or compressing. The removal of the force results in spring immediately recoiling back to its start position. All the deformation energy stored within the sample is used to return the spring to the original position, therefore the process is reversible with full recovery.

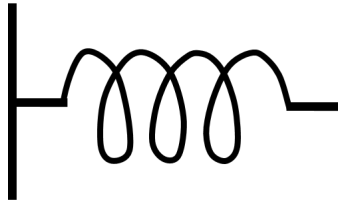


Figure 2.10: A perfectly elastic spring is an example of an ideal elastic solid.

2.7.2.1 Viscoelastic Solids and Viscoelastic Liquids

Viscoelastic behaviour in materials combines the properties of a viscous liquid and elastic solid. To understand the behaviour of a viscoelastic material, a combination of a dashpot (Section 2.7.1) and spring (Section 2.7.2) can be used as a model.

Viscoelastic materials have a delayed response to deformation, with some energy lost upon loading. Viscoelastic liquids can be described by Maxwell's model where a dashpot and spring are in series [243]. The application of a constant force causes the spring to immediately deform until extended, after which the piston begins to move. The removal of the force results in spring recoiling to the original shape, but the piston remains in the

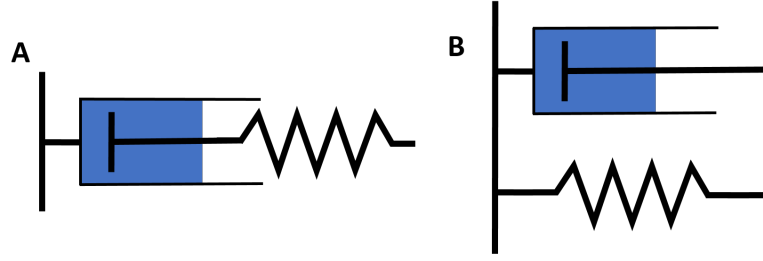


Figure 2.11: A) A viscoelastic liquid can be modelled by a dashpot and spring in series. B) A viscoelastic solid can be modelled by a dashpot and spring in parallel.

deformed position. Conversely for a viscoelastic solid, the Kelvin-Voigt model is used, where the dashpot and spring are in parallel [243]. When a force is applied, the spring and dashpot move together. As a result the deformation of the spring is slowed by the movement of the dashpot through the liquid. When the force is removed, the spring immediately will try to return to the original position but will be refrained from doing so quickly by the dashpot. Full recovery is eventually achieved, therefore the loading and unloading of force is reversible.

2.7.3 Oscillatory Shear Rheology

Oscillatory shear rheology is a technique used to characterise the mechanical properties of soft, viscoelastic materials involving a rheometer plate oscillating back and forth, creating a shear force on the sample [241]. In the previous section, we considered only linear shear strains but for an oscillating shear stress ($\sigma(t)$), the resultant shear strain (γ) is sinusoidal and is described in Equation 2.11:

$$\gamma = \gamma_0 \sin(wt) \quad (2.11)$$

where γ_0 is the amplitude of the oscillatory strain, w is the angular frequency and t is time. For an ideal elastic and viscous material, $\sigma(t)$ can be found by substituting Equation 2.10 and Equation 2.8 into Equation 2.11.

$$\sigma(t) = G\gamma(t) = G\gamma_0 \sin(wt) \quad (2.12)$$

$$\sigma = \eta \dot{\gamma}(t) = \eta \gamma_0 w \cos(wt) = \eta \gamma_0 w \sin\left(wt + \frac{\pi}{2}\right) \quad (2.13)$$

For an ideal elastic material (Equation 2.12), the response to force is immediate such that the $\sigma(t)$ and $\gamma(t)$ are in phase. The stress-strain phase angle (δ) describes the difference between the applied shear strain (γ) and the resultant shear stress (σ). For an ideal elastic material $\delta = 0$. For an ideal viscous material (Equation 2.13), the response to force is delayed such that $\sigma(t)$ and $\gamma(t)$ are out of phase by $\delta = \pi/2$. Viscoelastic materials have a phase difference that falls between $0 < \delta < \pi/2$, and an example of the relationship of the stress-strain behaviour for a viscoelastic materials is demonstrated in Figure 2.12. The application of an oscillatory shear strain on a viscoelastic materials therefore results in a stress response, determined by the phase angle:

$$\sigma = \sigma_0 \sin(wt + \delta) \quad (2.14)$$

where σ_0 is the amplitude of the oscillatory stress.

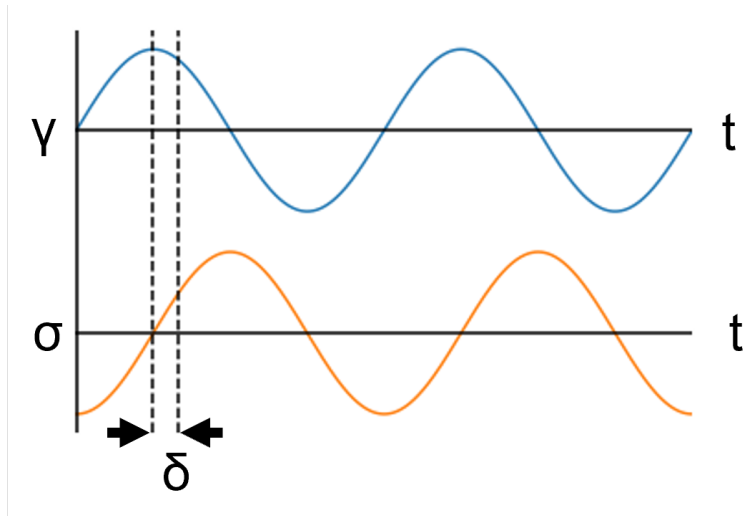


Figure 2.12: The graphs depict a phase difference (δ) between the applied shear strain (γ) and the resultant shear stress (σ) for a viscoelastic material.

The complex shear modulus (G^*) is used to describe harmonic periodic shear load that occurs in oscillatory shear rheology, combining viscous and elastic behaviours ($\sigma(t)$)

and $\gamma(t)$):

$$G^* = \frac{\sigma(t)}{\gamma(t)} = \frac{|\sigma_0|}{|\gamma_0|} e^{i\delta} = G' + iG'' \quad (2.15)$$

G^* is expanded using Euler's relation ($e^{i\delta} = \cos(\delta) + i\sin(\delta)$) splitting G^* into real and imaginary components [244]:

$$G' = \frac{|\sigma_0|}{|\gamma_0|} \cos(\delta) \quad (2.16)$$

$$G'' = \frac{|\sigma_0|}{|\gamma_0|} \sin(\delta) \quad (2.17)$$

G' is the in phase, elastic component of the complex shear modulus, and is known as the storage modulus. G' represents the energy stored upon deformation (Section 2.7.2). G'' is the out of phase, viscous component of the complex shear modulus, and is known as the loss modulus. G'' represents the loss upon deformation (Section 2.7.1)

The loss ratio, $\tan(\delta)$, is used to quantify whether the elastic component ($\tan(\delta) < 1$) or viscous component ($\tan(\delta) > 1$) is more dominant in a material, defined in Equation 2.18.

$$\tan(\delta) = \frac{G''}{G'} \quad (2.18)$$

2.7.4 Anton Parr Pseudo-Strain Controlled Rheometer

Oscillatory shear rheology measurements were performed on an Anton Paar MCR 302 stress-controlled rheometer, in pseudo-strain controlled mode. A parallel plate (flat base) was chosen over a cone plate to prevent the light source from inhomogeneous penetration through the sample. In oscillatory shear rheological measurements, the sample is assumed to be cylindrical, and rotates back and forth, where the torque (M) provides the force for the sample deformation and is driven by the rotatory actor (Figure 2.13). To determine σ and γ for a parallel plate, Equations 2.19 and 2.20 are used.

$$\sigma = \frac{2M}{\pi r^3} \quad (2.19)$$

$$\gamma = \frac{r\theta}{h} \quad (2.20)$$

where r is the radius of the parallel plate, h is again the sample height and θ is the angle that the sample moves upon deformation. The complex shear modulus (G^*) can be determined by substituting Equations 2.19 and 2.20 into Equation 2.10.

$$G^* = \frac{\sigma^*}{\gamma^*} = \frac{2M^*h}{\pi\theta^*r^4} \quad (2.21)$$

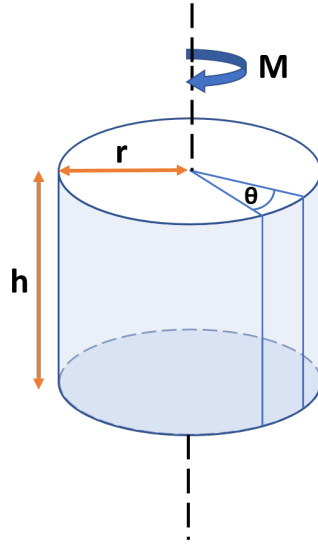


Figure 2.13: Parallel plate configuration on the rheometer, where the enclosed sample has a height, h , and radius, r . A torque, M , is applied which shears the sample, resulting in an angular deformation of θ

A light source for photo-activating the BSA pre-gel solution was designed to act as the bottom plate, such that the cross-linking of BSA is initiated while viscoelastic measurements were taken. This custom designed lighting rig was designed by Professor Nikil Kapur for the Anton Paar MCR 302 rheometer, and was produced by Keith Dredge, Mark Scott from the School of Physics and Astronomy's mechanical and electronic workshop. The blue prints and images of the set up is shown in Fig 2.14. The lighting rig consists of a base plate which sits on the rheometer. A blue LED ($\lambda = 452 \text{ nm}$) sits in the base layer, illuminating the upper layer, where the sample sits on a transparent glass plate as demonstrated in 2.15.

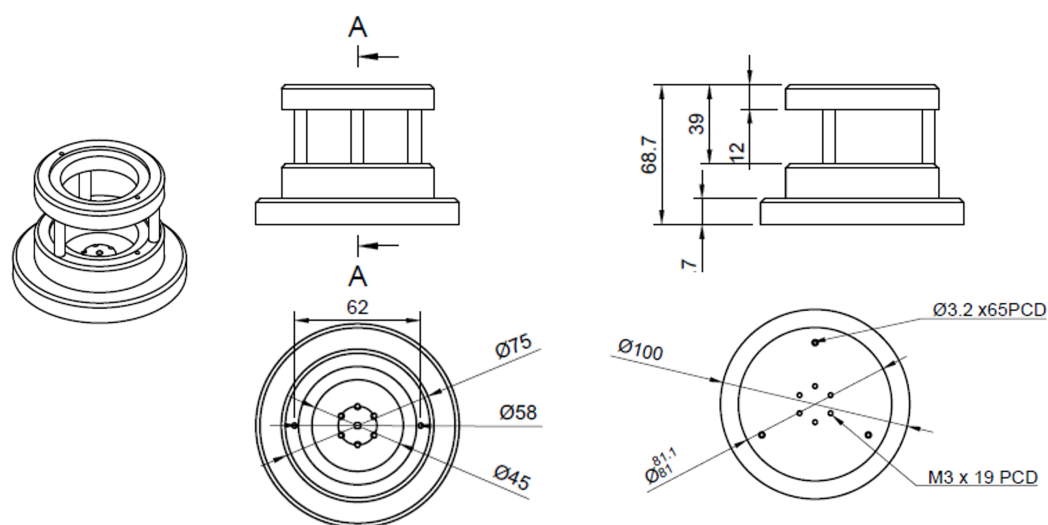


Figure 2.14: Schematic of the lighting rig used to illuminate a sample while sitting in rheometer by Mark Scott, where the distances are in mm and the angles in degrees Fusion 360® Autodesk screen shots reprinted courtesy of Autodesk, Inc. Diagram to scale.

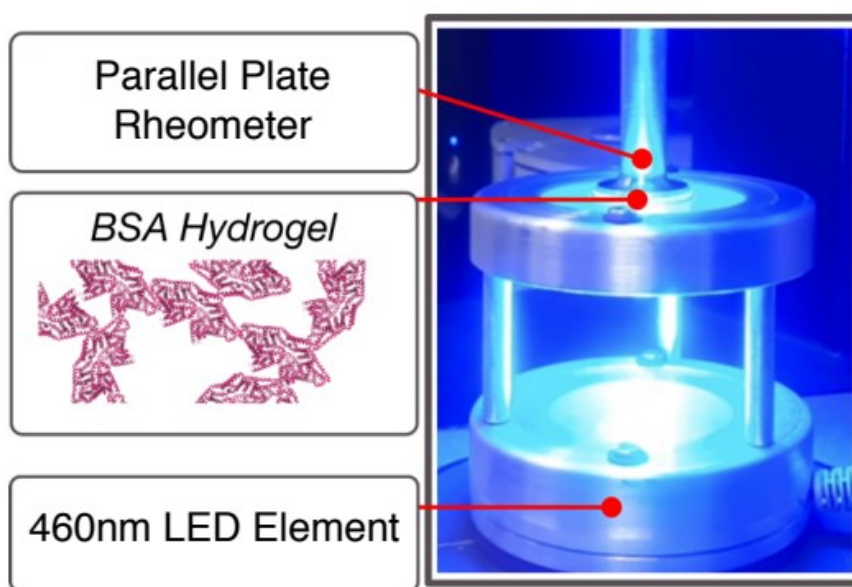


Figure 2.15: Image of the set-up for the LED lamp onto the Anton Paar rheometer to allow for photo-initiated chemical cross-linking. The LED is situated in the base. The sample is between top plate (a glass or translucent plastic plate) and the parallel plate geometry attached to the rheometer. Reprinted with permission from [123]. Copyright 2022 American Chemical Society.

An 8 mm diameter, parallel plate with gap height between 0.8-0.9 mm was used. The gap edges were coated with a thin layer of low viscosity silicone oil (≈ 5 ct) to

prevent dehydration of the sample over the course of the experiment. Four different measurements were performed on the BSA hydrogels, and are described below:

1. Gelation curves are measured by applying small amplitude oscillations to the sample, while measuring the viscoelastic properties for long time periods in order to monitor the kinetics of the network formation and the relaxation post gelation. The measurements were performed at a frequency of 1 Hz and shear strain of 0.5 % for 66 minutes. The pre-gel solution was loaded before the measurement started, and the samples illuminated after 1 min with the blue light for 5 mins for sufficient cross-linking and monitored for further 60 mins.

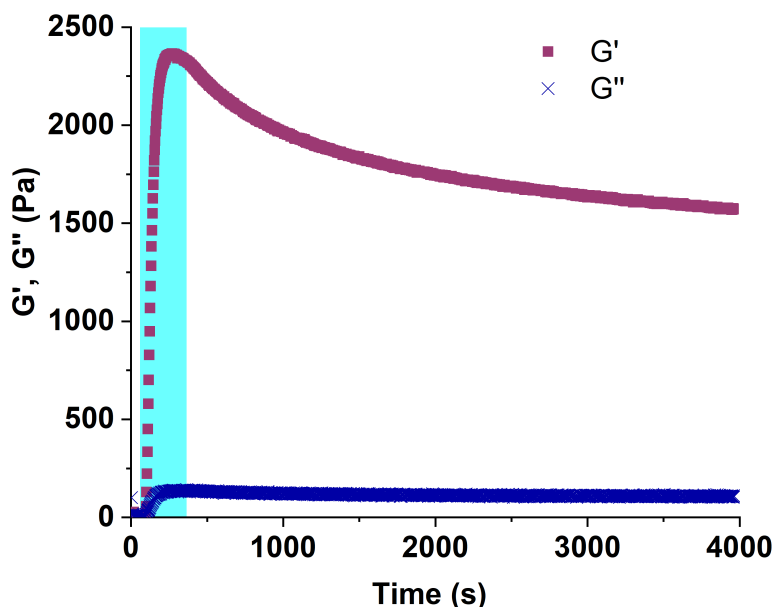


Figure 2.16: Example of a gelation curve for BSA protein hydrogels, showing the storage modulus, G' , in the purple curve and the loss modulus, G'' , in the dark blue curve. From $t = 60 - 360$ s the LED lamp illuminates the pre-gel solution (highlight in the light blue region on the graph), which photo-initiates the chemical cross-linking. G' quickly dominates over G'' , showing that dityrosine cross-links are forming between the proteins and that network formation is occurring. G' reaches a maximum and begins to decrease, associated with network relaxation.

2. Frequency sweep measurements are performed after the gelation curve, such that the gel has already relaxed for 1 hour post gelation. The storage and loss moduli are measured for a range of frequencies, under logarithmic ramp from 10 to 0.1 Hz. The

shear strain is kept constant at 0.5%.

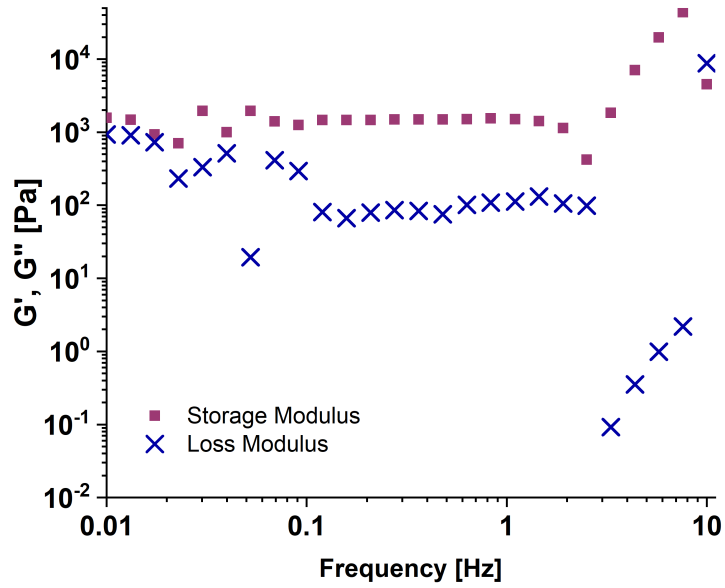


Figure 2.17: Example of a frequency sweep for BSA protein hydrogels, showing the storage modulus, G' , in the purple curve and the loss modulus, G'' , in the dark blue curve over a range of frequencies (f). In the linear region ($f = 0.12 - 1.45$ Hz) G' is greater than G'' , indicating the sample is a solid like behaviour. For frequencies greater than 2 Hz the torque required to move the parallel plate at high frequencies is contributing to the signal.

3. Stress-strain measurements investigate the loading and unloading behaviour of the protein hydrogels under strain. The gels are firstly loaded to a shear strain of 10% at a rate of shear strain increase of 1%/s and unloaded at 1%/s to 0% strain. After allowing the gel to relax for 5 mins the gels were loaded to a shear strain of 30% and 50% following the same process.

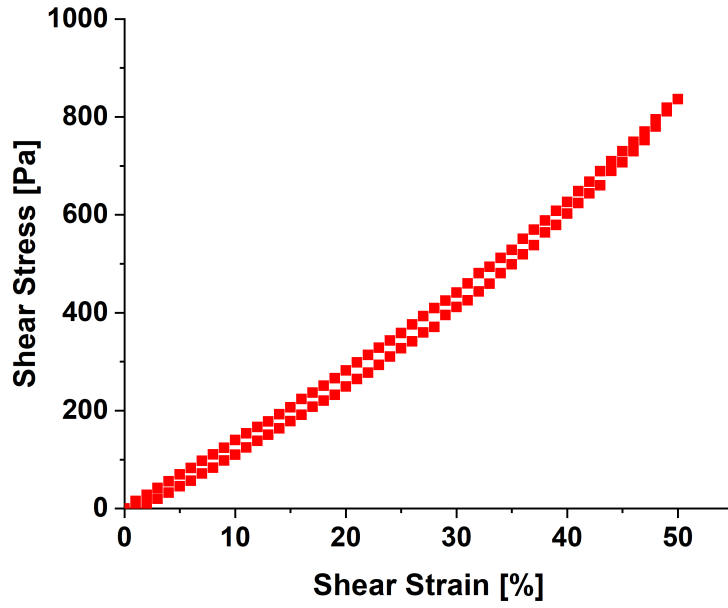


Figure 2.18: Example of a stress-strain curve for BSA protein hydrogels. The sample is loaded up to a shear strain of 50% at a rate of $1\%s^{-1}$ while the shear stress is measured. The sample was then unloaded to a shear strain of 0% at $1\%s^{-1}$. Information can be extracted from the stress-strain hysteresis. The gradient of the linear region (0 - 20%) can be used to determine G' . The area under the hysteresis is used to determine the efficiency of the loading-unloading cycle.

4. To investigate the non-linear behaviour and test the breaking point of the gel, the shear strain is increased logarithmically from 1 to 1000% at a constant frequency of 1 Hz.

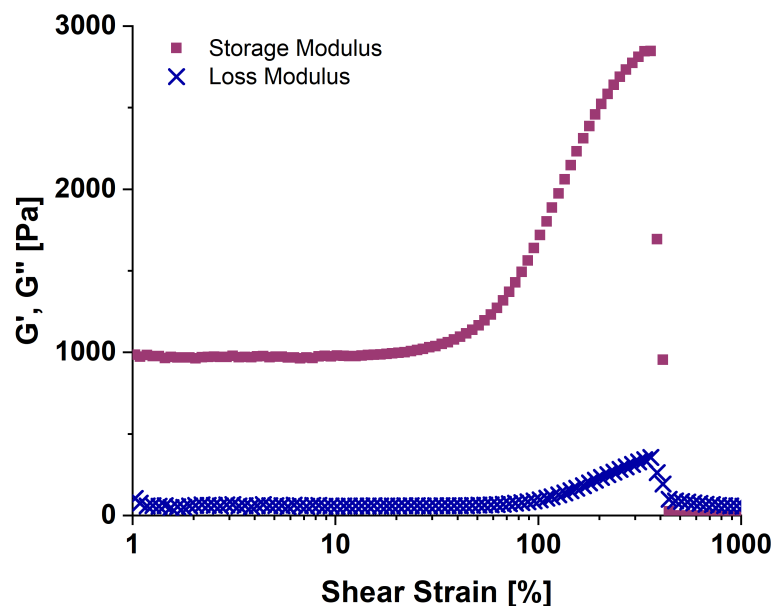


Figure 2.19: Example of a strain amplitude curve for BSA protein hydrogels, showing the storage modulus, G' , in the purple curve and the loss modulus, G'' , in the dark blue curve. The shear strain is increased from 1 - 10³%, while the G' and G'' . At low shear strain, the BSA hydrogel is in the linear viscoelastic region. Between shear strains of 10 to approximately 300%, G' increases indicating non-linear mechanics and the hydrogel is shear stiffening. At high shear strain (> 300% , G' suddenly decreases implying that the hydrogel has fractured.

2.8 Characterisation of Dityrosine Cross-links

Dityrosine covalent bonds cross-link BSA monomers into its fully cross-linked hydrogel network (Section 2.3), and due to its fluorescent properties the concentration of dityrosine bonds can be quantified from the intensity of the fluorescent emission. This is achieved by exciting the dityrosine at 315 nm, resulting in an emission peak at 410 nm. To quantify the number dityrosine bonds in the BSA and BSA:MB hydrogels, the hydrogels were first prepared as described in section 2.3.4, and then hydrolysed [245]. To hydrolyse the BSA hydrogel, firstly 4 μ L of the pre-gel solution is pipetted into a 2 mL Eppendorf with Pipetman Diamond (D10) pipette tips to ensure accurate pipetting of low volumes. The pre-gel solution is irradiated under lamp for 5 mins to gelate the sample. To digest the hydrogel, 2 mL of 6 M HCl is added and boiled at 105°C for 2 hours. The acid

hydrolysis product was neutralised by adding 120 μL of 5 M NaOH to 100 μL of the protein-acid solution, and diluted in Na_2CO_3 - NaHCO_3 buffer.

Cuvette-based steady-state fluorescence emission spectroscopy measurements were performed on an Edinburgh Instruments FLS980 fluorescence spectrophotometer. All samples were temperature controlled to 20°C during the measurements. Quartz cuvettes were used to hold 3 mL of the sample. Samples were excited with a 450 W Xenon lamp at 315 nm, the excitation wavelength for dityrosine bonds. An emission spectrum was produced from collecting the emission from 330 - 600 nm with a red-sensitive photomultiplier tube (PMT, Hamamatsu R928 PMT) used for detection of light. A 4 nm bandwidth was used for both excitation and emission slits, which controls the amount of light passing through from the Xe lamp and to the PMT. The emission data was collected in steps of 1 nm, with a dwell time of 0.1 s, with 5 scans averaged for each sample to improve the signal to noise ratio.

2.9 Small Angle Neutron Scattering

The structure of the protein hydrogel networks were investigated with small-angle neutron scattering (SANS) [123, 136, 245–248]. SANS allows for the investigation of structure over the range of 10s to 100s of angstroms. This section highlights the key theory of neutron scattering relevant for this thesis. The reader is directed to further relevant references for more details [249–252].

2.9.1 Diffraction for Structural Predictions

Diffraction is the phenomena whereby the passage of a wave from a single source, either through a small aperture of comparable size to the wavelength, or past a sharp edge causes the wave to bend or spread out. Diffraction considers how an individual wave changes when passing an object, however when two waves interfere with each other, the superposition of the two waves is used to predict the resultant wave. The Principle of

Superposition states that when two waves interfere, the resultant wave has an amplitude equivalent to the algebraic sum of the two individual, superposing waves [253]. Two examples of the superposition of two interfering waves is demonstrated in Figure 2.20. Figure 2.20A shows an example of destructive interference, where two interfering waves are 180° out of phase. Here, the the sum of the amplitudes of the superimposed waves equates to 0, therefore the amplitude of the resultant wave is also 0. Figure 2.20B shows an example of constructive interference, which occurs when two coherent waves interfere. In this case, the resultant amplitude is the sum of amplitudes of the two superimposed waves.

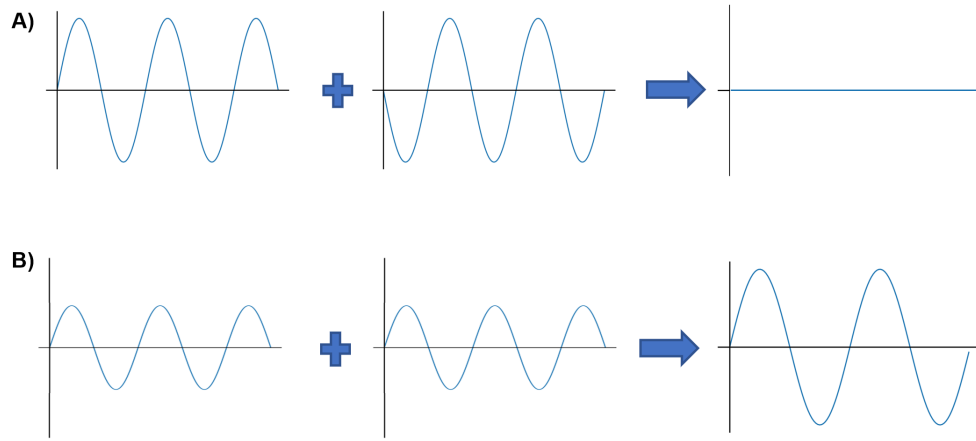


Figure 2.20: Schematics showing the principle of superposition: A) Constructive interference occurs when two in-phase waves interfere, where the final wave has an amplitude equal to the sum of the two incoming waves. B) Destructive interference occurs when two out-of-phase waves interfere, resulting in no wave.

Constructive and destructive interference can be defined by Equations 2.22 and 2.23:

$$\text{Constructive Interference } n\lambda = X \quad (2.22)$$

$$\text{Destructive Interference } n\lambda = X + \frac{\lambda}{2} \quad (2.23)$$

where n is an integer, λ is the wavelength and X a derivable quantity such as the path length difference. Constructive and destructive interference is required to produce

a diffraction pattern in a diffraction experiment. For a crystal lattice of evenly spaced

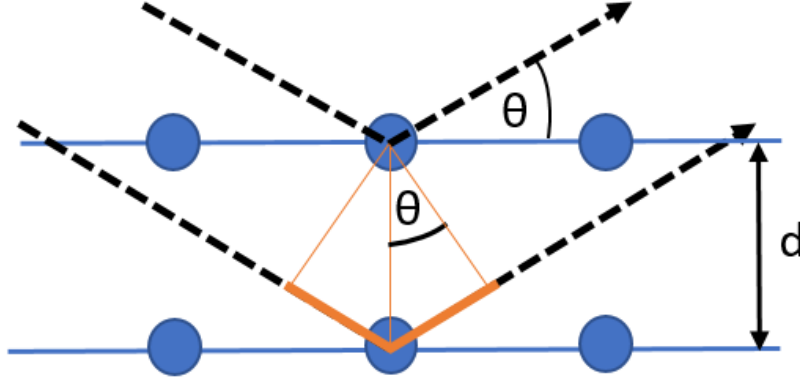


Figure 2.21: Bragg diffraction of waves from a plane of atoms, where θ is the angle of incidence and d is separation between planes of atoms. Bragg's Law is used to determine structural properties of the sample, using information from diffraction experiments. The orange highlighted section of the path defines the path length difference between the upper and lower waves.

planes of atoms separated by distance d , a wave entering the crystal incident to an atom with angle θ , will be scattered off the atom at the same angle (θ). The theory of diffraction and superposition can be used to determine the structural information of a lattice consisting of planes of atoms. Consider two parallel, coherent waves that scatter off atoms in two different planes, as depicted in Figure 2.21. The wave scattered off the top plane of atoms travels at a shorter distance compared to the wave scattered off the bottom plane. The extra distance travelled by the bottom wave is defined as the path length difference between the two reflected waves. X from Equation 2.23 can therefore be attributed to the path length difference, and is characteristic of the structure of the atoms. Trigonometry allows half of the path length difference to be calculated by $d\sin(\theta)$. Finding the total path length difference requires understanding of how the two scattered waves interact. As the waves are parallel and in-phase, the waves will constructively interfere, and Equation 2.22 can be used. The total path length difference of the two reflected waves can therefore be defined as:

$$n\lambda = 2d\sin\theta \quad (2.24)$$

which is known as Bragg's Law [254]. Bragg's Law is fundamental to understanding diffraction patterns and relating them to structures of a vast range of materials. In diffraction experiments, the angle of the incoming waves and scattered waves are quantified, where the angle of scattering is measured and equal to 2θ (Figure 2.21). In Equation 2.24 the angle of scattering is inversely proportional to d , therefore in diffraction experiments the position of detectors is key to probing structure. When conducting diffraction experiments, wide angles are therefore required to investigate small-scale structures and small angles are required to investigate large scale structures.

2.9.2 Reciprocal Space

This section will cover the basic theory involved in scattering experiment, using the example of an ideal elastic scattering experiment. Figure 2.22 shows a particle or wave that is incident to the sample, with wavevector $\bar{k}_i$. The wave is scattered by the sample, and the resultant wave is described to have a wavevector $\bar{k}_f$ and is detected for analysis [249]. A general equation for a wavevector, k , is defined as:

$$k = \frac{2\pi}{\lambda} \quad (2.25)$$

where λ is the wavelength of incident light.

In a scattering event, the waves incident to, and scattered by the sample can be expressed with as a conservation of momentum:

$$\bar{P} = \hbar\bar{k}_i - \hbar\bar{k}_f = \hbar\bar{q} \quad (2.26)$$

where $\bar{P}$ is the momentum, $\hbar = h/2\pi$ and h is Planck's constant, q is exchanged wavevector, determined from the difference between the incident and scattered wavevector, q :

$$q = \bar{k}_i - \bar{k}_f \quad (2.27)$$

For simplicity, a scattering experiment with elastic scattering is considered. In elastic

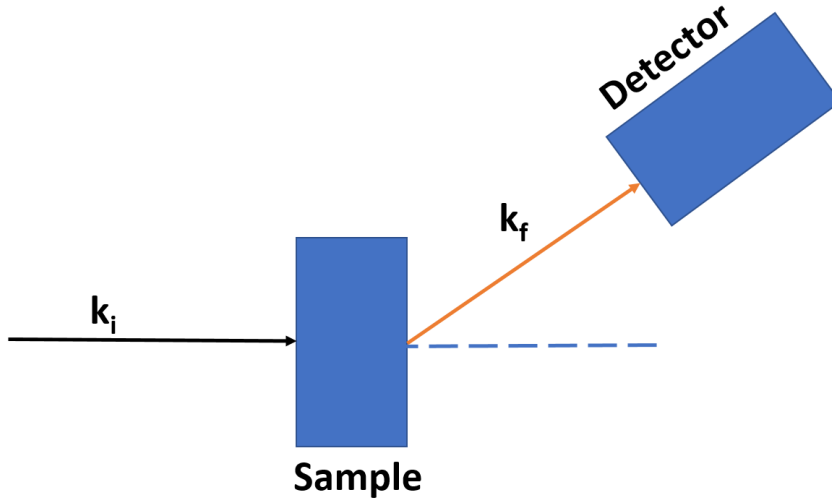


Figure 2.22: Schematic of a particle incident to the sample (k_i) being scattered by the sample (k_f)

scattering, the particle incident to the sample will have the same momentum and energy as the particle after scattering, as a result wavevector will not change after scattering allowing Equation 2.26 to be simplified:

$$|\bar{k}_i| = |\bar{k}_f| \quad (2.28)$$

Figure 2.23 shows an Ewald sphere, demonstrating how the wavevectors can be used to predict diffraction waves, whilst also proving Bragg's Law (Equation 2.24). The Ewald sphere consists of a sample crystal at the centre of a circle, where the incident particle (k_i) is scattered by an angle of 2θ . To determine the length of q , trigonometry is used to determine q from wavevectors k_i and k_f , and equation 2.25 for wavevectors k_i and k_f is substituted in Equation 2.27.

$$q = 2 \left(\frac{2\pi \sin\theta}{\lambda} \right) = \frac{4\pi \sin\theta}{\lambda} \quad (2.29)$$

Equation 2.27 demonstrates how the momentum, wavelength and scattering angle are related, but not how the spatial information is related. Using Bragg's Law for the

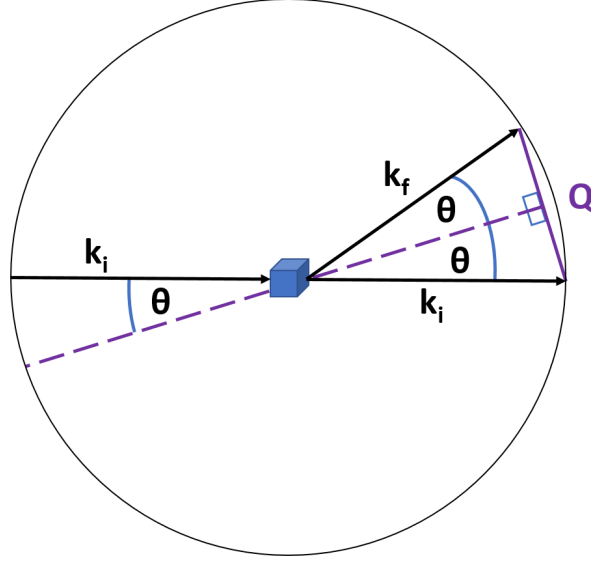


Figure 2.23: Schematic of a particle incident to the sample (k_i) being scattered by the sample (k_f)

definition of d (Equation 2.24) is therefore substituted in Equation 2.29, the relation between q and d can be determined:

$$q = \frac{2\pi}{d} \quad (2.30)$$

Equation 2.30 demonstrates that q is inversely proportional to d . This relationship allows q to be used in reciprocal space to measure real space distances. Considering the real space distances are on the order of 10^{-8} to 10^{-10} , the size would be difficult to determine in real-space. Measuring the distance in the reciprocal space (q -space), instead of the real space, is therefore more convenient to experimentally determine the structural information on small length-scales. Young's double slit experiment is an example of using the diffraction pattern to reveal information about the sample under investigation [255, 256].

In this case, light passes through two small slits, producing diffracted waves, as demonstrated in Figure 2.24. The diffraction pattern is produced from the diffracted waves undergoing constructive and destructive interference, producing a 1-D diffraction

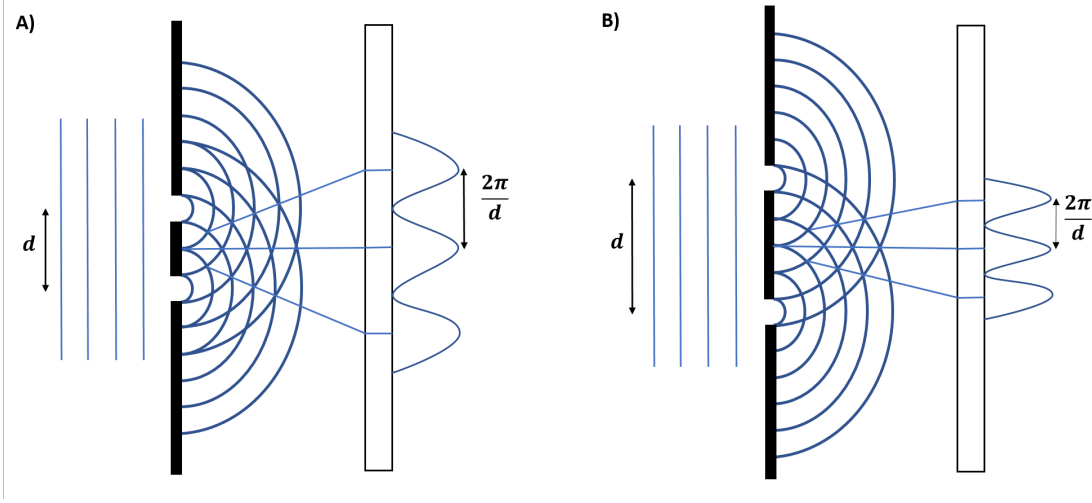


Figure 2.24: Young's double slit experiment, where d is the distance between slits, a is the slit width demonstrating constructive interference causes peaks in the diffraction pattern and destructive interference results a wave amplitude of 0. Furthermore, Young's double slit experiment shows how the change in the spacing between slits effects the resultant diffraction pattern. A) A small slit spacing results in a large distance between the peaks in the resultant diffraction pattern. B) A large slit spacing results in a smaller distance between the peaks.

pattern of light and dark fringes. Young's double slit experiment states that when the spacing between two slits (d) is small, the distance between peaks in diffraction peaks is large. Young's double split experiment demonstrates that diffraction patterns are controlled by the spacing between slits and that the knowledge of wavelength of the light incident to the slits is a valuable source of information when determining structural information. In terms of neutron diffraction, the distance between the diffraction peaks are equal to q from Equation 2.30. This has an inverse relationship between q and d . The Aperture function ($A(x)$) can be used to describe the relationship between the amplitude of light that passes through the slits, where the position of the slit is at x . In Young's double slit experiment, the $A(x)$ (Equation 2.31) is described by a pair of Dirac δ functions, with a distance $\frac{d}{2}$ from the centre.

$$A(x) = \delta\left(x - \frac{d}{2}\right) + \delta\left(x + \frac{d}{2}\right) \tag{2.31}$$

The diffraction pattern is determined by a Fourier transform, where the diffracted

wave function, $\psi(q)$ is described by Equation 2.32:

$$\psi(q) = \psi_0 \int_{-\infty}^{\infty} A(x) \exp(iqx) dx \quad (2.32)$$

where ψ_0 is the constant of proportionality, and q is defined in Equation 2.29. As the integral from $-\infty$ to ∞ of a Dirac delta function 1, Equation 2.32 can be simplified to:

$$\psi(q) = \psi_0 \left[\exp\left(iq\frac{d}{2}\right) + \exp\left(-iq\frac{d}{2}\right) \right] \quad (2.33)$$

$$\psi(q) = \psi_0 2 \cos\left(\frac{qd}{2}\right) \quad (2.34)$$

In diffraction experiments, the intensity $I(q)$ is observed, not the $\psi(q)$. Therefore to relate $I(q)$ to $\psi(q)$, the complex conjugate of $\psi(q)$, $\psi(q)^*$ is used:

$$I(q) = |\psi(q)|^2 = \psi(q)\psi(q)^* \quad (2.35)$$

$$I(q) = I_0 \cos^2\left(\frac{qd}{2}\right) \quad (2.36)$$

This demonstrates that to extract the information from the q -space, the Fourier Transform of the function of real space is used and vice versa. This is made possible due to the length-scales used for a function in real space being inversely related to its Fourier transform, as q -space and real space are inversely proportional [249].

2.9.3 Neutrons as a Diffraction Probe

In diffraction experiments it is possible to use different probes with wavelengths of a suitable length scale that allow the study of the structure. Commonly used probes include neutrons, X-rays and electrons. For the diffraction experiments in this thesis neutrons were selected as the most appropriate probe, and this section sets out to describe the motivation for studying the structure of protein hydrogels with neutrons.

Table 2.1 contains information about different probes, comparing neutrons to protons and photons. Neutrons (n) have a half life of roughly 15 mins, after which they decay

Table 2.1: Properties of different probes (neutrons, protons and photons) that are commonly used for diffraction experiments.

Property	Neutron	Proton	Photon
Mass (MeV/c ²)	939.6	938.3	0
Charge (e)	0	+1	0
Spin	$\frac{1}{2}$	$\frac{1}{2}$	1
Classification	Baryon	Baryon	Boson

into a proton (p), electron (e^-) and an anti-neutrino ($\bar{\nu}_e$):

$$n \rightarrow p + e^- + \bar{\nu}_e \quad (2.37)$$

Neutrons are subatomic particles, classed as fermions, made up of 3 quarks, two up and 1 down. Each up quark has a charge of $+2/3$ and the down quark has a charge of $-e/3$, making the charge of neutron zero. Due to having no charge neutrons are highly penetrating, only weakly interacting with matter, making neutrons a desirable probe for diffraction. Neutrons are therefore a non-destructive probe, making them advantageous to X-rays that can damage biological samples. Additionally, they are able to pass through the electron cloud surrounding a nuclei without interacting with the electron cloud. Instead, neutrons scatter off the nuclei due to strong force interactions. This leads to diffraction patterns based off the structure (Section 2.9.2). X-rays on the other hand interact with the electron cloud surrounding atoms. Interestingly for work not covered in this thesis, the neutron has a half integer spin allowing for the use of neutron scattering for studies with magnetism.

Furthermore, neutrons allow for a technique known as contrast matching which is not possible with X-rays (see Figure 2.25). Contrast matching takes advantage difference in scattering length densities (SLD) between an object and the surrounding medium [249]. Hydrogen and deuterium have very different SLDs, such that an object can be deuterated (replacing hydrogen with deuterium) or protonated (replacing deuterium with hydrogen) to match the surrounding medium. This allows the object to blend with the surrounding medium. For example in the case of particle with a shell submerged in a deuterated solvent, the particle could be deuterated to matching the contrast of the

deuterated solvent. This would provide the shell to have a strong difference in contrast to the deuterated solvent and particle, and simplifying the analysis of systems made up of at least 3 components [257].

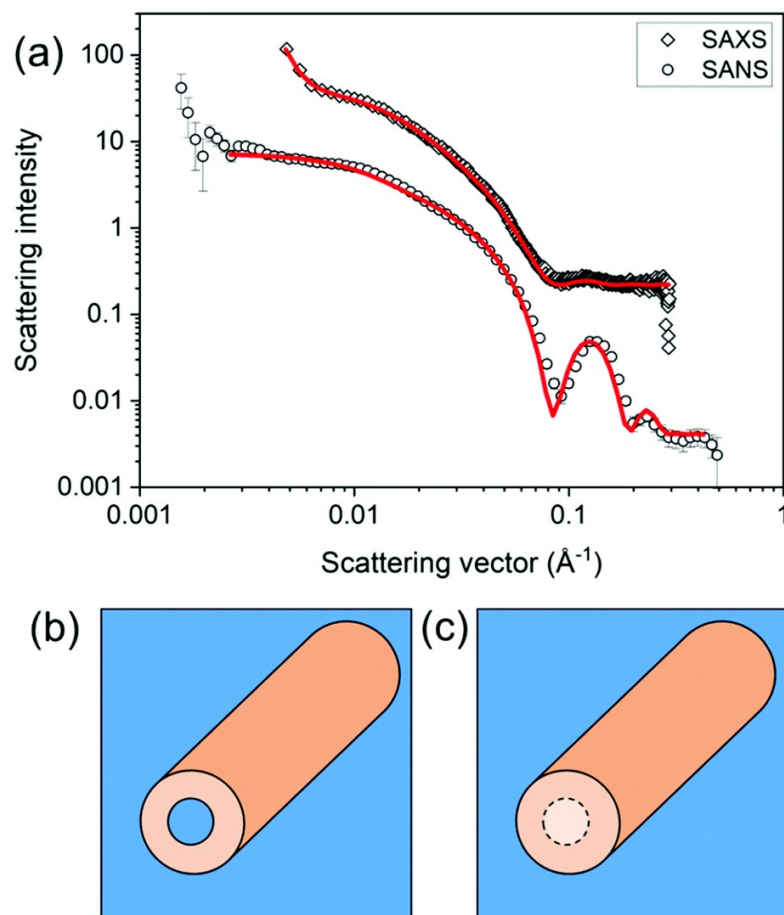


Figure 2.25: A) Different scattering intensity curves ($I(q)$) as a function of scattering vector (q) for a hollow cylindrical rod in a solvent, measured with small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS). B) SANS allows for contrast matching of core in the cylinder to the solvent surrounding the cylinder. C) SAXS does not have the ability to contrast match the core to the surrounding solvent. Reused from [257] with permission under the Creative Commons Attribution 3.0 Unported Licence.

2.9.4 Scattering Data Analysis: Form and Structure factor

This section outlines how the scattering data is converted to useful parameters, in order to interpret the results from a scattering curve. The structure of a material in real space,

$\mathcal{S}(r)$, can be summarised with the following expression:

$$\mathcal{S}(r) \propto P(r) * g(r) \quad (2.38)$$

where $P(r)$ is the pair-pair distribution function, and $g(r)$ is the radial distribution function, The $P(r)$ describes the distances between pairs of particles contained within a given scattering object, which is used to provide information on the shape of a single scattering object. $g(r)$ gives a measure of distribution of particles with respect to how far each particle is from defined reference particle. This function describes the distribution of the scattering objects in space. Equation 2.38 shows that $P(r)$ and $g(r)$ are that both functions are proportional to $\mathcal{S}(r)$, and also shows that $P(r)$ and $g(r)$ are convoluted together. Convolution is a mathematical term describing the operation on two individual, general functions ($f(x)$ and $g(x)$) that results in a new function ($f(x) * g(x)$), and can be used to solve complex interactions. By changing $f(x)$ or $g(x)$, the result of $f(x) * g(x)$ will also be changed. The structure of the material in real space in Equation 2.38 therefore shows both that the shape of the scattering object and the distribution of the scattering objects both affect the resultant structure. Furthermore, separating the $P(r)$ information from $g(r)$ is difficult due to being convoluted together. In order to separate the convoluted functions, the convolution theorem allows the use of a Fourier transform of $P(r) * g(r)$ to separate the two functions:

$$\mathcal{F}\{f(x) * g(x)\} = \mathcal{F}\{f(x)\} \cdot \mathcal{F}\{g(x)\} = \hat{f}(t) \cdot \hat{g}(t) \quad (2.39)$$

Equation 2.39 shows that by the Fourier transform, ($\mathcal{F}$), of $P(r) * g(r)$ is equivalent to the product of the Fourier transform of $P(r)$ and the Fourier transform of $g(r)$. Note that the order in which the convolution between two functions operated does not affect the resultant new function, i.e. $f(x) * g(x) = g(x) * f(x)$ Applying the convolution theorem in Equation 2.39 to Equation 2.38 results in:

$$\hat{\mathcal{S}}(q) = I(q) \propto P(q) \cdot g(q) \quad (2.40)$$

where $I(q)$ is the scattering intensity of the sample. Section 2.9.2 discussed that using the Fourier transform on structural information from real space to reciprocal space, or q-space. Applying the convolution theorem to the real space structure, $\mathcal{S}(r)$, therefore results in the structure in q-space, $\mathcal{S}(q)$. Equation 2.40 shows that $\mathcal{S}(q)$ is proportional to the product of $P(q)$, the form factor, and $S(q)$, the structure factor. The form factor is a function in reciprocal space, defined by the shape, or form, of the scattering object. The structure factor, also a function in reciprocal space, defined by the spacial arrangement of scattering objects. The interactions between scattering objects will determine the arrangement of the scattering objects. In reciprocal space, $P(q)$ and $S(q)$ are multiplied together, making the two functions easier to separate in reciprocal space, than in real space where the two functions are convoluted together. This allows the structural information to be determined by having separate information on the both the shape of the scattering objects and how they are arranged. In experiments a 2D scattering pattern is produced from the scattering data. This scattering pattern is integrated to produced a curve of $I(q)$ vs q , an example of which is shown in Figure 2.25 In contrast matching experiments described in Section 2.9.3, then scattering intensity can be related to the SLD of the object (SLD_{object}) and the SLD of the surrounding solvent ($SLD_{solvent}$):

$$I(q) \propto Mc(SLD_{object} - SLD_{solvent})^2 F(q) S(q) \quad (2.41)$$

where M is the molecular weight of the object and c is the concentration of the object. Equation 2.41 shows how the information about the object of interest, its size and shape is all accounted for in $I(q)$ [257].

This overview of how the structural characterisation is achieved with neutron scattering, highlights the value of using the reciprocal space to determine the structure of a system. The structure factor and the form factor are used in Chapters 3 and 5 for structural analysis from the scattering curves of folded protein hydrogels, allowing the arrangement and topology of the protein hydrogel to be predicted.

2.9.5 SANS Experimental Set-up

Small angle neutron scattering (SANS) experiments were performed at ISIS Neutron and Muon Source (STFC Rutherford Appleton Laboratory, Didcot, UK) using time-of-flight instrument Sans2d with the rear detector at 12 m and the front detector at 5 m from the sample to give a wavenumber range of $0.0015 \leq q \leq 1 \text{ \AA}^{-1}$. The pre-gel solution in 98% D₂O were loaded into quartz cuvettes with 1 mm path length. 98% D₂O was used as a result of the lipid preparation in H₂O buffer, to keep the lipid preparation the same in all samples. The pre-gel solutions in 98% or 20% D₂O were loaded into quartz cuvettes with 1 mm path length. 20% D₂O was used to contrast match the lipids to the buffer and 98% was used as a result of the lipid preparation in H₂O buffer. The scattering from an empty quartz cuvette was measured and used for accurate buffer subtraction of the sample. Experiments were executed at 20°C, controlled by a thermal bath. The raw data was reduced and corrected for transmission and detector efficiencies and normalised on an absolute scale with scattering from a partially deuterated polystyrene standard using the Mantid framework [258, 259]. The resultant, absolute scattering curves were fitted with SASview [260] by Dr Matthew Hughes.

2.10 Microfluidic Formation of Microgels

Microfluidic devices were used to produce microgels, this section outlines microfluidic background and theory, how the microfluidic devices were designed and produced, and how the microgels were formed. The production of the devices involves firstly designing the device in AutoCAD software, followed by the fabrication of the master using photolithography, and the final step of soft lithography to produce the poly-dimethyl siloxane (PDMS) devices.

2.10.1 Microfluidics and Laminar Flow

Microfluidics is defined as fluid flow through microscale devices, where the fluid behaviour differs to macroscale fluid dynamics. Microscale systems allow for precise control and manipulation over fluids due to the behaviour of flow known as laminar flow. This behaviour is achieved by combination of small sized channels, small volumes of fluid typically on the order of nano or picoliters, and low viscosity fluids. The low sample volumes required for microfluidics is advantageous, offering low cost system due to the small volumes of reagent used. Additionally, microfluidics is advantageous for its potential to scale up production, with automated and high throughput production.

2.10.1.1 Navier-Stokes Equation

The Navier-Stokes equation (Equation 2.42) is used to describe the flow of viscous liquids. For an incompressible Newtonian fluid, the Navier-stokes equation represents the conservation of momentum of a fluid in motion [261]:

$$\rho \frac{\delta u}{\delta t} + \rho(u \cdot \nabla)u = -\nabla p + \eta \nabla^2 u + F \quad (2.42)$$

where ρ is the density of the fluid, u is the fluid velocity, μ is the viscosity of the fluid (μ) and p is the fluid pressure. The left hand side of Equation 2.42 represents the inertia of the fluid, while the right hand side is the sum of the forces per unit volume. The first term on the right hand side represents the force due to pressure drops, the second term is due to viscous forces and is diffusion dependant, and the third term represents external forces due to gravity or electromagnetism. The conservation of mass is applied to the Navier-Stokes equation, allowing the continuity equation (Equation 2.43) to be used in order to solve the Navier-Stokes equation:

$$\frac{\delta \rho}{\delta t} + \nabla \cdot (\rho u) = 0 \quad (2.43)$$

As the fluid is assumed to be incompressible, the control is constant, leading to a constant fluid density, such that Equation 2.43 becomes $\nabla \cdot u = 0$.

2.10.1.2 Reynolds Number

To predict fluid flow, the flow can be characterised by different dimensionless quantities. The Reynolds number, (Re), is a dimensionless number used to describe the ratio between inertial forces (due to the momentum of the fluid through the channel) and viscous forces (due to the fluid's resistance to flow) [262]. This provides a quantitative range within which Navier-Stokes in microfluidic devices are valid.

$$Re = \frac{\text{Inertial Forces}}{\text{Viscous Forces}} = \frac{\rho u D_H}{\mu} \quad (2.44)$$

where D_H is the cross-sectional geometry of the channel, defined for a rectangular channel in Equation 2.45:

$$D_H = \frac{4A}{P} = \frac{2wh}{w+h} \quad (2.45)$$

where A is the cross-sectional area of the channel, P is the channel perimeter, h is the channel height and w is the channel width. High viscous forces result in low Re , a regime known as laminar flow. Laminar flow is defined as the fluid flowing in parallel continuous layers, with a lack of turbulence resulting in little mixing between the layers. Laminar flow allows for predictable flow behaviour and control over the flow. For a circular channel, the critical Re for achieving laminar flow is defined as $Re < 2300$. As Re increases, the inertial forces become more dominant and more mixing occurs between the layers due to more turbulent and chaotic flow. The flow behaviour becomes less predictable and more difficult to control. Microfluidic devices take advantage of flow behaviour in the laminar flow regime. Equation 2.44 shows that laminar flow is achievable in microfluidic devices with the use of microscale channel geometries, slow fluid velocities and fluid with high viscosity.

2.10.1.3 Capillary Number in Microfluidic Droplet Formation

In droplet formation, two fluids meet and the instability at the interface of fluids. Rayleigh-Plateau instability states that a pressure difference between two liquids at their interface that can result in the detachment of liquid in the dispersed phase, forming a

droplet. The capillary number (Ca) is used to characterise the relationship between the viscous forces and surface tension when two liquids that cannot mix (immiscible) meet at an interface [263].

$$Ca = \frac{\mu v}{\gamma} = \frac{\mu Q}{\gamma A} \quad (2.46)$$

where μ is the dynamic viscosity, v is the velocity of flow, γ is the surface tension, Q is the volumetric flow rate and A is the area of the channel.

In flow-focusing devices, the continuous phase meets the dispersed phase at an angle, forcing the dispersed phase into a thin stream, which at a high pressure disrupts the stream in droplets. Common regimes for droplet formation are dripping and jetting, where in flow-focusing devices a lower Ca number is associated with the dripping regime and a high Ca is associated with the jetting regime ($> 10^{-1}$) for flow-focusing devices) due to a large viscous force creating a pressure between the two liquids, such that that flow in the dispersed phase is disrupted [264]. The instability of the interfacial tension and viscous forces govern the droplet production.

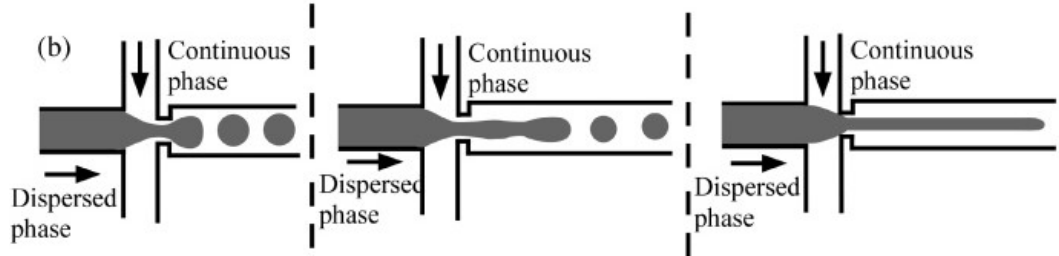


Figure 2.26: Schematic of different regimes in flow-focusing microfluidic devices. The dripping regime (left) and jetting regime (centre) result in droplet formation due to instability in pressure and interfacial tension where the two immiscible liquids meet. When the liquid is in stable (right), then droplets are not produced. Reused from [264] with permission from the Royal Society of Chemistry.

2.10.2 Master Fabrication

The masters for the devices were fabricated using 3-inch silicon wafers and negative photoresist SU-8 2075. Before use, the wafers were cleaned with acetone, isopropanol (IPA), and Milli-Q. Wafers were then put in an oven for 30 mins at 150°C, to ensure evaporation of any residual liquid then left to cool to room temperature. The cooled

wafers were coated with SU-8 2075 and spun using a spin coater (EMS 6000) and accelerated to 200 rpm for 10 s, followed by 400 rpm for 10s, 600 rpm for 10 s each, and finally at 2000 rpm for 40 s in order to achieve a SU-8 height of 120 μm . To solidify the SU-8, the solvent was evaporated off with a soft bake involving placing the wafer on a hot plate (EMS 1000-1, Salisbury, UK) at room temperature, and slowly ramping the temperature to 55°C. Once the temperature reached 55°C, the wafer was baked for 1 hour, after which the hot plate was turned off and the wafer left on the hot plate to cool to room temperature. Direct-write laser photolithography (MicroWriter MLTM, Durham Magneto Optics) was used to etch the designs into the SU-8 using a 2 μm laser, with a wavelength of 375 nm. A dose of 400 mJ/cm² was therefore selected to minimise overexposure, and to maximise the resolution of the channels when creating channels on the order of 50 μm . The SU-8 exposed to the laser cross-links, while the remaining SU-8 is soluble. The motivation for the dose selection is dependant on the device design, using a low dose allows for better resolution of smaller features, which can result in adhesion problems between the SU-8 and wafer due to insufficient cross-linking. On the other hand, if a high dose is used, then this can lead to a reduction in resolution that results in “bell-shaped” channels that are wider than designed. This occurs due to overexposure from the laser causing more cross-linking outside the design. Dose selection therefore considers the channel thickness, as a thicker channel could require a higher dose.

After etching was complete, the SU-8 underwent a post exposure bake to provide energy to complete the cross-linking of the SU-8 polymer matrix. where the wafer was heated again on a hot plate to 55°C for 1 hour. After cooling, the uncross-linked SU-8 was then washed away with ethyl lactate (EC) solvent for 5 mins, followed by washing in IPA for 1 min. Finally, SU-8 and wafer was hard baked at 200°C for 30 mins in order to anneal cracked SU-8, completing the master fabrication. The master is now reusable for the fabrication of the PDMS device.

The height of the channels was determined by scanning the master with Stylus Profiler (Bruker Detak). A probe is physically moved across the surface, therefore any change in force is part of a feedback loop such that the topography of the surface is measured.

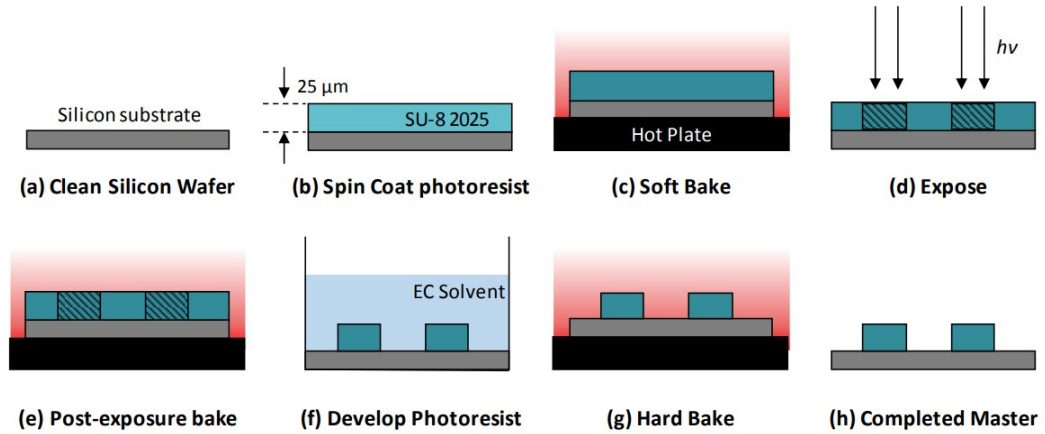


Figure 2.27: Schematic illustrating the steps to produce the a SU-8 silicon master in chronological order (a-h), used for microfluidic device production. Reused with permission from [265] under a CC BY-NC-SA 2.5 licence.

2.10.2.1 Soft Lithography

The fabrication of the microfluidic devices was achieved by using PDMS to create a negative copy of the SU-8 on the master. The master with the SU-8 is hydrophilic, therefore silanization of the master, which leaves the surface of the master hydrophobic, helps the hardened PDMS to peel off the master [266]. To silanize in gas, the wafer was placed in a petri dish with 3 drops of trimethylchlorosilane for 15 mins. To prepare the PDMS device, the PDMS base and cross-linker, were mixed in a 10:1 ratio. The uncured PDMS was centrifuged for 1 min at 4000 rpm to remove bubbles and poured onto the master in the petri dish. The PDMS and master were left under vacuum for 1 hour to remove any remaining gas in the PDMS mixture, before baking at 75°C for 1 hour to cure the elastomer. The now solid layer of PDMS was peeled away from the master and holes punched with a 1 mm Biopsy Puncher to allow for fluid flow through the inlet and outlet channels as outlined in Figure 2.28.

Glass slides form the base of the microfluidic device and were cleaned with IPA, Milli-Q and dried with nitrogen gas before use. Any dust fallen onto the PDMS and glass slides were removed with Scotch tape and then plasma treated for 1 minute, at 0.3-0.6 mbar of O_2 . The O_2 plasma treatment involves replacing the $-\text{Si}-\text{CH}_3$ on the glass and

PDMS surfaces with reactive gas, resulting in -Si-O-H- covered surfaces. This decreases the hydrophobicity of PDMS and glass, and when the two surfaces are pressed together, -Si-O-Si- covalent bonds in between the PDMS and the glass [267]. The bonded PDMS and glass slide are baked at 75°C for 30 mins, completing the device production.

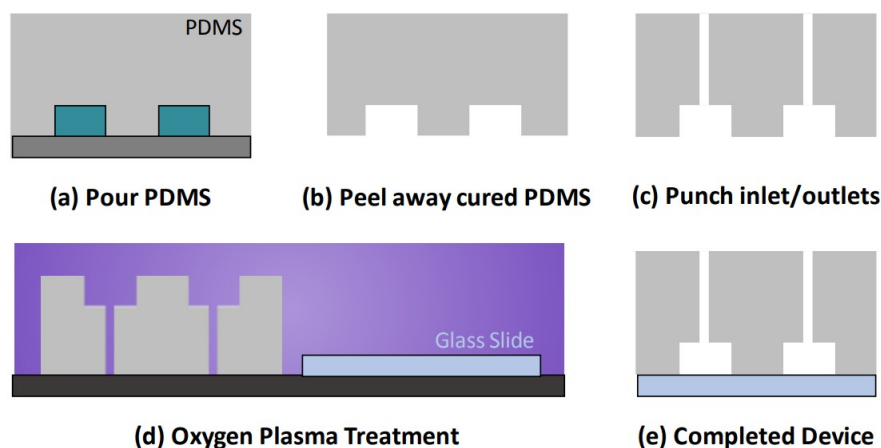


Figure 2.28: Schematic illustrating the steps in chronological order (a-e), to produce a PDMS microfluidic device. Reused with permission from [265] under a CC BY-NC-SA 2.5 licence.

2.10.3 Microfluidic Device Designs

Two different microfluidic designs were used in the project, and the device designs are outlined here. The flow-focusing method was used for droplet production [268–270]. This involved a middle channel containing the first liquid that the droplets are made from (dispersed phase) and two outer channels containing a second liquid (continuous phase). The two liquids must be immiscible, such that when the two liquids meet they do not mix. The continuous phase pressure and viscous stresses applied onto the dispersed phase breaks the stream of the dispersed phase, creating the ‘pinch-off’ of the dispersed phase to produce a droplet. Figures 2.29 and 2.30 show schematics of the flow-focusing designs used in this study. Each inlet channel is immediately followed by a grid of PDMS traps to prevent any large contaminants or aggregates from reaching the nozzle, reducing the chance of the nozzle becoming blocked. In both designs, two outer, continuous phase channels meet the inner, dispersed phase channel at a 90° angle. The

fluid passes through a small nozzle of $50\mu\text{m}$, where droplet formation occurs downstream in the dripping regime. The droplets then flow through a straight outlet channel. The first design (Figure 2.29) has a post-nozzle expansion channel with a width of $600\mu\text{m}$.

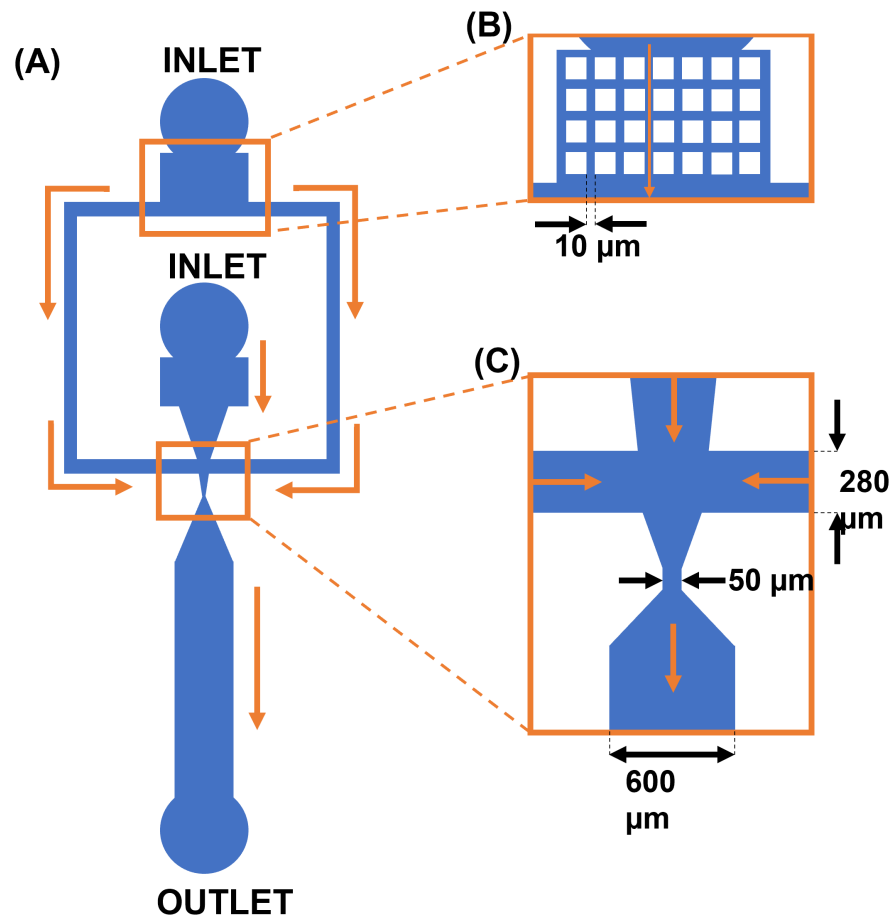


Figure 2.29: Schematic of the first microfluidic device for droplet formation. Orange arrows depict the direction of fluid flow. A) Flow-focusing device formed of three inlet channels that meet at a nozzle. The fluid leaves the channel at the outlet. B) Inset of the grid of traps designed to prevent any large aggregates from reaching the small nozzle. C) Inset of the nozzle, where the fluid is focused into the nozzle with a diameter of $50\mu\text{m}$, followed by an expansion in the outlet channel of $600\mu\text{m}$.

The second design (Figure 2.30) has a reduced post-nozzle expansion channel with a width of $90\ \mu\text{m}$ and longer outlet channel length to reduce microgel coalescence.

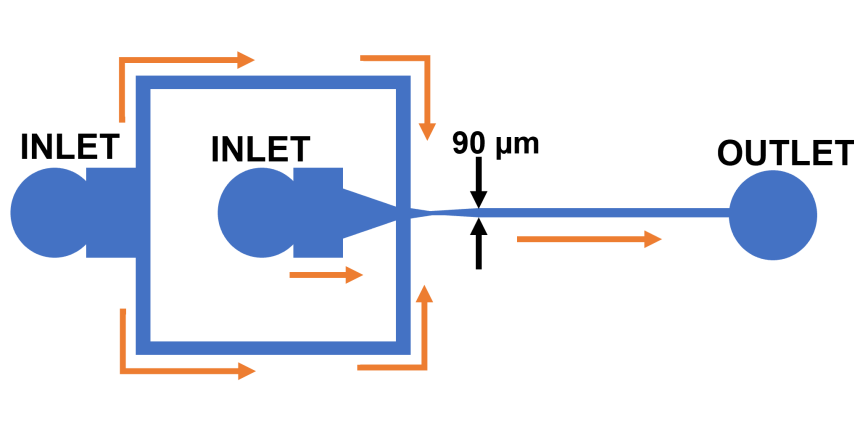


Figure 2.30: Schematic of the second microfluidic device used for droplet formation, where the orange arrows depict the direction of fluid flow. The fluid is focused into a small nozzle with a diameter of $50\ \mu\text{m}$, followed by an expansion in the outlet channel of $90\ \mu\text{m}$.

2.10.4 Microgel Production

To form droplets with the PDMS devices, designs from both Figures 2.29 and 2.30 were used. 0.25 mL glass syringes were cleaned with IPA and Milli-Q water before use. PDMS devices were connected to the syringes with fine bore polythene tubing (Smiths MedicalTM, UK), with an inner diameter of 0.36 mm and an outer diameter of 1.09 mm. Tubing was inserted into the 1 mm diameter holes punctured into the PDMS, such that fluid could flow in and out of the inlet and outlet channels of the PDMS devices. The pre-gel solution used in the dispersed phase was 200 mg/mL of BSA, 50 mM NaPS and 0.1 mM $\text{Ru}(\text{bpy})_3^{2+}$. The syringe that was loaded with the pre-gel solution was covered in foil, to prevent illumination of the pre-gel solution initiating gelation in the syringe. The continuous phase contained grapeseed oil mixed with a 2 wt% ABIL[®] EM 90 (Evonik). ABIL[®] EM 90 is a non-ionic emulsifier composed of Cetyl PEG/PPG-10/1 Dimethicone used to stabilise water/oil emulsions. The flow rates used were $0.1\ \mu\text{L min}^{-1}$ for the dispersed phase and $1\ \mu\text{L min}^{-1}$ for the continuous phase, controlled by syringe pumps (PhD Ultra and Pump Elite Harvard Apparatus). Droplets were collected in a centrifuge tube via the outlet tubing, where the set up is shown in Figure 2.31. The droplets were

illuminated with a blue LED light for 5 mins to initiate gelation of the BSA protein.

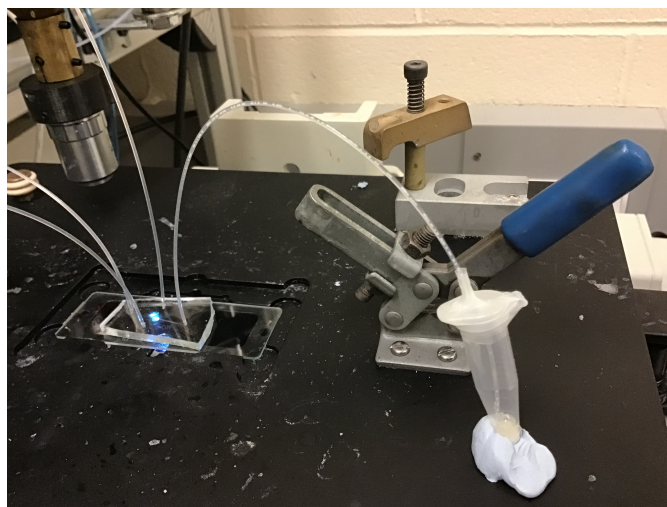


Figure 2.31: Set-up for microgel production. The PDMS devices with inlets and outlet channels inserted. A syringe pump was used to control the flow of the continuous and dispersed phase for on-chip droplet production. Droplets were collected via outlet in a centrifuge tube.

High speed microscopy was performed with a FASTCAM SA-X camera (Photron, Japan) observe droplet production. Bright field microscopy was used to image the microgels after production with an inverted microscope (Eclipse Ti-U Nikon, Japan). Imaging software *ImageJ* [271] was used to analyse the high speed videos of microfluidic droplet production.

2.11 Cell Lines and 2D Cell Culture

This section outlines the steps followed to grow the human pancreatic epithelial ductal adenocarcinoma cells (PANC-1) and human pancreatic stellate cells (PSC), used for the pancreatic ductal adenocarcinoma (PDAC) cultures [272]. PDAC cells were cultured on both the BSA hydrogel and a control hydrogel, Cultrex[®] Basement Membrane Extract (BME) hydrogel. The cell viability of the PDAC cells grown on both the gels were compared. Cell culture and preparation of the BME gel was completed by Dr. Delanyo Kpeglo.

2.11.1 Culturing PANC-1 and PSC cells

The PANC-1 cells were maintained in culture medium composed of Dulbecco's Modified Eagle Medium (DMEM), 10% foetal bovine serum (FBS), 1% Penicillin Streptomycin (PS) and 1% GlutaMAX Supplement, referred to as DMEM/FBS culture medium. PSC cells were maintained in culture medium (Stellate media) composed of Stellate Cell Medium (SteCM), 10% FBS, 1% Stellate Cell Growth Supplement and 1% PS, referred to as Stellate/FBS culture medium.

PANC-1 and PSC cells were cultured in a 25 or 75 cm² culture flask under humidified conditions (95 – 99%) at 37°C and 5% CO₂. The PSC cells were cultured in culture flasks coated with poly-l-lysine for at least an hour. For both cell lines, the cells were passaged from culture with 1 mL TrypLE Express Enzyme (1×) no phenol red. Cells were used only when ≥ 70 % cell confluence was achieved. For PSC cells, passages between 2 and 6 were used. Cells were counted by adding 20 μ L of Trypan Blue-treated cell suspension to a haemocytometer.

2.11.2 Seeding of PDAC Cells onto Hydrogels

The BSA hydrogels were prepared, as described in Section 2.3.4, in a 96 well plate, filling each well with 50 μ L. The well plate containing the BSA hydrogel was sterilised under UV light ($\lambda = 265$ nm) for 30 mins. Some of the well plates had an additional step of washing the BSA hydrogels with DMEM/FBS culture medium 3 times. This involved plating 100 μ L of sterile DMEM/FBS culture medium on the surface of the BSA hydrogel and incubating the DMEM/FBS culture medium on the BSA hydrogel for 5 mins, after which the DMEM/FBS culture medium was pipetted off and replaced with fresh DMEM/FBS culture medium. Between the 5 min washing steps for the BSA hydrogel, the BME (R&D Systems, Bio-Techne) hydrogel was prepared, ready to be pipetted into the 96-well plate. To prepare the BME hydrogel, BME was mixed with DMEM/FBS culture medium media in a 3:1 ratio, such that the final BME was 6 - 9 mg/mL. Both BME/DMEM and BSA hydrogels were incubated at 37°C for 30 mins to

gelate the BME.

To culture the PDAC cells, PANC-1 and PSC cells were seeded together in a 1:3 ratio, into the 96-well plate. The seeding density was 1×10^5 cells mL^{-1} per well. PDAC cells were cultured in the DMEM/FBS culture medium under humidified conditions (95-99%) at 37°C and 5% CO_2 for 48 hours. Media was replaced when the DMEM cell media turned to an orange-yellow colour, due to changes in pH of the DMEM/FBS medium solution. Images of the PDAC cells were taken using the inverted microscope (VWR VisiScope[®] IT404).

2.11.3 ATP Viability Assay

The metabolic activity of the PDAC cultured cells grown on the BSA and BME gels was compared. To assess the metabolic activities, the adenosine triphosphate (ATP) content of the cultures was quantified with CellTiter-Glo[®] 3D reagent. The ATP content of the cells is proportional to the luminescence signal of the CellTiter-Glo[®], and can therefore be used to quantify the viability of the cultured cells. To prepare the cells for the viability assay, the culture media in the wells of the 96-well plate was removed, leaving the PDAC cell behind. A 1:1 ratio of DMEM/FBS culture media was mixed with CellTiter-Glo[®] 3D reagent, and added to the wells in the 96-well plate. The plate was incubated for 20-25 mins to lyse the cells. The cells and DMEM/FBS/CellTiter-Glo[®] mix was transferred by pipetting into a 96-well opaque walled plate (BD Falcon) for reading the luminescence signal with a SpectraMAX M2 microplate reader (Molecular Devices).

Chapter 3

Characterisation of BSA Hydrogels in the Presence of Embedded Microbubbles

This chapter covers an introduction to the literature on bovine serum albumin (BSA) protein hydrogels and the results from a range of methods to characterise the effect that including microbubbles (MBs) within folded protein hydrogels has on the protein and the protein hydrogel network formed. Characterisation of the folded protein hydrogel across a range of length scales aims to help understand how folded globular proteins can be used for creating functional, responsive biomaterials for MB aided drug release, without compromising on the mechanical properties of the protein hydrogel. Folded protein hydrogels have hierarchical behaviour, meaning that changes made to the protein monomer can translate through to the differences in the mesoscale structure defined by the network of protein monomers formed, through to the bulk mechanical properties of the hydrogel [123, 136, 245–248]. It is therefore important to characterise the properties from the nanoscale, mesoscale structure, through to the macroscale properties, in order to gain a full understanding of the multi-composite system for controlling drug delivery in later stages.

Understanding the mechanical properties when designing a new hydrogel system is key, as tissues in the body cover a wide range of stiffnesses, from 1 kPa up to 1 GPa [98]. When considering the biomedical applications of the hydrogel it is therefore vital to understand the mechanical properties. The presence of MBs will create a volume where the hydrogel network cannot percolate. A fundamental question to understand is how the inclusion of an embedded microbubble will impact the mechanical properties of folded protein hydrogels. The microbubbles will create a void within the cross-linked hydrogel network and may impact the gelation process. The inclusion of particles dispersed into hydrogel networks (filler particles) has been shown to affect the mechanical properties of the hydrogel, heavily influence the filler particle and interactions between the filler particle with the network. Key properties of the filler particles are the filler size, volume fraction and stiffness relative to the surrounding network [273, 274]. Cases where the filler particles interact with gel can result in an increase in the shear modulus of the hydrogel. Conversely, the inclusion of non-interacting filler particles can cause a decrease in the shear modulus [275–277]. The presence of MBs in the gelation of BSA hydrogels therefore may impact the resultant network, therefore the viscoelastic and structural properties of BSA hydrogels in the presence of MBs (BSA:MB hydrogels) were characterised. Characterisation of the mesoscale structure will allow the impact of the MBs on the properties of the network of proteins formed to be determined. Previous studies of folded protein hydrogel structures has predicted fractal-like clusters of proteins that are connected by chains of proteins in the inter-cluster space [119, 123, 135, 136]. Figure 3.1 shows a schematic of how different protein building blocks are predicted to form different size clusters, determined from SANS. This chapter studies the mechanical properties and structure of the protein network in BSA:MB hydrogels and compares to BSA hydrogels.

The amount of unfolding of proteins in protein hydrogels can influence the viscoelastic properties of the resultant protein hydrogel, with further details described in Chapter 1. Hughes et al. (2021) showed that the chemical cross-linking of BSA can cause unfolding of protein, shown in Figure 3.2. The process of chemically cross-linking protein and the relaxation of the network (network rearrangement to find a less energetic state), was

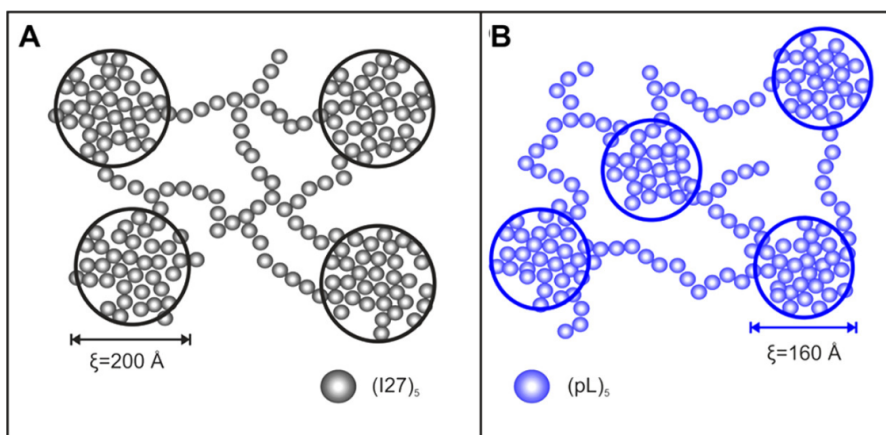


Figure 3.1: Schematic of the protein network structure for A) I27 (small black spheres) and B) (pL)₅ (small blue spheres), based off SANS predictions. Large circles highlight the correlation length (ξ) associated with the size of the fractal-like clusters. Reproduced from da Silva et al. (2017) [119] under a CC BY 4.0 licence.

suggested to generate forces which can induce the unfolding of proteins in the final BSA hydrogel network. The fraction of the folded protein in the BSA hydrogel formed in the presence of MBs was studied in this chapter. Furthermore, this underpins the end goal of using proteins as building blocks for hydrogels: to utilise functional proteins for improved therapeutic treatment.

3.1 BSA: A Model Protein

The model protein for this study is required to have several surface exposed tyrosine residues to form dityrosine bonds between BSA monomers, in order for successful cross-linking via photo-chemical initiation (see Section 2.3.1). Furthermore, the model protein is required to be soluble in water for hydrogel production. BSA is a well characterised protein in terms of the secondary structure and what causes BSA to unfold [278, 279]. Folded BSA hydrogels have previously been studied, which provide key information for comparison with the data presented in this chapter. Additionally, BSA hydrogels have previously demonstrated success in proving a tuneable protein hydrogel network in the mechanical and structural properties, controlled by both chemical and physical differences to the preparation of the BSA hydrogel [115, 119, 123, 136, 280]. This makes

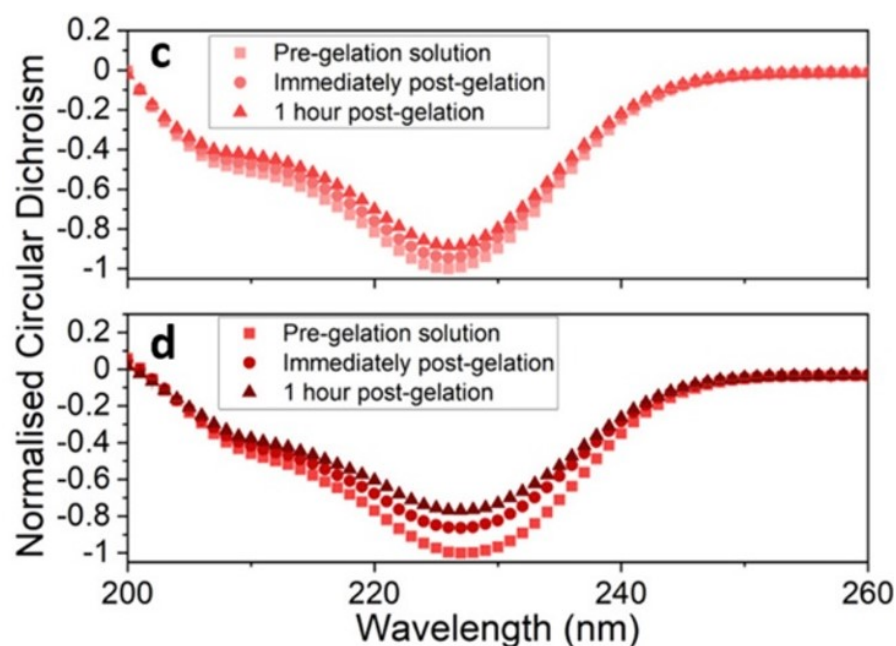


Figure 3.2: Circular dichroism before, immediately after and one hour after chemical cross-linking between BSA monomers is initiated. Top: BSA hydrogel, Bottom: BSA in the presence of dithiothreitol that induces partial unfolding of BSA. Reproduced from Hughes et al. (2021) [136] with permission from the Royal Society of Chemistry.

BSA hydrogels an interesting system to investigate for biomedical applications, as the methods to control the network properties could be beneficial to applications in tissue engineering and drug delivery.

Bovine serum albumin (BSA) is an α -helical structured blood protein (Figure 3.3), with the function of carrying steroids, fatty acids and thyroid hormones [281]. BSA contains 583 amino acids, and has a molecular weight of 66.5 kDa [282]. The secondary structure of BSA 67% α -helical, 10% β turn, 23% extended chains, and has a melting temperature of 60°C [278, 283, 284]. BSA was selected to form chemically cross-linked hydrogels due to the access of 18 surface exposed tyrosine residues. Serum albumin proteins are the most abundant proteins in humans and bovine [285]. They can be extracted from blood serum using the fractionation of plasma, a method first developed using the Cohn process in 1946, involving high concentrations of ethanol to fractionate the protein [286]. This is possible due to serum albumin proteins being significantly more stable than other plasma proteins, such that albumin proteins can be heated without

denaturing. Heat-shock fractionation takes advantage of this, resulting in the extraction of BSA at a purity of 98% and available in large quantities [281, 287].



Figure 3.3: Structure of BSA molecule (PDB code: 3V03) BSA molecule with the tyrosine residues highlighted in orange.

3.2 Biological Functionality of BSA

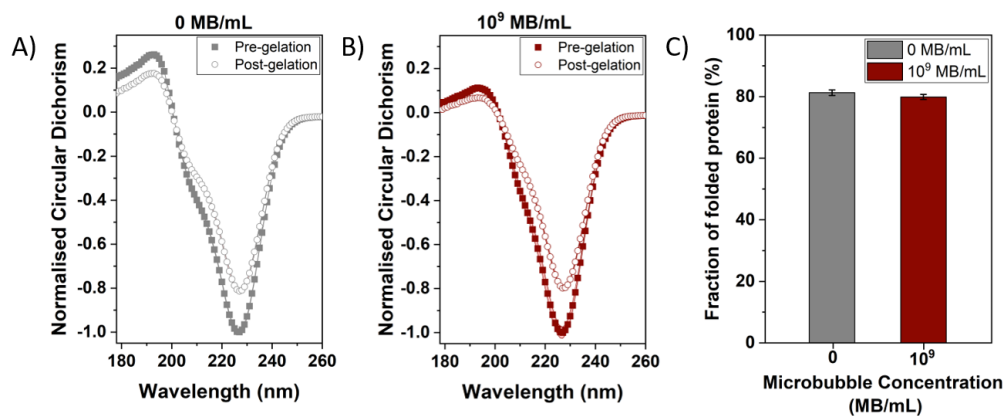


Figure 3.4: Normalised circular dichroism spectra of BSA for A) the standard BSA gel and B) BSA:MB hydrogel with encompassed microbubbles at a concentration of 10⁹ MB/mL with spectra included for both before gelation (squares) and post-gelation (open circles). C) Comparison of the peak at 222 nm associated with the α -helical structure of the BSA and BSA:MB hydrogels.

The gelation of BSA has been previously shown induce the unfolding of proteins in the final network [136]. Reducing the folded fraction of protein can contribute to a

loss of functionality of the protein, therefore quantifying the folded fraction of protein post-gelation is important. Changes in the proportion of folded protein can result in different mechanical and structural properties, therefore it is crucial to understand how the inclusion of MBs affects the folded fraction of protein post-gelation [123, 135, 136]. To quantify the amount of folded BSA that remains in the BSA and BSA-MB hydrogels, CD spectroscopy was used to measure the secondary structure of the BSA monomers in the pre-gel and post-gel system. Figures 3.4A and 3.4B shows the normalised spectra from the CD experiments for the BSA hydrogel and BSA:MB hydrogel for both pre-gelation (closed circles) and post-gelation (open circles) systems. The CD data is collected from 3 repeats of the BSA and BSA:MB hydrogels. The raw data is normalised to the 222 nm peak in the pre-gelation spectra, to produce Figures 3.4A and 3.4B. Both BSA and BSA:MB hydrogels show a α -helical structure, with well characterised negative peaks at 222 and 209 nm [236, 288]. In Figures 3.4A and 3.4B at the 209 nm peak is in fact weaker than the 222 nm, and a slight shift in the spectra from the expected 222 nm and weaker 209 nm for both BSA and BSA:MB hydrogels. BSA typically presents CD spectra with more prominent peak at 209 nm and weaker 222 nm peak. The observed differences to the typical CD spectra for BSA can be attributed to the high protein concentration (100 mg/mL) used to produce the protein hydrogels. The high protein concentration causes a higher proportion of photons to be absorbed, weakening the signal at low wavelengths. This leads to a lower signal to noise ratio, which is characterised by the high tension voltage observed with the CD spectrometer.

Figure 3.4C compares the folded fraction of BSA at the 222 nm peak in Figures 3.4A and 3.4B for BSA and BSA:MB hydrogels in the post-gelation state. This allows the amount of unfolding due to cross-linking procedure to be directly compared for both BSA and BSA:MB hydrogels. Figure 3.4C shows the remaining folded fraction of BSA after gelation in the BSA hydrogel was $81.3 \pm 0.9\%$ and for the BSA:MB hydrogel (10^9 MB/mL), the remaining folded fraction of BSA post-gelation was $79.9 \pm 0.8\%$. Using the analysis of variance (ANOVA) test in Appendix C Table C.1, the remaining folded fraction of BSA post-gelation in BSA and BSA:MB hydrogels was found to not be statistically significant. Comparing this to a previous study on CD

with BSA hydrogels, Hughes et al. found 91% of BSA remained folded upon gelation [136]. BSA is expected to remain folded after gelation due to the majority of the 17 intramolecular disulphide bonds being buried within the BSA in conditions, at neutral pH and room temperature [289]. The disulphide bonds, that act as staples holding the structure of BSA together, creating a high energy barrier to unfold BSA [136]. Only a small portion of the BSA proteins was shown to unfold upon gelation for both BSA and BSA:MB hydrogels (18.7% and 20.1%), suggesting that a high proportion of BSA in the hydrogel would remain functional both in the BSA and BSA:MB hydrogels. The folded fraction of BSA protein for hydrogels with and without microbubbles showed no significant change. This suggests that the inclusion of MBs in a protein hydrogel matrix does not negatively impact the functionality of the protein after cross-linking, over the concentration range of MBs used here (10^7 - 10^9 MB/mL).

3.3 Rheological Characterisation

The viscoelastic properties of BSA and BSA:MB protein hydrogels, for MB concentrations of 10^7 , 10^8 and 10^9 MB/mL, were measured using applied shear rheology, a technique for the characterising the mechanical properties of viscoelastic solids. Hydrogel mechanics is an important property to consider for biomedical applications as tissues in the body have a wide range of mechanical stiffnesses, and different locations in the body will require different abilities to withstand high stresses or frequent movement [85, 290].

3.3.1 Gelation Curve

The gelation curve in Figure 3.5A shows the storage (G') and loss (G'') moduli during photo-initiated chemical cross-linking activation of the pre-gel solution and relaxation of the cross-linked network for BSA and BSA:MB hydrogels for a range of MB concentrations (10^7 , 10^8 and 10^9 MB/mL). At $t = 60 - 360$ s the lamp to photo-initiate cross-linking formation was turned on. Figure 3.5B shows the associated $\tan(\delta)$ (where $\tan(\delta) = G''/G'$) for the gelation curve in Figure 3.5A.

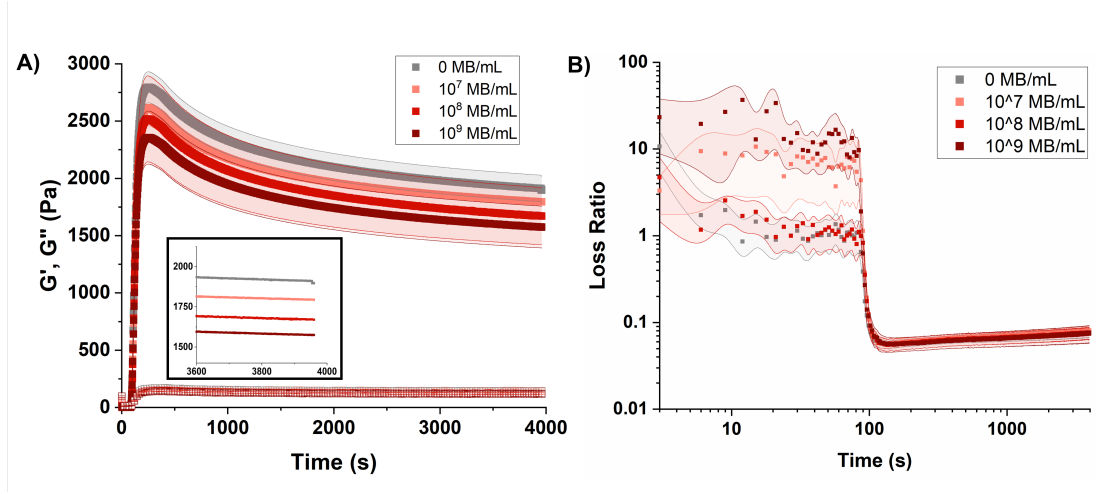


Figure 3.5: A) Gelation curve for BSA and BSA:MB hydrogels, where closed squares is G' and open squares is G'' , inset highlights the final G' in the final 400 s of the experiment. B) Loss ratio ($\tan(\delta)$) calculated from the gelation curve.

In Figure 3.5B at $t = 0$ s before cross-linking was initiated, all samples had loss ratio in the range of $40 > \tan(\delta) > 1.2$, indicating the samples were a viscoelastic fluid as $\tan(\delta) > 1$. The lamp was turned on at $t = 60$ s for 5 mins to give sufficient time for cross-linking to be initiated [123, 136]. After the lamp is turned on, G' and G'' rapidly increase (Figure 3.5A) and at the same time, $\tan(\delta)$ dramatically decreases to less than 1 (Figure 3.5B). A $\tan(\delta) < 1$ shows that viscoelastic liquid has become a viscoelastic gel ($\tan(\delta) < 1$) as a result of the formation of covalent dityrosine cross-links between BSA molecules. Figure 3.5A shows that G' rapidly increases and reaches a maximum peak demonstrating network formation at $t \sim 300$ s, and begins to decrease, indicating the rate of network formation is slowing down. After the lamp is switched off, the G' continues to decrease, implying that the network was relaxing due to network rearrangement [136].

The gelation curves in Figure 3.5A can be fitted with Equation 3.1, previously used to fit gelation curves of protein hydrogels [135, 136].

$$G'(t) = \frac{1}{1 + e^{-C(t-t_0)}} \cdot (G'_\infty + B_1 e^{-t/\tau_1} + B_2 e^{-t/\tau_2}) + G_0 \quad (3.1)$$

Where C is the rate of increase of G' after the photo-chemical cross-linking has started, t_0 is the midpoint of the rate of increase of G' , G'_∞ is the relaxed G' extrapolated to

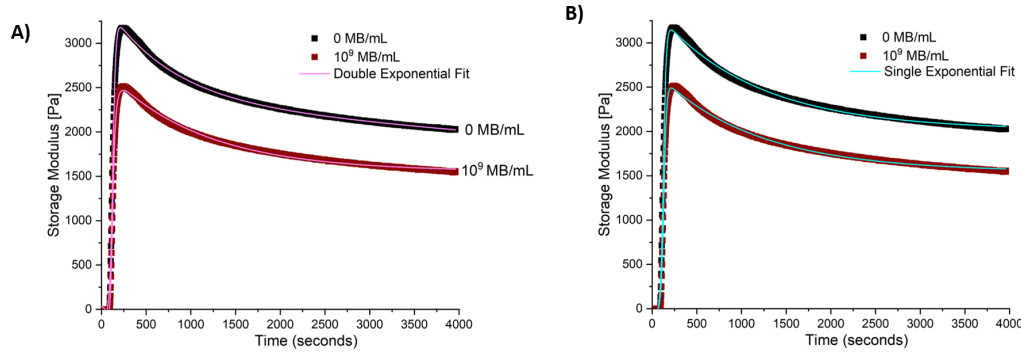


Figure 3.6: Example curves for BSA hydrogel (black curve) and BSA:MB hydrogel with 10^9 MB/mL (red curve) A) a double exponential fit (pink line) and B) a single exponential fit (blue line).

Table 3.1: Results of fitting the gelation curve with one and two exponential decay components, where Exp is the number of exponential decay components, G'_∞ is the relaxed G' extrapolated to $t = \infty$ s, B is the coefficient of relaxation, τ the time constant of relaxation and R^2 a measure of the goodness of the fits.

	BSA hydrogel	BSA:MB hydrogel	BSA hydrogel	BSA:MB hydrogel
Exp	1	1	2	2
G'_∞	2084 ± 7	1593 ± 5	$-3.3 \times 10^5 \pm 8 \times 10^5$	1513 ± 7
B_1	1385 ± 4	1163 ± 4	1155 ± 9	$-4 \times 10^5 \pm 2000$
B_2	0	0	$330,000 \pm 800,000$	1192 ± 3
τ_1	1112 ± 9	1113	740 ± 14	33.9 ± 0.5
τ_2	1	1 ± 4	$4 \times 10^6 \pm 10^7$	1061 ± 7
R^2	0.99625	0.99617	0.9971	0.99763

$t = \infty$ s, B is the coefficient of relaxation, τ is the relaxation constant and G_0 is the G' before photo-initiation. Previous studies have shown that a double exponential fit describes a network relaxation process which displays both network rearrangement and protein unfolding [135, 136]. Figure 3.6 shows an example of the fitting of both one (Figure 3.6A) and two (Figure 3.6B) exponential components in the BSA and BSA:MB hydrogels. When fitting the gelation curve with Equation 1 for BSA and BSA:MB hydrogels, the best fit for both curves was found with one exponential decay constant, rather than two, as demonstrated in Table 3.1. Figure 3.6A shows that G' is plateauing at 1580 Pa, however fitting two exponential decay constants resulted in a negative G'_∞ for the BSA hydrogel therefore does not represent the data. The values of B and τ

describe the exponential decay from the peak G' . A positive B describes an exponential decay, however Table 3.1 shows that the value of B for the BSA:MB hydrogel is negative despite the curve in Figure 3.6B decay. This suggests that relaxation is only due to the rearrangement of the network, with no significant affect on the relaxation from any unfolding of protein. This result, in combination with of the CD spectra in Figure 3.4, shows that the addition of MBs does not result in additional protein unfolding.

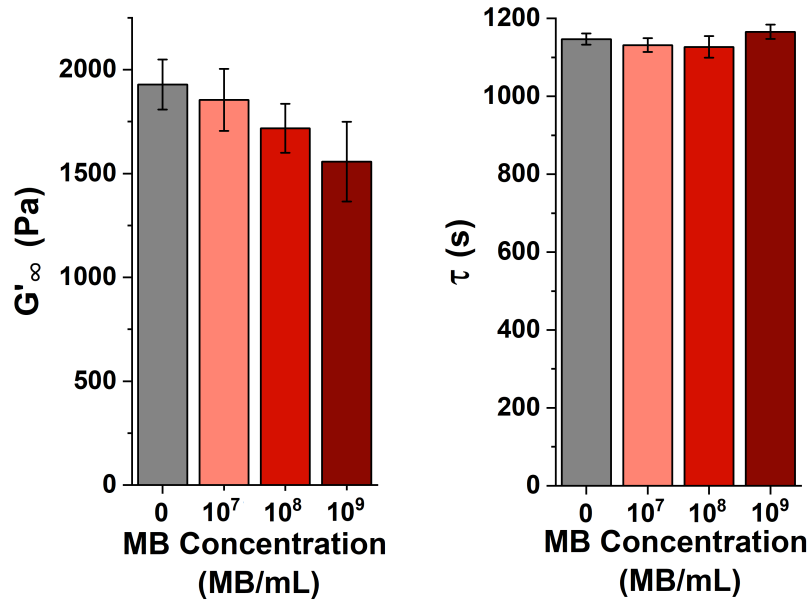


Figure 3.7: Parameters extracted from the gelation curve in Figure 3.5 for BSA hydrogels and BSA:MB hydrogels with 10^7 , 10^8 and 10^9 MB/mL. A) the relaxed storage modulus (G'_∞) and B) the relaxation time constant (τ).

Several parameters of interest can be extracted from the gelation curves in Figure 3.7A. Firstly, the storage modulus at $t = \infty$, (G'_∞), was determined using Equation 3.1 to fit the gelation curve in Figure 3.5. Not that the reproducibility of the relaxed data in Figure 3.5A is more reproducible than the peak, therefore the G'_∞ is compared rather than the maximum G' . Figure 3.7A shows G'_∞ for the BSA hydrogel without MBs was 1990 ± 110 Pa. The G'_∞ reported here is consistent with previously published data on the G' of BSA hydrogels [123, 136]. The results from the BSA hydrogel was compared to BSA:MB hydrogels for a range of MB concentrations (10^7 , 10^8 and 10^9 MB/mL). A decrease in G'_∞ from the BSA hydrogel was observed when encompassing 10^8 and 10^9 MB/mL (1710 ± 120 and 1560 ± 190 Pa). By using the ANOVA test with a significance

level of 0.05 (Appendix C Section C.1.2), the BSA:MB hydrogel with 10^9 MB/mL had a statistically significant G'_∞ to the control BSA hydrogel, whereas 10^7 and 10^8 are not statistically significant. Secondly, the relaxation time constant (τ) was also extracted from Equation 3.1 and compared for the BSA hydrogel with and without MBs, where the results of the control BSA hydrogel and 10^7 , 10^8 and 10^9 MB/mL are not statistically significantly, determined by the ANOVA test in Appendix Table C.3.

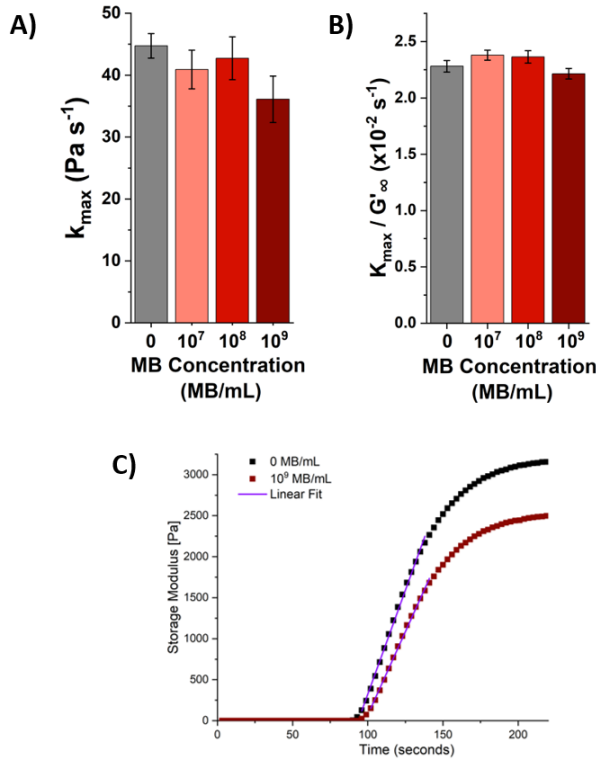


Figure 3.8: A) The kinetics of gelation (k_{max}) for the BSA hydrogels and BSA:MB hydrogels (10^7 , 10^8 and 10^9 MB/mL), B) k_{max} normalised to the G'_∞ and C) an example of fitting the gelation curve with a linear fit for BSA hydrogel (black curve) where k_{max} was determined to be $50 \pm 0.8 \text{ Pa s}^{-1}$. An example of the linear fit of the BSA:MB hydrogel curve (10^9 MB/mL) calculated $k_{max} = 40.6 \pm 0. \text{ Pa s}^{-1}$.

Finally, the kinetics of gelation is extracted from Figure 3.5A by comparison of the maximum derivative, k_{max} , taken from linear region that occurs immediately after photo-initiation. k_{max} describes the rate of gelation as a result of the photo-chemical cross-linking reaction and is compared for the BSA and BSA:MB hydrogels (10^7 , 10^8 and 10^9 MB/mL). An example of the linear fit is shown in Figure 3.8C for both BSA

hydrogels and BSA:MB hydrogels 10^9 MB/mL. Figure 3.8A shows that k_{max} with increasing concentration of MBs in the BSA hydrogel. The reduction k_{max} from the BSA hydrogel ($45 \pm 2 \text{ Pas}^{-1}$) to the BSA:MB hydrogel with 10^9 MB/mL ($36 \pm 4 \text{ Pas}^{-1}$) was found to not be statistically significant from the ANOVA test in Appendix C, Table C.4. Figure 3.8B shows that k_{max} with respect to G'_{∞} is not significantly changing with the addition of MBs (ANOVA test of significance results shown in Appendix C Table C.5)

Aufderhorst-Roberts *et. al.* [123] observed that intensity of light irradiating the pre-gel solution to initiate the photo-activated chemical cross-linking increased k_{max} from 20 - 80 Pas^{-1} . This was suggested to be a result from changing the rate of cross-link formation. In this study, only small reductions were observed in both G'_{∞} and k_{max} in all three BSA:MB hydrogels. This implies that increasing concentrations of MBs has a subtle affect on the network formation, where a more significant affect on the G'_{∞} and k_{max} would be observed at higher MB concentrations.

3.3.2 Frequency Sweep

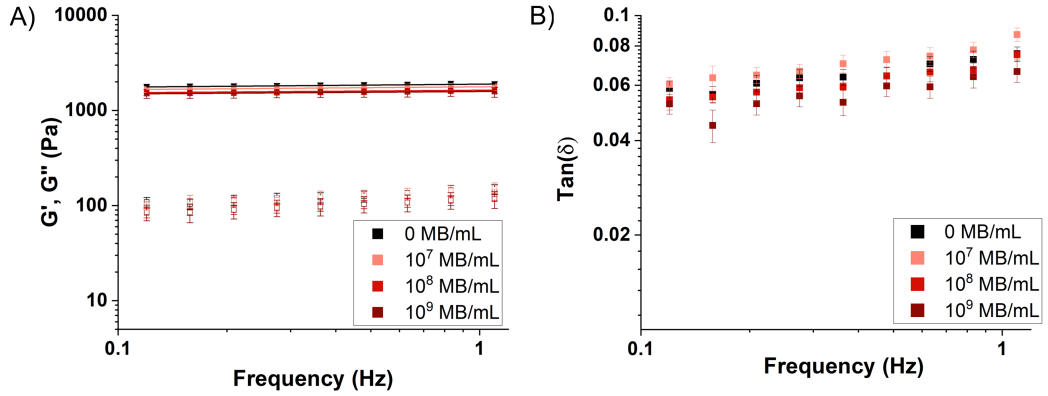


Figure 3.9: A) Frequency sweep for BSA with and without MB for both G' (closed squares) and G'' (open squares). B) The loss ratio calculated from the frequency sweep, for the hydrogel with and without MB for both G' .

A frequency sweep provided insight into the post-gelation viscoelastic properties of BSA protein hydrogels with increasing concentrations of MBs. Figure 3.9A shows for all concentrations of MBs in the BSA:MB hydrogel, G' is greater than G'' . Across all

frequencies, G' was greatest for the BSA hydrogel. Increasing the concentration of MBs in the BSA hydrogel reduced G' , following the same trend as the gelation curves in Figure 3.9A. The G' at 1.13 Hz for the BSA hydrogel was ~ 300 Pa higher than the BSA:MB hydrogel with 10^9 MB/mL. Figure 3.9a was used to determine the loss ratio, $\tan(\delta)$, calculated from Equation 2.18. For all hydrogel conditions investigated, $\tan(\delta)$ was less than 1 (see Figure 3.9B) and therefore classed as a viscoelastic solid. The loss ratio was lowest for the BSA:MB hydrogel with 10^9 MB/mL (0.066 ± 0.005), where a decrease of 12% between the BSA and BSA:MB hydrogel at 1.1 Hz was observed, suggesting this composite system has a slightly lower viscosity component than the BSA hydrogel.

3.3.3 Stress Strain Curve

The BSA hydrogels were loaded and unloaded with an applied shear strain of 50%, as for this range of shear strain BSA hydrogels, the mechanics remains in the linear regime, and the bulk BSA hydrogel does not break. As the shear strain is increased, the shear stress measured for each hydrogel system to understand the response to shear force in the linear regime. This mimics the strain that hydrogels experienced in practice for biomedical applications. Figure 3.10A shows the stress-strain curve for the BSA hydrogel and BSA:MB hydrogels for 10^7 , 10^8 and 10^9 MB/mL. A linear relationship between the shear strain and shear stress occurs for all curves up to a shear strain of 20%. The shear strain was increased to a maximum of 50%, and the shear stress compared for each hydrogel system. The shear stress at 50% for the control BSA hydrogel (978 ± 48.7 Pa) was showed statistically significant decrease (915 ± 55 Pa) in the presence of 10^9 MB/mL for the BSA:MB hydrogels (demonstrated in Appendix C, Table C.6). The results from BSA:MB hydrogels with 10^7 and 10^8 MB/mL (830 ± 79.6 and 834 ± 75.7 Pa) showed no statistically significant changes (see Appendix C, Table C.6). The linear region of Figure 3.10A was used to determine G' from the area within the hysteresis curves. Figure 3.10B and C shows the G' calculated from gradient of a linear fit of linear region (0-20%) of the stress-strain curve. The G' of the BSA hydrogel calculated from Figure 3.10A was 1800 ± 150 Pa. The BSA:MB hydrogels measured all resulted in a decrease in the G' , with the largest decrease observed for the BSA:MB with 10^9 MB/mL of 21 %. Increasing

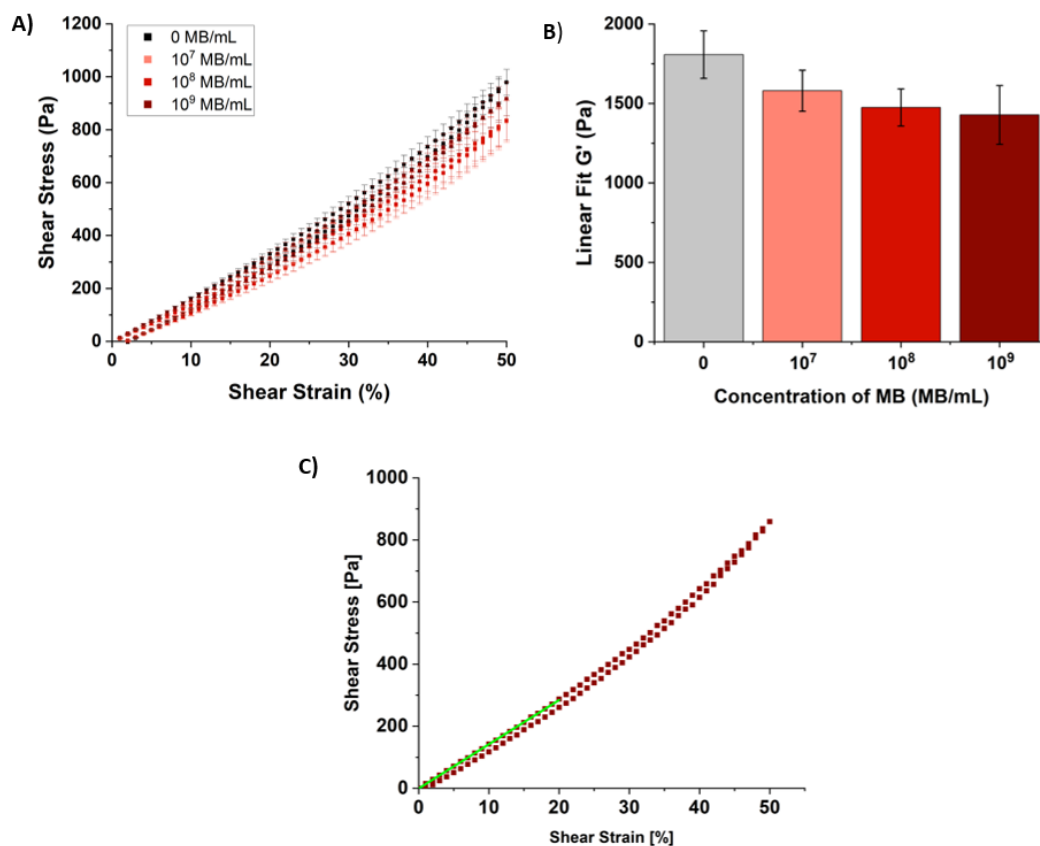


Figure 3.10: A) Stress-strain curve in the BSA hydrogel and BSA:MB hydrogels for 10^7 , 10^8 and 10^9 MB/mL. A shear strain of 50% was applied and the shear stress measured. B) Storage modulus calculated from the linear region of the stress strain curve. C) An example of the stress-strain curves for BSA:MB hydrogels with 10^9 MB/mL, with a linear fit (green) of shear strains 0 - 20%. The gradient of the linear fits are used to calculate an additional value for G' (1420 ± 0.04 Pa).

the concentration of MBs encompassed in the BSA hydrogel decreases the G' , following the same trend observed in the shear modulus calculated from the gelation curve in Figure 3.10B. The ANOVA test (Appendix C, Table C.7) further confirming that 10^9 MB/mL encompassed in BSA:MB hydrogel had a statistically significant reduction in the stiffness compared to the BSA hydrogel, while encompassing 10^7 and 10^8 MB/mL were not statistically significant to the control BSA hydrogel.

From the area of stress-strain hysteresis in Figure 3.10A, the energy of dissipation can be determined. Equation 3.2 can then be used to determine the efficiency of loading and unloading shear force, providing an insight into how the BSA hydrogel recovers from

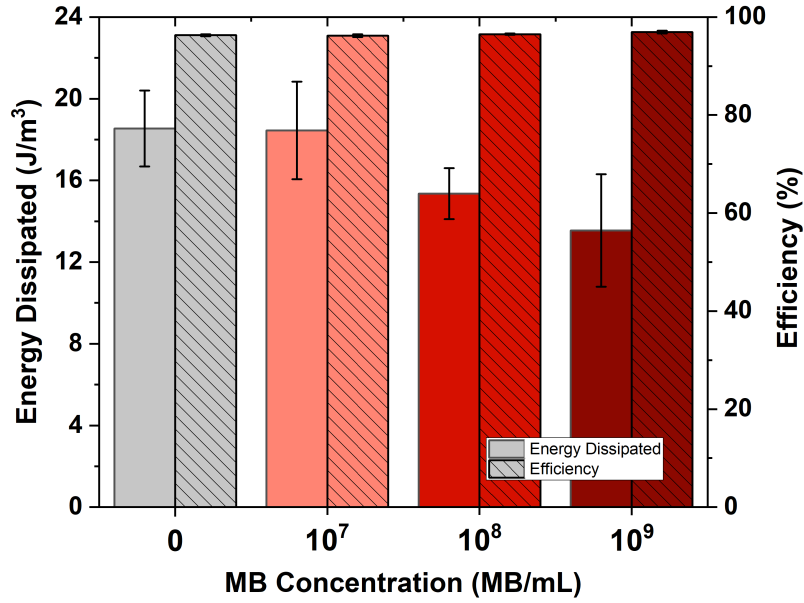


Figure 3.11: Extracted parameters from the stress-strain curves for the energy dissipation (no stripes) and efficiency of loading (stripes) for BSA hydrogels and BSA:MB hydrogels with 10^7 , 10^8 and 10^9 MB/mL.

the loading of strain. Figure 3.11 shows that the energy dissipation from loading and unloading, and the efficiency of the loading. The energy dissipated from loading and unloading slightly decreases with increasing concentration of MBs. Interestingly, this leads to no significant changes in the efficiency of loading and unloading, confirmed by the ANOVA test in Appendix C, Table C.8. The maximum shear strain ($Strain_{Max}$) at 50% for each measurement, and the maximum shear stress measured ($Stress_{Max}$) were used to determine the efficiency of loading and unloading shear stress using Equation 3.2. The results of the efficiency, shown in Figure 3.11 found no statistically significant changes with the addition of MBs to the BSA hydrogel, confirmed by the ANOVA test in Appendix C, Table C.9, suggesting that the inclusion of MBs does not impact the ability of the hydrogel to recover from an applied stress.

$$Efficiency = 1 - \frac{Energy\ Dissipated}{Strain_{Max} \cdot Stress_{Max}} \quad (3.2)$$

3.3.4 Non-Linear Behaviour

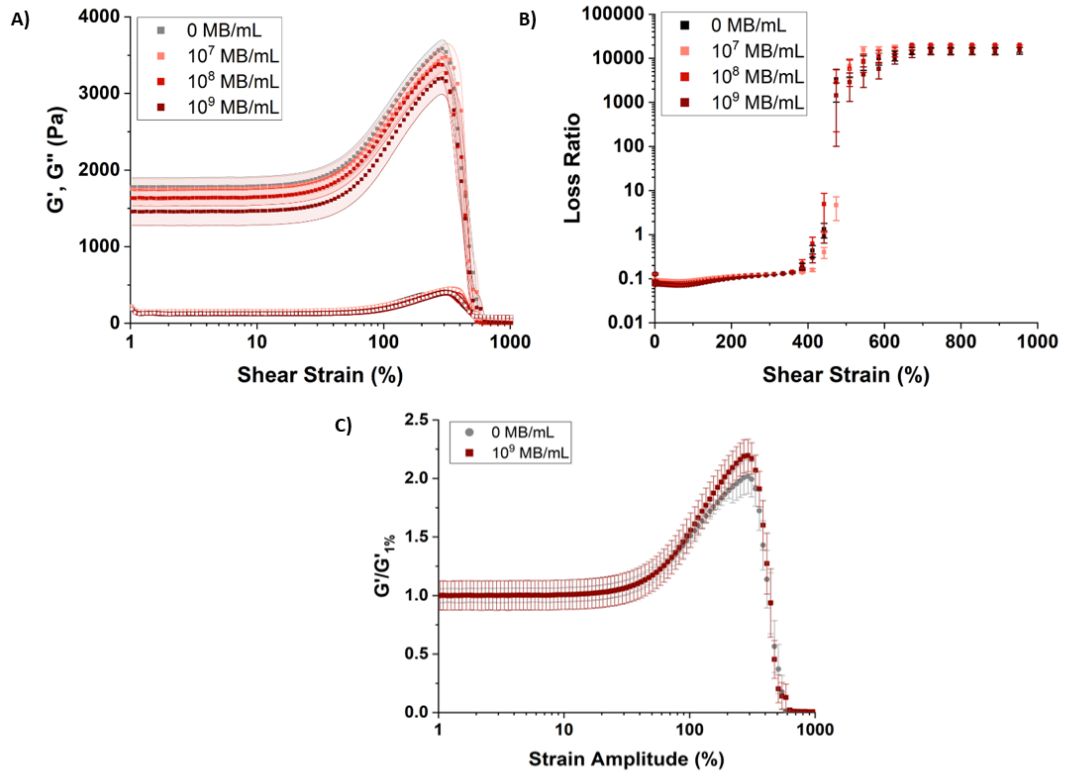


Figure 3.12: A) Strain amplitude for the BSA hydrogel and BSA:MBs hydrogel with 10^7 , 10^8 and 10^9 MB/mL for both G' (closed squares) and G'' (open squares). B) The loss ratio calculated from the strain sweep, for the BSA and BSA:MB hydrogels. C) The G' with respect to the initial G' at strain amplitude of 1 % ($G'_{1\%}$) for the BSA hydrogel and BSA:MB hydrogel with 10^9 MB/mL embedded.

The non-linear behaviour of hydrogels determines the strain required to fracture the hydrogel. Figure 3.12A shows the non-linear behaviour of BSA and BSA:MB hydrogels. For all the BSA hydrogels systems measured, linear deformation occurs up to a strain amplitude of 25%, after which the hydrogels shear stiffen and eventually break. All hydrogels start to break at a shear strain of 290%, demonstrating that the presence of MBs in BSA hydrogels of up to 10^9 MB/mL can be used without concerns of weakening the hydrogel. Figure 3.12C shows BSA hydrogels normalised to $G'_{1\%}$, in presence of the 10^9 MB/mL. At high strain amplitudes of greater than 50%, $G'/G'_{1\%}$ is lower for the BSA hydrogel than the BSA:MB hydrogel with 10^9 MB/mL. This implies that the BSA:MB hydrogel is experience a larger amount of strain stiffening due to the presence of

the MBs. Previously, atomic force microscopy force deformation found that the Young's modulus of phospholipid MBs was on the order of 1 GPa [162]. This suggests that at high strain amplitudes the MBs are supporting the network.

3.4 Dityrosine Cross-Linking Efficiency

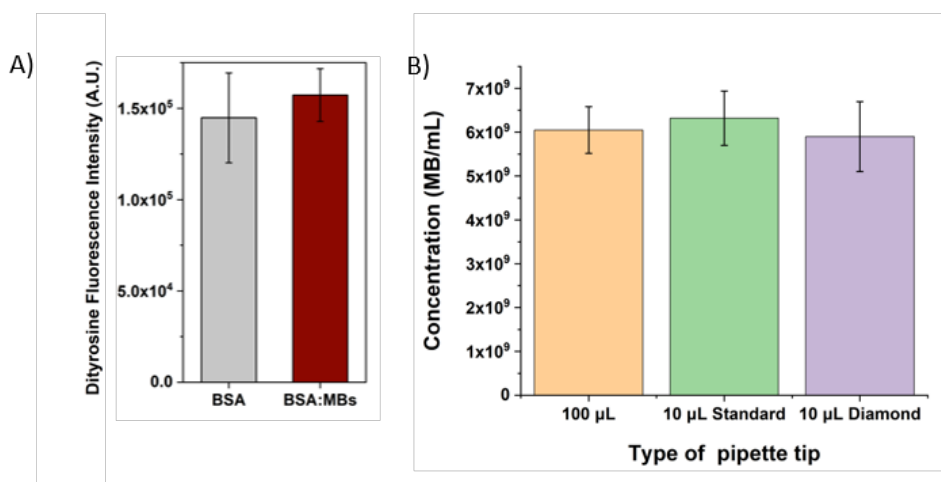


Figure 3.13: Hydrolysed BSA hydrogel (grey) and BSA:MB hydrogel with 10^9 MB/mL (dark red) both excited at 315 nm and emission taken at 415 nm characteristic of the excitation and emission from dityrosine covalent bonds. The fluorescence emission intensity at 415 nm is compared. The error bars are the standard error of three experimental repeats. B) Comparison of the concentration of MBs from pipetting the MBs through different pipette tips.

To understand how many proteins are cross-linking in the network an assay was run to determine the fluorescent emission from dityrosine bonds, where a higher fluorescent emission describes a higher concentration of dityrosine bonds. The number of dityrosine cross-links was therefore quantified for the BSA hydrogel and BSA:MB hydrogel with 10^9 MB/mL, by comparing the fluorescence emission of the dityrosine bonds.

The assay to determine the dityrosine bonds requires pipetting small volumes ($> 10 \mu$ L) of the pre-gel solution. Pipetting small volumes results in larger errors therefore thin pipette tips (Diamond) were used to accurately pipette small volumes of the pre-gel solution. The 100μ L pipette tip is the standard pipette tip used for pipetting

MBs, however the Diamond pipette tips have a higher gauge compared to standard 100 μL pipette tip. Using a higher gauge results in higher pressures experienced by the sample during pipetting [291], which could lead to rupturing of MBs from pipetting. To understand if the MBs were being ruptured during pipetting through higher gauge needles, the concentration of the same population of MBs was compared for 3 different pipette tips was compared. Figure 3.13B compares the 10 and 100 μL pipette tips that are normally used for pipetting MBs, to the thinner, higher gauge Diamond pipette tips. There was no significant change in the concentration of MBs observed when comparing thinner Diamond pipette tips to the 10 and 100 μL pipette tips. This results shows that the Diamond pipette tips can be used with MBs without concerns rupturing of MBs.

The subtle changes in the mechanical properties of MBs in BSA hydrogels, determined from the rheological characterisation in Section 3.3, could be linked to the rate of dityrosine cross-link formation between BSA monomers. Figure 3.13A shows that no significant differences in the number of cross-links were observed, however it should be noted that the errors are significant (2.5×10^4 and 1.4×10^4 A.U. for the BSA and BSA:MB hydrogel with 10^9 MB/mL). The CD results showed no significant changes to the folded fraction of BSA in the BSA and BSA:MB hydrogel with 10^9 MB/mL, suggesting the building block of the protein hydrogel remains unchanged. The reduction in G' in the all the BSA:MB hydrogels relative to the BSA hydrogel could be attributed to multiple properties of the MBs. Previous studies have demonstrated that the inclusion of filler particles in soft viscoelastic materials can result in a decrease or no change in material stiffness. This is suggested to be due to the filler particle being unbound to the network, or that the filler particles are at such a low volume fraction there is no measurable effect [277, 292]. In the composite system investigated here the volume fraction of MBs is comparatively low relative to the volume fraction of BSA monomers, approximately 1% MBs compared to 7% BSA. A study by Genot et al. showed that the inclusion of multilamellar vesicles (MLVs) in gelatin gels resulted in a 13.8% decrease in the stiffness of the gel, despite the low volume fraction of 1.25% of MLVS in the gel. The conclusion for the relatively large decrease in the stiffness was attribute to the size of filler particle and interaction between the filler particle and gelatin is important [293]. The size

of MLVs was 5 μm , composed of egg-PC lipids, therefore both the size (1.5 μm) and composition of the lipids (95 mol% DPPC) in Genot et al was similar to the MBs in this study. Furthermore, in this study PEGylated lipid (DSPE-PEG-2000) are included in the lipid shell at 5 mol%, which may influence interaction behaviour between the MBs and the BSA network. Low molecular weight PEG molecules, such as PEG-2000, have shown in both theoretical and experimental studies, to have energetically unfavourable interactions between human serum albumin proteins, predicted to be due to the hydrophilicity and flexibility of PEG chains[294]. This has led to the inclusion of PEG into many drug delivery systems, and has improved the stability and lifetime of MBs and liposomes by reducing the removal rate from the blood stream via the mononuclear phagocyte system[163, 295, 296]. This, with the reduction in G' with the addition of MBs in Figures 3.5A and 3.7A suggests that the MBs are not bound to the BSA network, which could lead to changes in the structure of the protein network formed while in the presence of MBs. This is suggested by a simple thermodynamic expression of the shear modulus, G , of a cross-linked network [238, 297]:

$$G = nC \frac{dF^2}{dx^2} \quad (3.3)$$

where n is the number of bonds per unit area, F is the Gibbs free energy, x is the cross-link length. C describes the structure of the network and how the clusters are linked together. How the presence of MBs in the BSA hydrogel is changing the network formed has not been determined from comparing the number of cross-links in the BSA and BSA:MB hydrogels, therefore further studies into the mesoscale structure are required.

3.5 Mesoscale Structure of Protein Network with Embedded Microbubbles

The previous section showed that the presence of MBs in the BSA:MB hydrogel was affecting the mechanical strength of the hydrogel. This section studies the structure of

the BSA and BSA:MB hydrogels with small-angle neutron scattering (SANS). The aim for studying the structure is to link the mechanical properties of the BSA:MB hydrogels to the structure of the protein network. In this section the fitting of SANS data with SASview was performed by Dr Matthew D.G. Hughes.

Figure 3.14 shows the intensity of scattering as a function of q for BSA and BSA:MB (10^9 MB/mL) hydrogels, along with the fits. The fitted curves were used to extract the fractal dimension and correlation length from the scattering data. The intensity of scattering curve for the BSA hydrogel shows a similar curve to previous SANS curves of BSA hydrogels, where the structure of BSA hydrogels was predicted from SANS and SAXS, to be formed of fractal-like clusters connected by chains of BSA proteins[136].

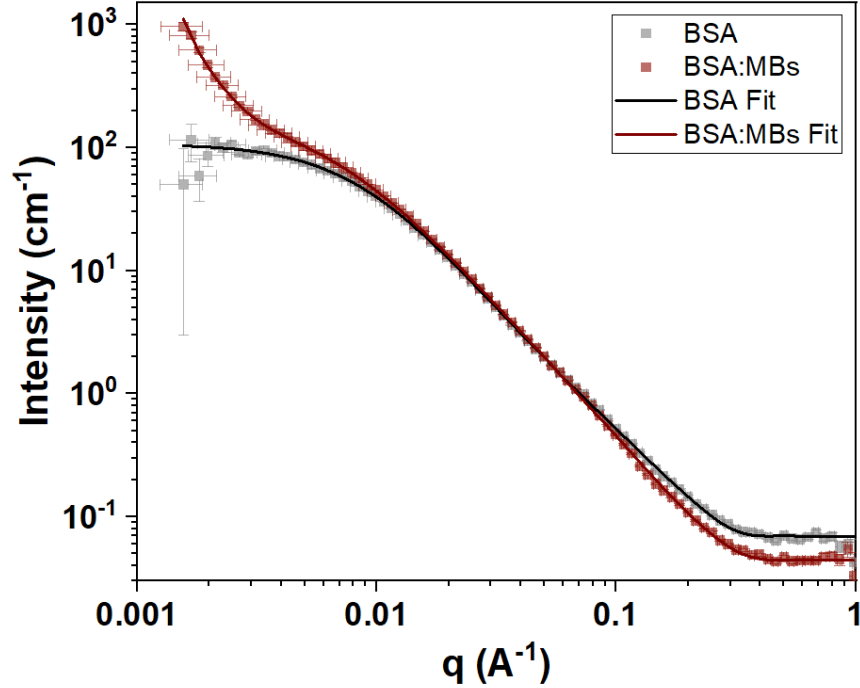


Figure 3.14: SANS curves of folded BSA (grey) and BSA:MB hydrogels with 10^9 MB/mL (red). Fits included from Equations 3.4 and 3.5

In the low- q region of Figure 3.14, the scattering curve shows an upturn in scattering intensity in the BSA:MB which does not occur in the BSA hydrogel. This higher scattering intensity and inflexion in the curve for BSA:MB hydrogel appears in the region where large objects such as the MBs would be expected, confirming the presence of the MBs in the hydrogel. For MBs of size $1\ \mu\text{m}$, the plateau in the low- q region would be

expected at $q = 6.28 \times 10^{-3}$ from predictions with SASview [260], therefore not in the detectable range of the instrument. In the mid-q region that correlates to length scales that define the geometry and topology of BSA hydrogel networks, both curves have similar structural behaviour for both BSA and BSA:MB hydrogels. The high-q region correlates to the size of clusters, where the shape of curve for both BSA hydrogels is similar.

Equation 3.4 was first used by da Silva et al (2017) to fit SANS curves for folded protein hydrogels for I₂₇ and (pL)₅, which has since been applied to BSA protein hydrogels [119, 136] In this study, Equation 3.4 was therefore used to provide information about the structures formed:

$$I(q) = \phi_p V_p \Delta \rho_p^2 P_p(q) \cdot [(1 - p_c) + p_c S(q)] + background \quad (3.4)$$

where $I(q)$ intensity of the scattered neutrons, ϕ_p is the volume fraction of the protein, V_p is the volume of the protein, $\Delta \rho_p^2$ is the difference in contrast between the protein and the buffer, $P_p(q)$ is the ellipsoidal form factor of the building block [298], p_c is the amount of protein in clusters, and $S(q)$ is the fractal structure factor. For the BSA:MB hydrogel, an adjustment to the Equation 3.4 was made to incorporate the MBs into the fit where an additional spherical form factor term for the MBs, $P_b(q)$, can be added to the model in Equation 3.5.

$$I(Q) = \phi_b V_b \Delta \rho_b^2 P_b(q) + \phi_p V_p \Delta \rho_p^2 P_p(q) \cdot [(1 - p_c) + p_c S(q)] + background \quad (3.5)$$

where ϕ_b , V_b and $P_b(q)$ are the the volume fraction, volume and the ellipsoidal form factor of the MBs, chosen to consider to deformation in MB shape through gelation. The structure factor, $S(q)$, used for modelling objects that cluster.

$$S(q) = \frac{D_f \Gamma(D_f - 1)}{\left(1 + \frac{1}{(q\xi)^2}\right)^{\frac{D_f - 1}{2}}} \cdot \frac{\sin[(D_f - 1)\tan^{-1}(q\xi)]}{(qR_0)^{D_f}} \quad (3.6)$$

where D_f is the fractal dimension, ξ is the correlation length, R_0 is the minimum cut-off length scale defined by $P(q)$. Previous studies on the structure of folded protein based hydrogels shows that the protein network is formed of clusters containing cross-linked folded proteins, where the clusters have fractal-like behaviour [119, 123, 135, 136] This model is used as it takes into consideration that objects can be fractal-like.

The D_f can be defined as a measure of how the mass of protein in a fractal cluster of proteins changes with volume. In this context can be thought of as how the geometry of the cluster relates to the density of the cluster. The results in Figure 3.15A shows that the presence of MBs in the BSA hydrogel slightly decreases ($D_f = 2.44 \pm 0.01$ for BSA hydrogel, $D_f = 2.41 \pm 0.02$ for BSA hydrogel in the presence of MBs), suggesting slightly sparser clusters due to the presence of MBs. ξ is the maximum length scale that a fractal-like cluster reaches, giving an upper limit on the size of the fractal-like protein clusters. The inclusion of MBs in the protein hydrogel results in an increase in ξ , ($\xi = 138 \pm 31 \text{ \AA}$ for BSA hydrogel, $\xi = 160 \pm 4 \text{ \AA}$ for BSA hydrogel in the presence of MBs), as shown in Figure 3.15B. The results from 3.15 suggests that the size of the clusters of proteins increases, without any change to the density of packing of the proteins in the clusters.

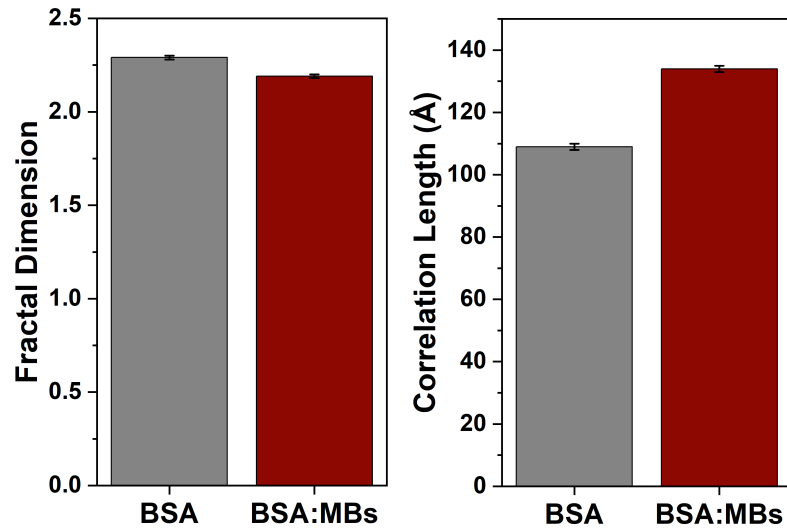


Figure 3.15: A) Fractal dimension and B) Correlation length determined from SANS of clusters of protein in BSA hydrogels (grey) and BSA:MB hydrogel with 10^9 MB/mL (red).

Previous studies have shown that the G' of a system composed of a network of clusters is not dependent on the size of the cluster, but is dominated by large changes in the volume fraction of the clusters and interactions between clusters[299–301]. This suggests that the changes in G' measured the protein hydrogels in Figure 3.7A is not due to the change in cluster size observed in Figure 3.15B. To determine the origin of the changes in the mechanical properties observed to the BSA hydrogel in the presence of MBs, further analysis was applied as used in previous studies.[123, 136] This analysis uses the fractal radial distribution function, $g(r)$, first used by Teixeira in Equation 3.7 to determine the structure factor[302, 303].

$$g(r) = \frac{\rho_k D_f}{4\pi\phi r_0^{D_f}} r^{D_f-3} e^{-r/\xi} \quad (3.7)$$

where ρ_k is the maximum packing density of the system, r is the radius of the BSA monomer. The radius of the BSA monomer used is $33\text{\AA}$, determined from the previously characterised form factor in SAXS experiments.[123]

The number of monomers in a cluster, $N(r)$, can be determined from multiplying the radial distribution function (3.7) and the volume fraction of the fractal system, and integrating over r , with derivation outlined in Section B [135, 136, 304].

$$N(r) = \rho_k D_f \left(\frac{\xi}{r_0} \right)^{D_f} \gamma \left(D_f, \frac{R}{\xi} \right) \quad (3.8)$$

where $\gamma(D_f, r/\xi)$ is the lower incomplete gamma function (Appendix A) and R is the radius of the cluster.

The number of BSA protein monomers as a function of r is shown in Figure 3.16. In both the BSA and BSA:MB hydrogels, $N(r)$ increases with radius of the cluster, where both curves have have similar gradients, and then plateaus to a maximum $N(r)$. The plateau for BSA hydrogel occurs at a lower $N(r)$. This shows that the maximum number of proteins in a fractal-like clusters is lower for the BSA hydrogel network formed compared to the BSA:MB hydrogel. The average size of the cluster is $190 \pm 1 \text{\AA}$ for

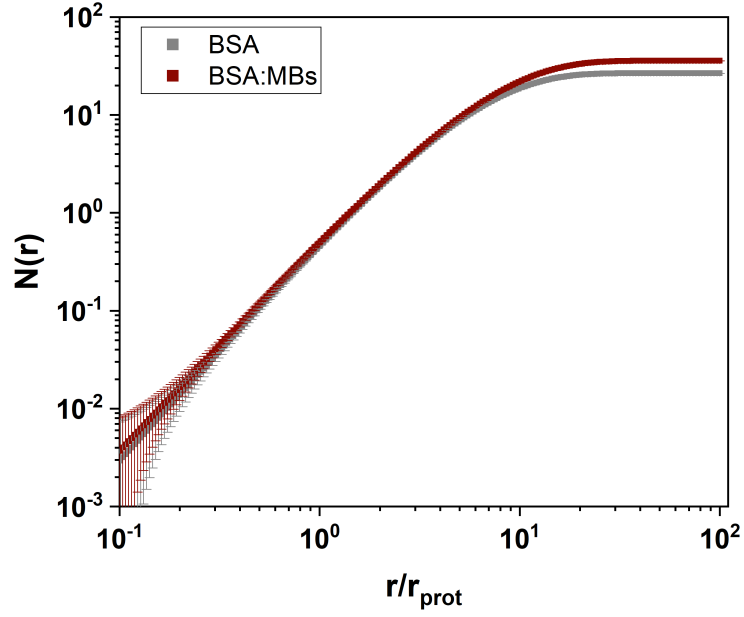


Figure 3.16: $N(r)$, the number of proteins in each fractal-like cluster as a function of r , the distance from the centre of the cluster, with respect to r_{prot} , the radius of a BSA protein monomer.

the BSA hydrogel and $223 \pm 1 \text{ \AA}$ for the BSA:MB hydrogel, in agreement with the correlation length in Figure 3.15B.

From $N(r)$ in Figure 3.16 a more in depth analysis allows the average volume fraction of the proteins in clusters (ϕ_c) and inter-cluster (ϕ_{ic}) regions to be determined, from which the average number of proteins in a cluster to be predicted. This ϕ_c and inter-cluster ϕ_{ic} are determined from the average cluster size and the number of BSA monomers in the fractal-like clusters as described in Equations 3.9 and 3.10.

$$\phi_c = N_{tot}^c \left(\frac{R_{BSA}}{R_{cluster}} \right)^3 \quad (3.9)$$

$$\phi_{ic} = \frac{\phi_{system} - p_{Vol}^c}{1 - p_{Vol}^c} \quad (3.10)$$

where N_{tot}^c is the total number of protein monomers in a cluster, R_{BSA} is the radius of a BSA monomer, $R_{cluster}$ is the radius of the cluster, ϕ_{system} is the total volume

fraction of protein (7%), p_{Vol}^c is the defined in Equation 3.11.

$$p_{Vol}^c = \frac{4\pi R_{cluster}^3}{3} \cdot \frac{p_c n_d^{system}}{N_{tot}^c} \quad (3.11)$$

where n_d^{system} is the number density of protein monomers in the system.

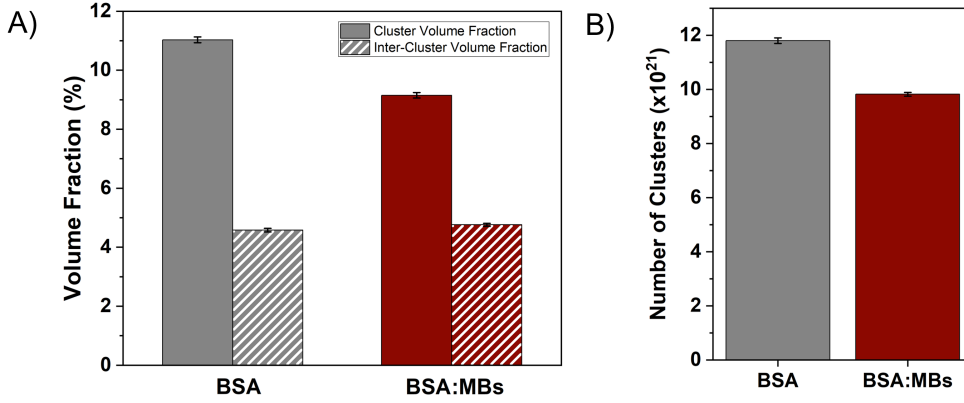


Figure 3.17: A) Volume fraction a cluster and inter-cluster space, and B) the estimated number of clusters in the BSA and BSA:MB hydrogels.

Figure 3.17A shows that the network formed in both systems with and without MBs, ϕ_c is greater than ϕ_{ic} . This suggest that a higher proportion of the proteins is in fractal-like clusters, with a smaller proportion of the proteins in the inter-cluster region, which is consistent with the expected structure of folded BSA hydrogels from previous studies.[135, 136, 304] Comparing the structures in the BSA and BSA:MB hydrogels, ϕ_c is lower when the network is formed in the presence of MBs ($11.0 \pm 0.1\%$ in BSA hydrogel, $9.2 \pm 0.1\%$ in the BSA:MB hydrogel), while ϕ_{ic} has a very minor increase in the presence of MBs ($4.58 \pm 0.06\%$ in the BSA hydrogel, $4.76 \pm 0.05\%$ in the BSA:MB hydrogel). The predicted structure of BSA and BSA:MB hydrogels is displayed in the schematic in Figure 3.18, combining the information from CD and SANS on the folded state of the BSA monomers and the network.

Previous studies show that for clustered colloidal networks, small changes to ϕ_c alone do not significantly impact the mechanical properties.[136] For example in Zaccone et al. (2009), ϕ_c was changed by 2% without significant impact on the mechanical properties of

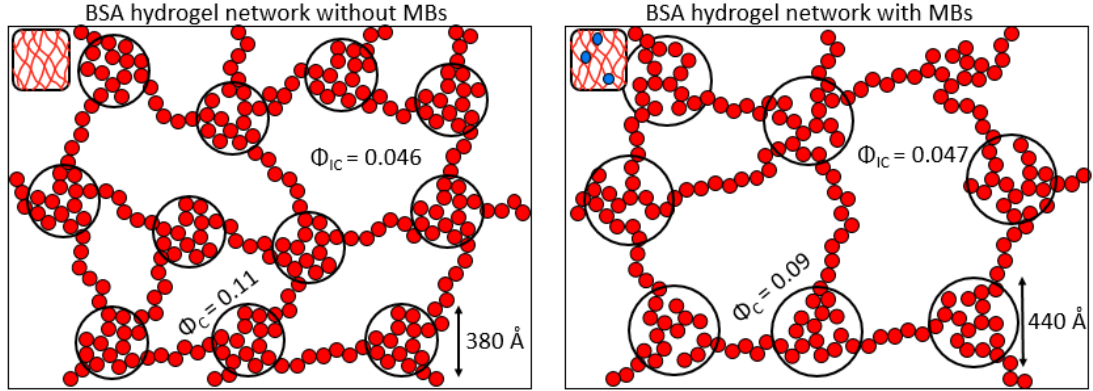


Figure 3.18: Predicted structure of the BSA hydrogel network, Left) in the BSA hydrogel, and Right) BSA:MB hydrogels. The smaller red circles represent folded proteins, the black circles represent the fractal-like clusters (ϕ_c) connected by proteins in the inter-cluster space (ϕ_{ic}).

the hydrogel (less than 100 Pa), whereas increasing the ϕ_c by 10%, a change of 1000 Pa was observed.[300] The number of clusters of protein in the hydrogel network, shown in Figure 3.17B, is estimated to reduce by 19% for the BSA:MB hydrogels ($n_c = 11.8 \pm 0.1 \times 10^{21} \text{ m}^{-3}$) for the BSA hydrogel and $n_c = 9.82 \pm 0.07 \times 10^{21} \text{ m}^{-3}$, correlating with the reduction in G' of approximately 20% in Figure 3.7. Classic affine network theory states that the stiffness of a system formed of junction points connected by chains, the number of connecting chains between junction points linearly depends on the number of junction points[305]:

$$G' \approx \mu k_B T \quad (3.12)$$

where μ is the number of junction points, k_B is the Boltzmann's constant and T is the temperature of the system. With less junction points and therefore connecting chains between junction points, the stiffness of the network will decrease linearly with the number of junction points. Applying this to the structure of the BSA hydrogels in this study, the clusters of proteins act as junction points between chains of cross-linked BSA monomers. Therefore the observations of the reduction in G' for the BSA hydrogel in the presence of MBs is suggested to be as a result of a reduction in the number of clusters. The increase in size and decrease in density of the protein clusters in the presence of MBs suggests that the reaction rate of BSA monomers forming clusters has

slowed. This could be attributed to the influence of interactions with PEG in the lipid shell of the MBs, where the PEG could be interacting with the BSA monomers BSA, leading to a reduction in the number of BSA monomers forming clusters, with less BSA monomers in the clusters.

3.6 Conclusion

MBs were embedded within BSA hydrogels to explore the impact of their insertion on the properties of both the protein and the protein network. The BSA hydrogels were embedded with MBs and characterised across a range of length scales to understand the resultant nanoscale, mesoscale and bulk properties. Given the protein fold is sensitive to mechanical and chemical changes, the folded fraction of the BSA after gelation was assessed to understand how the presence of MBs affects the BSA monomer. When comparing the BSA and BSA:MB hydrogels there was no significant change. This implies that the BSA remains functional in the presence of MBs.

The mechanical properties of the BSA hydrogel was characterised to understand if the presence of MBs would influence the mechanical properties of the hydrogel, as this would determine what tissues the hydrogel would be suitable for in applications. The BSA:MB hydrogel has a reduced G' relative to the BSA hydrogel by 19%. The measured reduction in observed in the mechanical properties are expected due to the low volume fraction of MBs in the composite system. Therefore, it is conclude that the impact of the MBs on the hydrogel mechanics is minor. The structure of the BSA hydrogel network with SANS, suggesting a the decrease in the number of fractal-like clusters of proteins.

This multi-length scale characterisation allows the use of MBs for drug delivery from folded protein hydrogels while understand that the hydrogel stiffness and mesoscopic structure will experience minor changes, when using the concentration of MBs investigated here of up to 10^9 MB/mL. Proteins have functions that are reliant on the fold of the protein, so this composite system has the potential to use the folded protein to complement the drug release. MBs can control drug delivery with ultrasound application.

This study implies that the functionality of BSA is not affected by presence of MBs, in addition to BSA:MB hydrogel maintaining mechanical strength. This stiffnesses observed for the BSA:MB hydrogel $G' = 1560 \pm 190$ Pa is a similar stiffnesses to tissues such as the brain, pancreas and kidney [98]. Therefore the composite BSA:MB hydrogels system could have an interesting future biomedical applications.

Chapter 4

Folded and Unfolded Protein Hydrogel Behaviour: Linking the Nanoscale, Mesoscale and Macroscale

4.1 Introduction

Proteins can become unfolded due to changes in the environment such as changes in heat, radiation, pH or the presence of certain chemicals and solvents, for example urea [306–309]. In the context of folded proteins hydrogels, the hydrogels could be subject to a change in environment that causes cross-linked proteins to unfold within the hydrogel [310]. The folded fraction of protein in protein hydrogels has previously been demonstrated to result in cross length scale changes to the protein hydrogel structure and bulk properties [124, 125, 136]. One of the strongest intramolecular bonds within proteins is the disulphide bond. Around 10% of proteins have disulphide, intramolecular bonds which are strong covalent bonds that form between cysteine side chains. The disulphide bonds help maintain the fold of the protein, due to being mechanically resistant [310].

SMFS studies have shown that disulphide bonds are mechanically resistant, due to the ability of covalent bonds to withstand high forces of 2nN [311]. Proteins that have their disulphide bonds removed have shown to be mechanically weaker, for example in disulphide bonded FimG protein, a pulling force of 440 ± 36 pN was required to unfold FimG [312]. After adding a reducing agent to break disulphide bonds, the pulling force required to unfold the reduced FimG was lower at 339 ± 27 pN. Disulfide bonds are used in cells for sensing oxidative stress, and the reduction of the disulphide bonds is linked to some extracellular signals [310, 313].

To understanding how unfolding of proteins can affect resultant protein hydrogel networks, Hughes *et al.* (2021) investigated with BSA protein hydrogel networks where reducing agent dithiothreitol (DTT) was mixed with the BSA pre-gel solution (BSA:pre-DTT hydrogel) [136]. DTT is a strong reducing agent, which reduces the cysteine disulphide bond in a two step thio-disulphide exchange, producing oxidised DTT and the reduced disulphide bond [314]. This mechanism is shown in Figure 4.1.

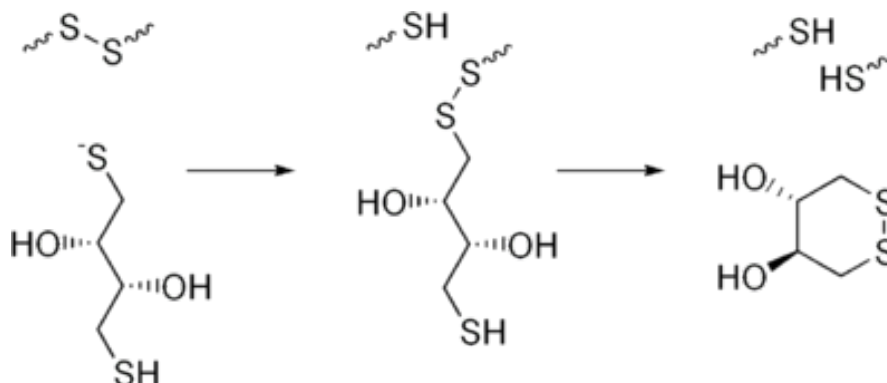


Figure 4.1: Dithiothreitol (DTT) reduces the cysteine disulphide bond in a two step thio-disulphide exchange. The final product is oxidised DTT and the reduced disulphide bond. Reproduced from Arruda *et al.* (2019) [315] with permission from Elsevier.

BSA has 17 disulphide bonds [289], and the arrangement of the those disulphide bonds are shown in 4.2. As DTT breaks the disulphide bonds that are present in BSA proteins, therefore a cross length scale study of the BSA:pre-DTT hydrogel was conducted to explore the affects of DTT.

Hughes *et al.* (2021) observed that the gelation of BSA in the control BSA hydrogel

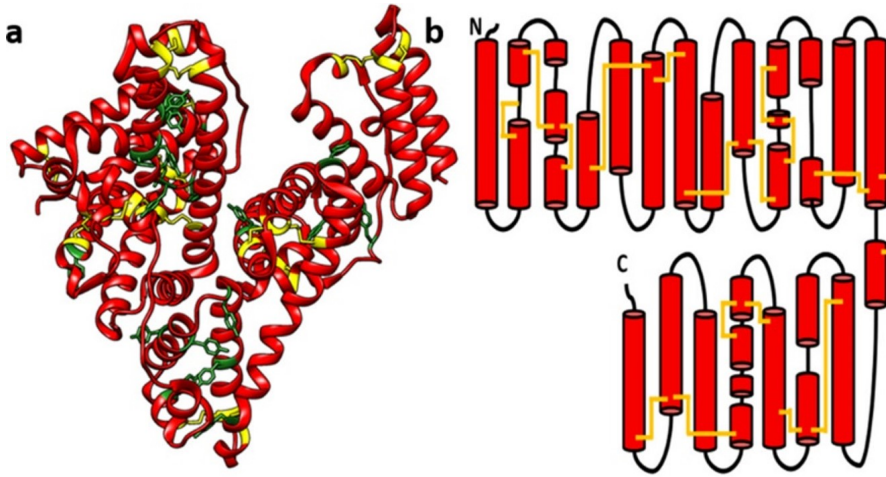


Figure 4.2: A) Crystal structure of BSA, where cysteine residues that have disulphide bonds are in yellow and tyrosine residues are in green and B) 2D schematic of BSA, where cysteine residues that have disulphide bonds are in yellow. Reused from Hughes *et al.* (2021) [136] with permission from CC BY 4.0.

resulted in a 9% decrease in the proportion of folded protein compared to the pre-gel solution, whereas in the BSA:pre-gel DTT hydrogel a 28% reduction in folded protein was observed. The disulphide bonds act as ‘nano-staples’ and by breaking those nano-staples, the unfolding of BSA protein is induced by the addition of DTT in the BSA pre-gel solution.

The properties of BSA hydrogels and the BSA:pre-gel DTT hydrogel were compared. As discussed previously in Chapter 3 Section 3.5, folded protein hydrogels are predicted by small-angle neutron scattering (SANS) to form a network of clusters of proteins connected by chains of proteins in the inter-cluster space. The addition of DTT to the BSA pre-gelation showed changes in the mesoscale structure. This included the unfolding of 28% of the BSA proteins in the BSA:pre-gel DTT hydrogel, resulted in a lower volume fraction of proteins in the inter-cluster ($\phi_{ic} = 5\%$) compared to the BSA hydrogel in the absence of DTT ($\phi_{ic} = 2\%$). The macroscale bulk properties were also compared with rheological characterisation showing that the BSA:pre-gel DTT hydrogel had a storage modulus (G') that was $\sim 2.5x$ larger than the BSA hydrogel in the absence of DTT [136].

Additionally, Hughes *et al.* investigated a different scenario with the addition of

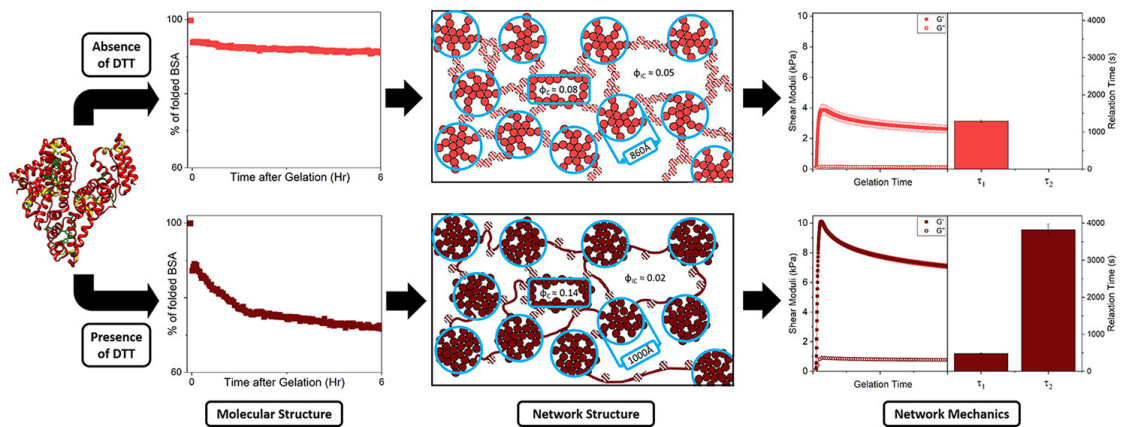


Figure 4.3: Left: a schematic of BSA with highlighted cysteine residues that have disulphide bonds are in yellow and tyrosine residues are in green. Top row shows different characterisation methods of the BSA hydrogel and the bottom row shows the same characterisation methods for BSA hydrogels where reducing agent dithiothreitol (DTT) was mixed with the BSA pre-gel solution before photo-activated chemical cross-linking (BSA:pre-gel DTT hydrogel). The molecular structure shows a normalised circular dichroism spectra at 222 nm of BSA, where the BSA hydrogel (top) has a greater fraction of folded protein than the BSA:pre-gel DTT hydrogel (bottom). The network structure shows a schematic based off predictions from small-angle neutron scattering data, where the BSA hydrogel (top) has structure of fractal-like clusters (clusters highlighted by blue circles) of folded proteins (red solid circles) connected by proteins in the inter-cluster region (red and white striped circles) with a volume fraction of ϕ_{ic} . The BSA:pre-gel DTT hydrogel (bottom) shows the network structure of fractal-like clusters connected unfolded proteins (dark red solid lines), resulting in a lower volume fraction of protein in the inter-cluster space (dark red and white striped circles). The network mechanics show that BSA hydrogels (top) have a lower storage (G') and a slower rate of relaxation (τ_1 and τ_2) compared to BSA:pre-gel DTT hydrogels (bottom). This shows that presence of DTT in the BSA pre-gel solution induces BSA protein unfolding, which results in a network with a stiffer hydrogel. Reused from Hughes *et al.* (2022) with permission from CC BY 4.0.

DTT, where a previously cross-linked protein hydrogel network BSA was soaked in DTT, post gelation (BSA:post-gel DTT hydrogel) [136]. Different observations were made in the macroscale bulk properties, as observed in Figure 4.4. In Figure 4.4, 3mM of dithiothreitol (DTT) was added to the BSA hydrogel at $t = 3960$ s, such that the BSA hydrogel was soaking in the BSA hydrogel. Figure 4.4 showed that BSA:post-gel DTT hydrogel experienced a sharp decrease in the G' was observed with the addition of DTT [136]. The rate of relaxation of the cross-linked network in the BSA:post-gel DTT hydrogel had also increased. However, how the structure of the network changes in response to breaking of the BSA nanostaples in the BSA:post-gel DTT hydrogel is unknown.

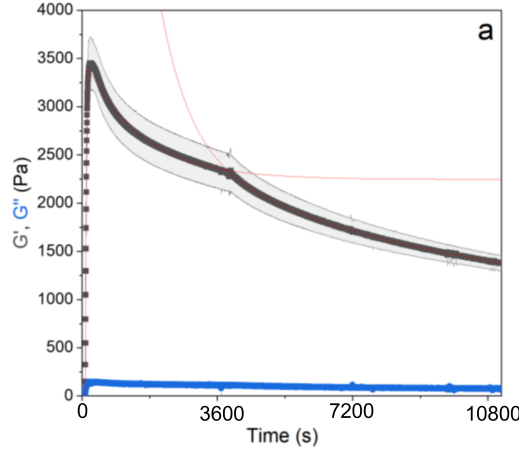


Figure 4.4: Time sweep of the gelation of BSA hydrogels with the storage modulus (G') in black and the loss modulus (G'') in blue. The BSA pre-gel solution is photo activated at $t = 0$ s, which initiates chemical cross-linking. The sharp increase in G' demonstrates rapid cross-link formation, after which network relaxation dominates shown by the decrease in G' . At $t = 3960$ s, 3mM of dithiothreitol (DTT) was added to the system and resulted in a further reduction in G' . Reused from Hughes *et al.* (2022) with permission from CC BY 4.0.

This work by Hughes *et al.* demonstrates that the nanoscale changes to the fraction of folded protein has the power to influence changes to the mesoscale and macroscale protein hydrogel that is formed [136]. Not only is the proportion of folded protein important to the bulk properties, but also the point at which the BSA unfolding occurs i.e. before or after protein gelation. It is therefore useful to understand what happens to the protein hydrogel mesoscale structure and macroscale viscoelastic properties, when unfolding of the proteins in the hydrogel is triggered. If unfolding causes significant changes to the mesoscale structure, this could influence the inter-cluster space between protein clusters in the network. In terms of biomedical applications, if the inter-cluster space increases significantly, the rate of diffusion of drug molecules could be affected.

This chapter presents studies of the rheological and structural properties of folded BSA protein hydrogels. Previously cross-linked folded BSA hydrogels were investigated in the absence and presence of microbubbles (MBs), which was reported in Chapter 3. The addition of DTT to the BSA hydrogel acts as a model for triggering protein unfolding in a folded protein hydrogel in biomedical applications, where changes to the protein hydrogel environment could induce unfolding.

4.2 Rheological Characterisation

This chapter investigates the rheology and structure of BSA hydrogels, and BSA hydrogels with microbubbles embedded in absence of DTT (BSA hydrogel and BSA:MB hydrogel). The cross-linked BSA and BSA:MB hydrogels were then soaked in DTT (BSA:post-gel DTT hydrogel and BSA:MBs:post-gel DTT hydrogel), and then measured with oscillatory shear rheology. The concentration of MBs in the BSA:MB hydrogels was 10^9 MB/mL.

4.2.1 Gelation Curve

To understand the affect that the unfolding BSA has on a cross-linked BSA hydrogel and BSA:MB hydrogel, in terms of the mechanical properties of the BSA hydrogel the following protocol was conducted. A gelation curve and time sweep was conducted, measuring recording the storage modulus, G' , and the loss modulus, G'' . An example of the measurements conducted for gelation and relaxation of the BSA and BSA:MB hydrogels is shown in Figure 4.5, and describes the sequence of events involved with the measurement. At $t = 0$ s, the BSA or BSA:MB pre-gel solution was exposed to a light ($\lambda = 452$ nm) from a blue LED lamp for 300s, in order to photo-initiate chemical cross-link formation of dityrosine bonds between BSA molecules. This is shown in the light blue region of Figure 4.5, where the G' rapidly increases due to BSA cross-linking and network formation. The G' then decreases as the BSA and BSA:MB hydrogels relax, shown by the purple region of Figure 4.5. At $t = 4000$ s the gelation curve was paused to take a frequency sweep of the BSA and BSA:MBs hydrogel, with the results shown in Section 4.2.2. At $t = 4480$ s the same BSA hydrogel or BSA:MB hydrogel is soaked in 3 mM of DTT for 3 hours giving time for the DTT to diffuse into the BSA hydrogel, this is highlighted by the green region of Figure 4.5.

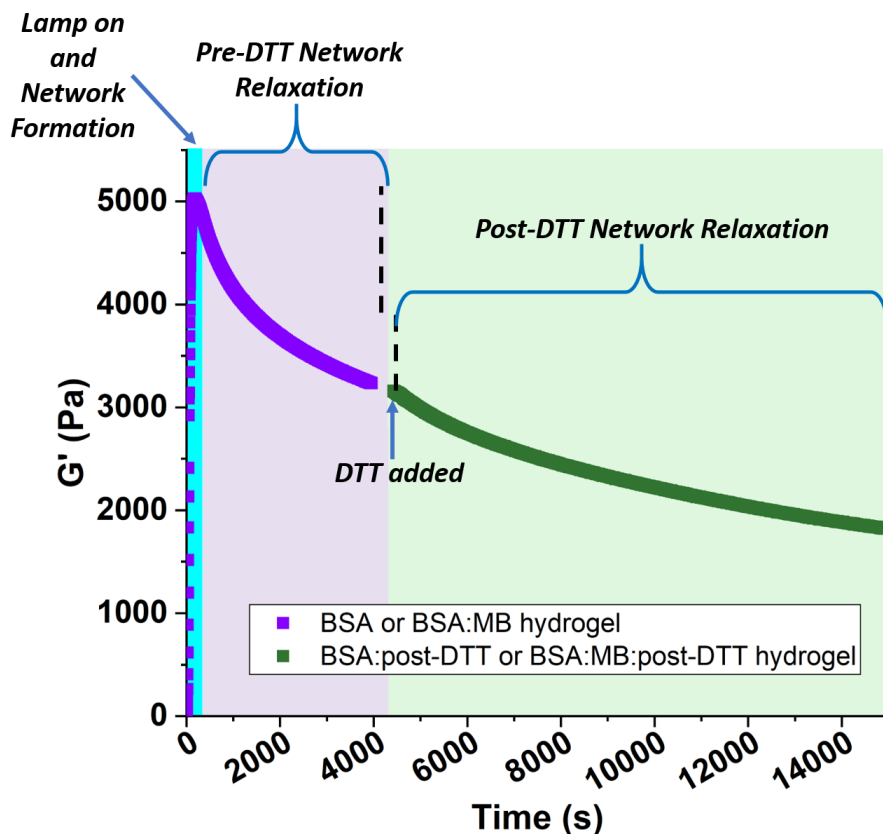


Figure 4.5: Time sweep of the gelation and relaxation of BSA hydrogels or BSA hydrogels with embedded microbubbles (BSA:MB hydrogels) measuring the storage modulus (G'). Photo-activated chemical cross-linking is initiated for pre-gel solution occurs at $t = 0$, where the lamp for the photo-activation is on for 5 mins (light blue region). This results in the BSA network formation, after which network relaxation dominates (purple region). At $t = 4450$ s, 3mM of dithiothreitol (DTT) was added to soak the BSA hydrogel for 10,800 s (180 mins). The post-DTT relaxation G' is measured (green region) for the hydrogels in the absence and presence of MBs (BSA:post-gel DTT hydrogel and BSA:MBs:post-gel DTT hydrogel).

The viscoelastic properties, G' and G'' of the BSA and BSA:MBs hydrogels were monitored through the gelation and relaxation, and for the post DTT relaxation for the BSA:post-DTT and BSA:MB:post-DTT hydrogels, shown in Figure 4.6. Figure 4.6A shows that after the lamp is turned on, G' of the BSA and BSA:MB samples rapidly increases to a larger value than G'' , due to the photo-activated dityrosine bond formation and gelation of the samples. Within the first 5 mins, the G' begins to decrease as the rate of cross-link formation decreases, and the network relaxation dominates. The gelation

curve was measured until $t = 4000$ s, and between $t = 350$ and 4000 s the G' continued to decrease with network relaxation, previously hypothesised to be associated with the rearrangement of the network [136]. Before the addition of DTT at $t = 4000$ s, the G' of the BSA hydrogel remains at higher stiffness than the BSA:MB hydrogel, which is consistent with the results shown in the previous Chapter 3 Section 3.3. At $t = 4480$ s,

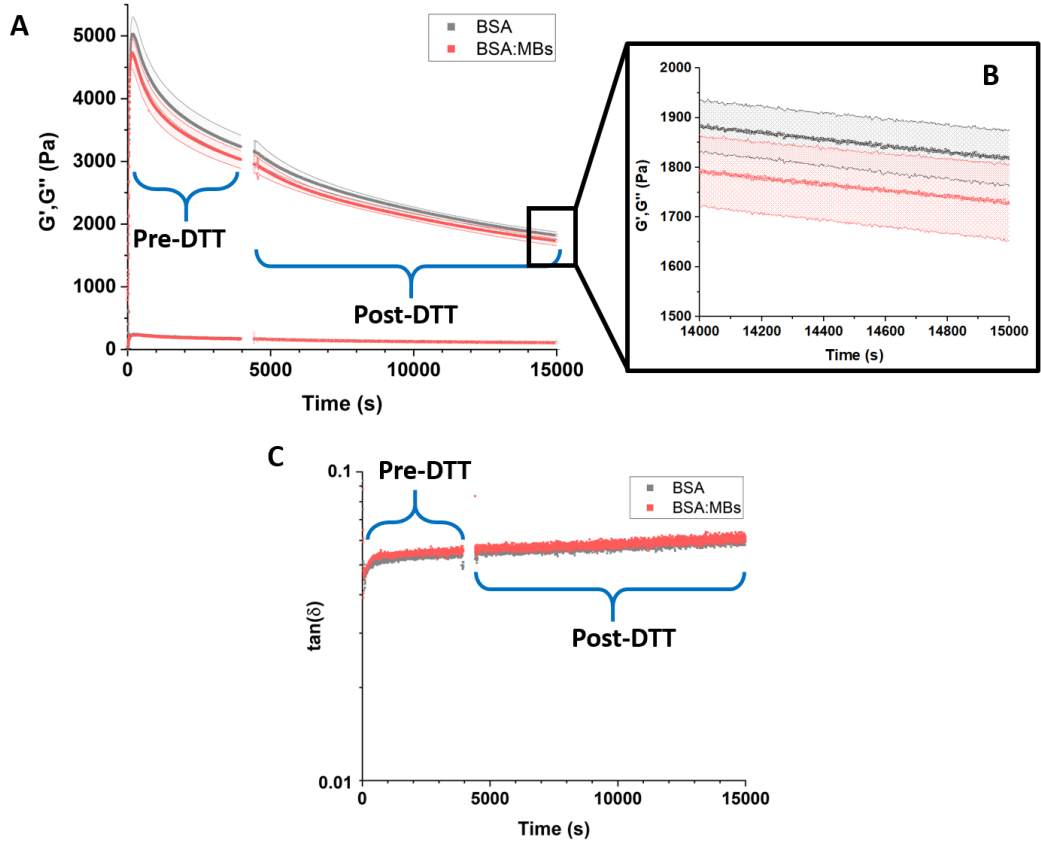


Figure 4.6: A) Time sweep of the gelation of BSA (grey) and BSA:MBs (pink) hydrogels with the storage modulus (G' , squares) and the loss modulus (G'' , closed squares). Photo-initiation of pre-gel solution occurs at $t = 0$ for 5 mins. At $t = 4480$ s, 3mM of dithiothreitol (DTT) was added to soak the BSA hydrogel for 180 mins. B) Inset of the final G' for the BSA:DTT and BSA:MBs:DTT hydrogels. C) Loss ratio ($\tan(\delta)$) for the BSA and BSA:MBs hydrogels, pre- and post DTT,

the BSA and BSA:MBs were soaked in DTT, where the post-DTT time sweep for the BSA:post-DTT and BSA:MB:post-DTT hydrogels were measured for 3 hours to monitor the network changes over time. Figure 4.6A shows the post-DTT relaxation and an inset of final measured G' in Figure 4.6B for the BSA:post-DTT and BSA:MB:post-DTT

hydrogels. The BSA:post-DTT and BSA:MB:post-DTT experienced a further decrease in the G' , following the same trend observed by Hughes *et al.* for the BSA:post-DTT hydrogels (Figure 4.4) [136]. This confirms that the addition of the DTT resulted in a decrease in the fraction of folded BSA proteins. The difference in G' between the BSA:post-DTT and BSA:MB:post-DTT hydrogels decreases after the addition of DTT (Figure 4.6B), suggesting that the DTT dominates the relaxation behaviour over the impact of the addition of MBs. The loss ratio ($\tan(\delta)$) in Figure 4.6C, shows that the BSA:MBs and BSA:MBs:post-DTT hydrogels remain more viscous over time than the BSA and BSA:post-DTT hydrogels.

The pre-DTT and post-DTT curves in Figure 4.6A can be fitted to give information on the network relaxation behaviour and the relaxed state of the four hydrogels [136]. Figure 4.7A shows an example of BSA and BSA:post-DTT hydrogels with fits. The BSA and BSA:MB hydrogels were fitted with Equation 4.1 using a single exponential decay component, while the BSA:post-DTT and BSA:MBs:post-DTT hydrogels required two exponential decay components to fit:

$$G' = B_1 \exp\left(\frac{-t}{\tau_1}\right) + B_2 \exp\left(\frac{-t}{\tau_2}\right) + G'_0 \quad (4.1)$$

The fit of the BSA (example in Figure 4.7A, pink curve) and BSA:MB hydrogels can be extrapolated to predict the final G' (G'_f) at $t = 15,000$ s and compared to the exponential decay fit for the BSA:post-DTT (example in Figure 4.7A, blue curve) and BSA:MBs:post-DTT hydrogels. Figure 4.7B compares the average G'_f for the four hydrogels measured. The G'_f of the BSA and BSA:MBs hydrogels (3130 ± 170 and 2970 ± 130 Pa) were significantly stiffer than post-DTT hydrogels, where the G'_f of the BSA:post-DTT hydrogel was 1800 ± 60 Pa and BSA:MBs:post-DTT hydrogels was 1710 ± 80 Pa). The statistical significance was confirmed via an ANOVA test in Appendix C, Table C.10 and C.11. There is a very small reduction in G'_f between the BSA:post-DTT and BSA:MBs:post-DTT hydrogels, which was determined to not be statistically significant (see Appendix C, Table C.12).

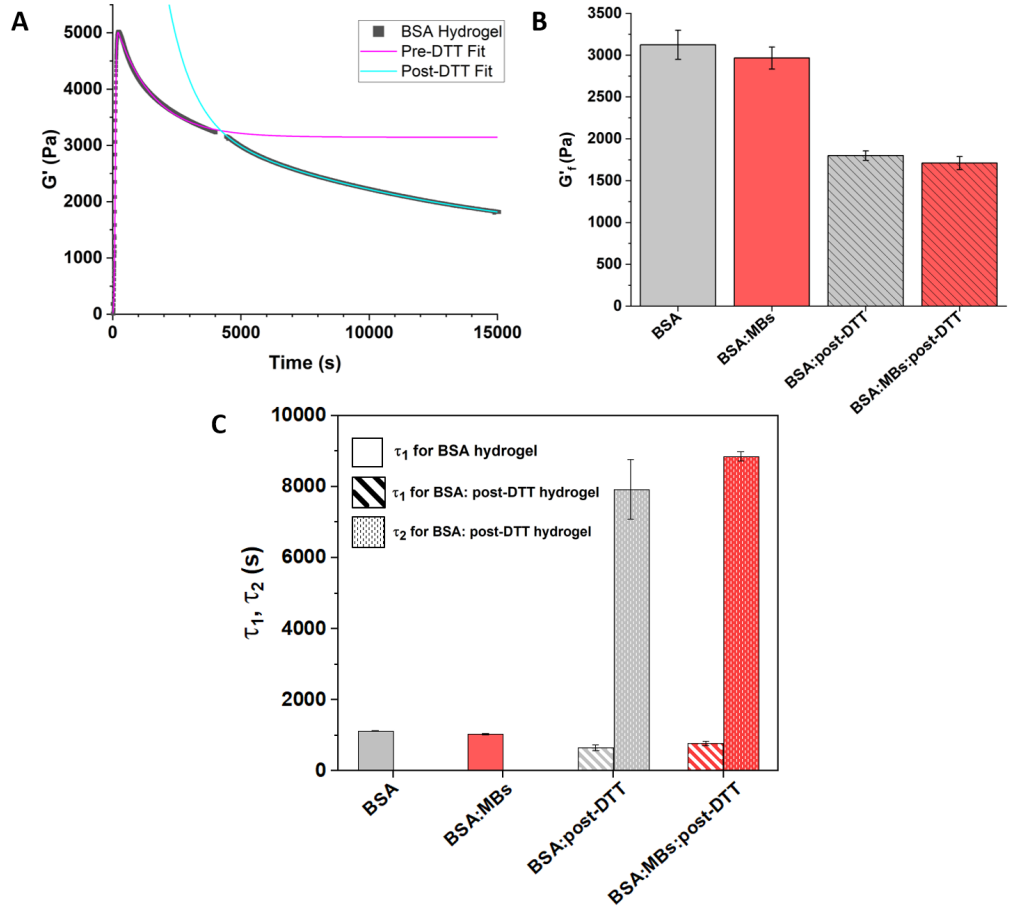


Figure 4.7: A) An example of a BSA hydrogel and a BSA:post-DDT hydrogel time sweep curve (black curve). The BSA hydrogel data was fitted using Equation 4.1 with only one exponential decay component (magenta curve) and the BSA:post-DDT hydrogel data (DTT was added at 4430 s) was fitted with Equation 4.1 with two exponential decay components (cyan curve). B) Values from for G' at $t = 15,000$ s from the average fits from Equation 3.1 for BSA (grey) and BSA:MBs (pink) and for BSA:post-DDT (grey, stripes) and BSA:MBs:post-DDT (pink, stripes) hydrogels. C) Exponential time constants, τ_1 and τ_2 taken from the relaxation fits where τ_1 for the BSA and BSA:MB hydrogels are solid, τ_1 for the BSA:post-DDT and BSA:MB:post-DDT hydrogels are striped, and τ_2 for the BSA:post-DDT and BSA:MB:post-DDT hydrogels are dotted.

The exponential decay constants (τ), describing how G' decreases over time, can reveal information about the relaxation of the BSA network. Figure 4.7C summarises the different τ for each of the hydrogel measured. The BSA and BSA:MB hydrogels have only one exponential decay constant ($\tau_1 = 1130 \pm 10$ and 1040 ± 20 s), which can

be assumed to be a result of rearrangement of the network in order to find less energetic state. For the BSA:post-DTT and BSA:MBs:post-DTT hydrogels, two exponential decay constant were extracted ($\tau_1 = 660 \pm 80$ and 70 ± 50 s, $\tau_2 = 7900 \pm 800$ and 8800 ± 100 s). The addition of DTT, for both BSA and BSA:MB hydrogels, resulted in a statistically significant decrease in τ_1 (Appendix C, Table C.13 and Table C.14). Furthermore, the relaxation times observed in τ_2 of the BSA:post-DTT and BSA:MBs:post-DTT hydrogels were similar to the timescales of the reduction in α -helical secondary structures of BSA in the presence of DTT [136], suggesting an additional relaxation mechanism associated with protein unfolding. By breaking the disulphide bonds in BSA, the unfolded protein may be able to explore new conformations and find states lower energy states. As τ_2 in both BSA:post-DTT and BSA:MBs:post-DTT hydrogels is approximately 8x larger than τ_1 in both BSA and BSA:MB hydrogels, this suggests that the unfolding of protein in a BSA hydrogel induces a faster rate of relaxation in comparison to the presence of MBs or the network rearrangement. The addition of MBs to the BSA hydrogel did not cause statistically significant results in τ_1 when comparing both BSA to BSA:MBs before the addition of DTT (Appendix C Table C.15), and when comparing τ_2 BSA:post-DTT to BSA:MBs:post-DTT (Appendix C Table C.16).

Table 4.1: Exponential decay constants τ_1 and τ_2 for the relaxation of BSA, BSA:MB, BSA:post-DTT and BSA:MBs:post-DTT hydrogels.

Hydrogel	τ_1	τ_2
BSA	1130 ± 10	-
BSA:MB	1040 ± 20	-
BSA:post-DTT	660 ± 80	7900 ± 800
BSA:MB:post-DTT	770 ± 50	8800 ± 100

Note that the stiffness observed here for BSA hydrogels is stiffer than observed in Chapter 3 Section 3.3, where the was $G'_f = 1560 \pm 190$ Pa, compared to $G'_f = 3130 \pm 170$ Pa in this study. This is presumed to be due to batch to batch variability in the purchased BSA from the supplier, where the purity is $98 \geq \%$.

4.2.2 Frequency Sweep

To investigate the storage (G') and loss (G'') moduli as a function of oscillation frequency, a frequency sweep was measured at $t = 4000$ s after photo-activated chemical cross-link initiation to allow time for network relaxation, before adding the DTT to the BSA and BSA:MB hydrogels.

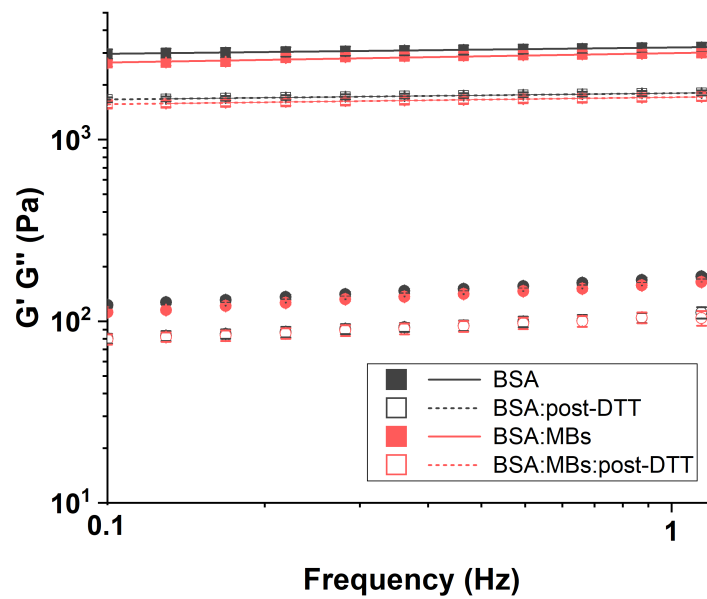


Figure 4.8: A) Frequency sweeps for BSA (grey, closed), BSA:MBs (grey, open), BSA:post-DTT (pink, closed), and BSA:MBs:post-DTT (pink, open) hydrogels for G' (squares) and G'' (circles). B) The loss ratio calculated from the frequency sweep, for the BSA, BSA:MBs, BSA:post-DTT, BSA:MBs:post-DTT hydrogels.

At $t = 4400$ s, DTT was added and a second frequency sweep was taken at $t = 15000$ s after photo-initiation to monitor relaxation, after the DTT was added, in the BSA:post-DTT and BSA:MB:post-DTT hydrogels. Figure 4.8A shows the frequency sweeps of the BSA and BSA:MBs hydrogels compared to the BSA:post-DTT and BSA:MB:post-DTT hydrogels. The frequency sweep shows for all hydrogels that G' is significantly greater than G'' , confirming that in the presence of MBs and addition of DTT the hydrogels remain in the gel state. Across all frequencies (f), the BSA hydrogel has the largest G' (at $f = 1.13$ Hz $G' = 3200 \pm 200$ Pa). For the BSA:MB hydrogels, the presence of MBs in

the pre-gel solution resulted in a subtle reduction in G' as seen previously in Chapter 3, where at $f = 1.13$ Hz the $G' = 3000 \pm 100$ Pa. The addition of DTT causes a reduction in the G' , where at $f = 1.13$ Hz the BSA:post-DTT hydrogel had a $G' = 1800 \pm 60$ Pa and the BSA:MBs:post-DTT had a $G' = 1700 \pm 90$ Pa. When comparing the BSA:MBs and BSA:post-DTT hydrogels, the addition of the DTT resulted in a greater decrease in G' , suggesting that DTT is therefore dominating the relaxation of the protein network. The DTT induces the unfolding of BSA proteins by breaking the disulphide bonds, which maintain the fold of the BSA. This implies that by breaking the disulphide bonds, the BSA proteins are able to explore a larger conformational space.

Figure 4.8B shows the loss ratio ($\tan(\delta)$), where the BSA:post-DTT and BSA:MBs:post-DTT hydrogels results in a highest $\tan(\delta)$ than the BSA and BSA:MB hydrogels. A higher $\tan(\delta)$ is associated with a more viscous hydrogel, which is consistent with the G' and G'' results Figure 4.8A. As the BSA:post-DTT and BSA:MB:post-DTT hydrogels show a lower stiffness than the BSA and BSA:MB hydrogels, this suggests that the unfolded BSA is not forming new dityrosine cross-links with the load-bearing BSA network (summarised in Figure 4.9). This correlated to the work shown by Hughes *et al.* found the same trend with BSA protein hydrogels, where the BSA:post-DTT hydrogels had a lower G' [136].

4.3 Structural Characterisation

Structural information is required to understand BSA hydrogel networks, as demonstrated in Chapter 3. The presence of both DTT and MBs changes the mechanical properties of the bulk BSA hydrogel. Small angle neutron scattering (SANS) is a tool that provides information on the resultant mesoscale structure. This will allow a more informed conclusion as to how the DTT and MB together control the properties of both the viscoelastic and structural properties of folded protein hydrogels. This section outlines the structural characterisation for the BSA, BSA:MB, BSA:post-DTT and BSA:MB:post-DTT hydrogels, to compare with the rheology. The same concentrations of BSA (7% volume fraction) and MBs (1×10^9 MB/mL) were used as in the rheological

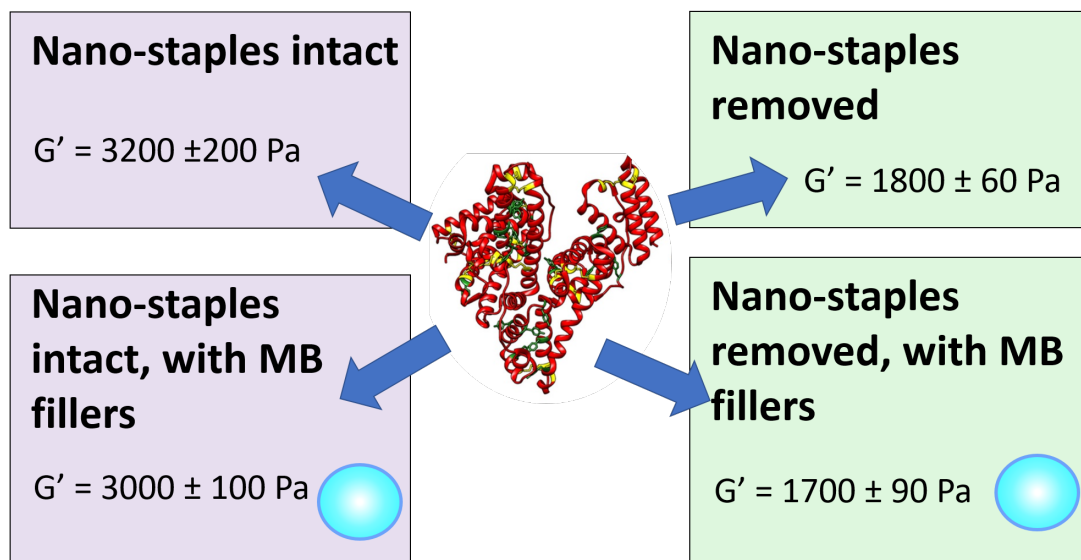


Figure 4.9: Summary figure of the rheology data, where the storage moduli (G') are quoted for the different measurements made in this study. The breakdown of disulphide ‘nano-staples’ resulted in weaker BSA hydrogels. Central BSA monomer adapted reused from Hughes *et al.* (2021) [136] with permission from CC BY 4.0.

characterisation. In this section the fitting of SANS data with SASview was performed by Dr Matthew D.G. Hughes.

4.3.1 Contrast Matching

In this chapter, the SANS experiments were conducted on SANS2d (ISIS muon and neutron facility), where the instrumental q -range was $0.0015 \leq q \leq 1 \text{ \AA}^{-1}$. Contrast matching was used for the BSA:MB hydrogels, such that the lipids in the MBs were constant matched to the buffer. By contrast matching out the lipids, it allowed the scattering information to be collect from primarily the proteins in the BSA:MB hydrogels. The scattering length densities (SLD) were calculated for lipids this experiment using SASview, based off the density of lipids in the BSA:MB hydrogels at a MB concentration of $1 \times 10^9 \text{ MB/mL}$. The SLDs were used to determine the match point of the lipids and the D_2O buffer, to find the ratio of D_2O to H_2O for contrast matching. This was calculated to be 20% D_2O to 80% H_2O . Figure 4.10 shows the intensity curves of the BSA:MB hydrogels prepared in 20% and 98% D_2O . A reduction in scattering intensity was observed when lowering the percentage of D_2O from 98% to 20%. This demonstrated

that using 20% D₂O successfully reduced the scattering contribution from the lipids, therefore the scattering data collected is presumed to be mainly from the BSA proteins. The high-q data was much noisier in the 20% D₂O, due to a lower flux of neutrons collected and less scattering from MBs than in the 98% D₂O BSA:MBs hydrogel.

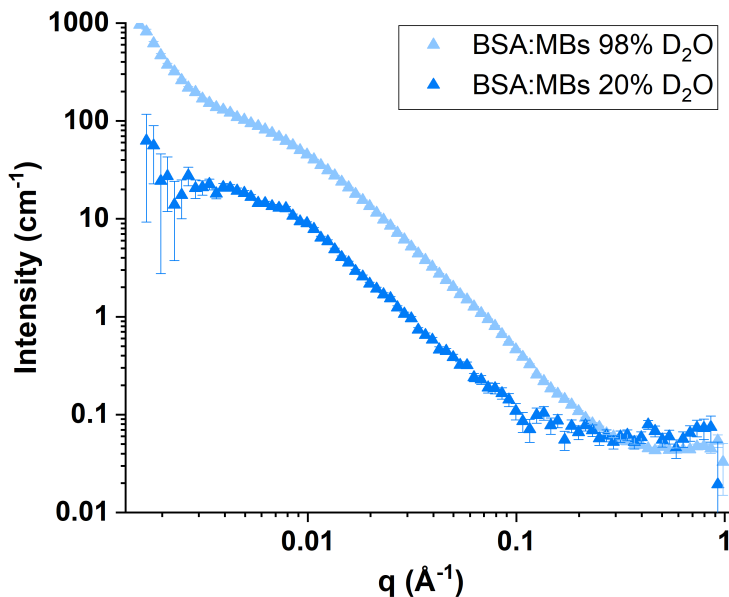


Figure 4.10: SANS intensity curves for BSA:MBs hydrogels prepared in 98% D₂O (light blue) and 20% D₂O (dark blue), used to contrast match the lipids in the MB shell to the buffer.

4.3.2 SANS Intensity Curves

Figure 4.12 shows the SANS intensity curves taken for the BSA, BSA:MB hydrogel, which were both soaked in DTT for approximately 3 hours before repeating the SANS experiment to mimic the rheology set-up in previous Section 4.2 of the BSA:post-DTT and BSA:MBs:post-DTT hydrogels. The intensity curves are consistent in shape with those in Chapter 3 Section 3.5 and previous publications of SANS on BSA hydrogels [136]. Qualitative observations can be made from the SANS intensity curves. The low-q region of Figure 4.11 shows a higher scattering intensity in the BSA hydrogels with MBs, due to larger errors in the low-q range and possibly due MBs scattering neutrons. The intensity

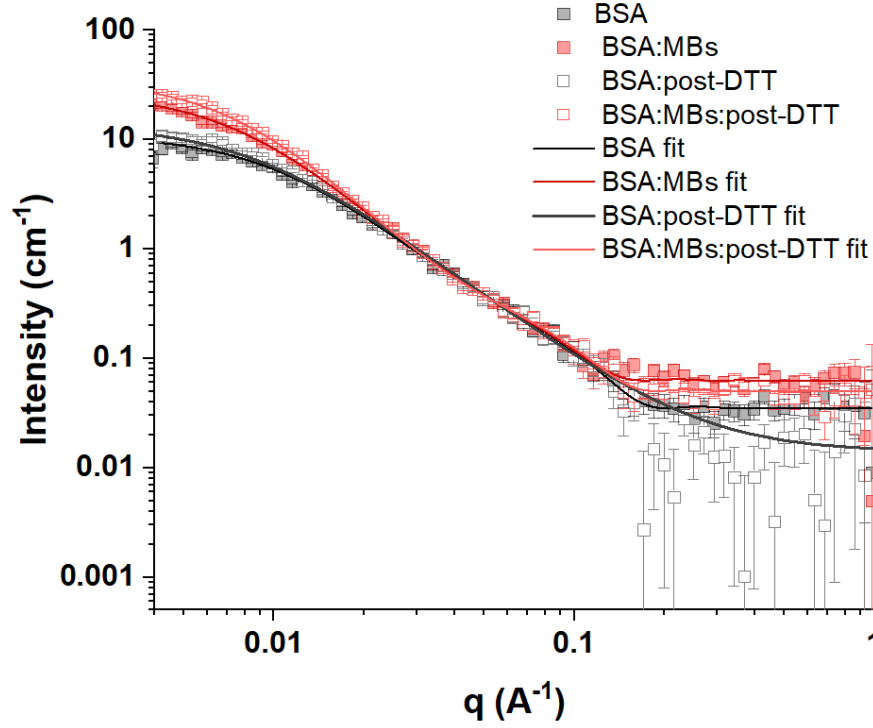


Figure 4.11: SANS intensity curves for BSA (grey closed squares), BSA:MBs (pink closed squares), BSA:DTT (grey open squares) and BSA:MBs:DTT (pink open squares) hydrogels. Fits of the intensity curves (solid lines) are used to extract the fractal dimension and correlation length

is slightly increased in the BSA:DTT and BSA:MBs:DTT hydrogels, compared to the BSA and BSA:MBs without DTT. The mid- q region, representing the geometry and topology of the protein network, are similar shapes for all curves, with a slightly steeper gradient for the BSA:DTT and BSA:MBs:DTT curves. The high- q region, representing the size of the protein clusters, have similar shaped curves, with larger error bars in the samples with DTT. The larger error bars indicate that in future studies, a higher number of neutrons collected for each sample would be preferable to improve the statistics and subsequent analysis.

4.3.3 Fractal Dimension and Correlation Length

From the intensity curves in Figure 4.11 and the fitting model previously described in Chapter 3 Section 3.5, the fractal dimension (D_f) and the correlation length (ξ) of the

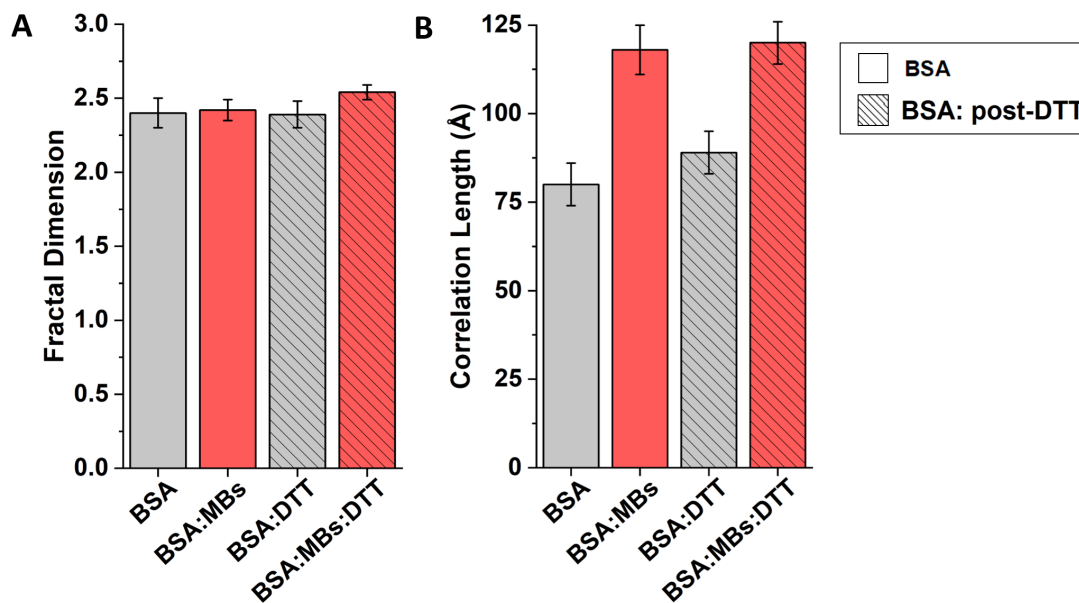


Figure 4.12: A) Fractal dimension and b) correlation length for BSA (grey), BSA:MBs (pink), BSA:post-DTT (grey striped) and BSA:MBs:post-DTT (pink striped) hydrogels.

protein clusters were calculated. The D_f of a cluster of proteins describes how the mass of protein packs into a certain cluster size. Previously, the clusters of proteins in folded protein hydrogels have been predicted to be fractal, where a high D_f describes a high density of proteins in a fractal protein cluster [123, 135, 136]. Figure 4.12A shows that for the all hydrogel samples the calculated D_f are similar: 2.4 ± 0.1 for the BSA hydrogel, 2.42 ± 0.07 for the BSA:MB hydrogel, 2.39 ± 0.09 for the BSA:post-DTT hydrogel, and subtly higher for BSA:MBs:post-DTT at 2.54 ± 0.05 . The similarity in D_f between the samples suggests that the presence of MBs, and the unfolding of cross-linked BSA does not significantly change the density of fractal-like clusters. The ξ describes how the size of the largest protein clusters changes. The BSA and BSA:post-DTT hydrogels had the smallest cluster sizes ($\xi = 80 \pm 6 \text{ \AA}$ for the BSA hydrogel and $\xi = 89 \pm 6 \text{ \AA}$ for the BSA:post-DTT hydrogel). ξ increased most significantly for the BSA hydrogels with MBs present ($\xi = 118 \pm 7 \text{ \AA}$ for the BSA:MB hydrogel and $\xi = 120 \pm 6 \text{ \AA}$ for the BSA:MBs:post-DTT hydrogel). The addition of DTT to the cross-linked hydrogels increased the size of the clusters, but the affect on ξ was not as significant as the presence of the MBs during gelation. The increase size of protein clusters for the BSA:MBs and BSA:MB:post-DTT is consistent with the work in Chapter 3 Section 3.5, where the

results from adding the DTT to the already cross-linked hydrogel network experienced a similar increase in ξ of 9 Å.

Hughes *et al.* (2021) showed that when adding 3 mM of DTT to the pre-gelation solution (BSA:pre-DTT hydrogel), a significantly higher D_f was observed (BSA hydrogel: $D_f = 2.17 \pm 0.01$. BSA:pre-DTT hydrogel: $D_f = 2.66 \pm 0.01$) [136]. This suggested that building a network with unfolded BSA resulted in a network with much denser clusters. In network formation with unfolded BSA, it was predicted that the BSA can form a higher number of cross-links, as a stiffer protein hydrogel network is formed [136]. The results presented here showed no large changes in D_f , with less densely packed clusters of proteins. Hughes *et al.* (2021) found that in the BSA:pre-DTT hydrogels the ξ of the clusters to increases by 8 Å compared to the BSA hydrogels, showing very similar increase in ξ reported here for BSA:post-DTT hydrogels.

4.3.4 Characterising the Clusters and Inter-Cluster Region

The previous work in Chapter 3 showed that further analysis can be used to calculate $N(r)$ which is the number of protein clusters in a sphere of radius, R , from the centre of the cluster where the derivation is shown in Chapter B [123, 136]. From the $N(r)$, the volume fraction of proteins in clusters, ϕ_c , and in the the cluster space, ϕ_{ic} can be calculated. This analysis was applied to the study presented in this section for BSA, BSA:post-DTT, BSA:MB and BSA:MB:post-DTT hydrogels. Figure 4.13 shows how $N(r)$ changes with distance from the centre of the average cluster, where all curves have similar gradients which plateau at similar distances. The D_f (Figure 4.12A) change is very similar for all the hydrogels, therefore similar gradients of $N(r)$ are to be expected. The radius at which the BSA and BSA:MBs hydrogels plateau is shorter than the BSA:post-DTT and BSA:MB:post-DTT hydrogels, following the same trend as the correlation length in (Figure 4.12B). As the ξ describes the largest cluster size, the number of proteins found in a cluster is expected to be lower for clusters with a lower ξ . Figures 4.14A and B compare the ϕ_c and ϕ_{ic} for the four hydrogels systems. In all hydrogels, ϕ_c is greater than ϕ_{ic} , predicting that the majority of proteins are situated in clusters, with less protein in

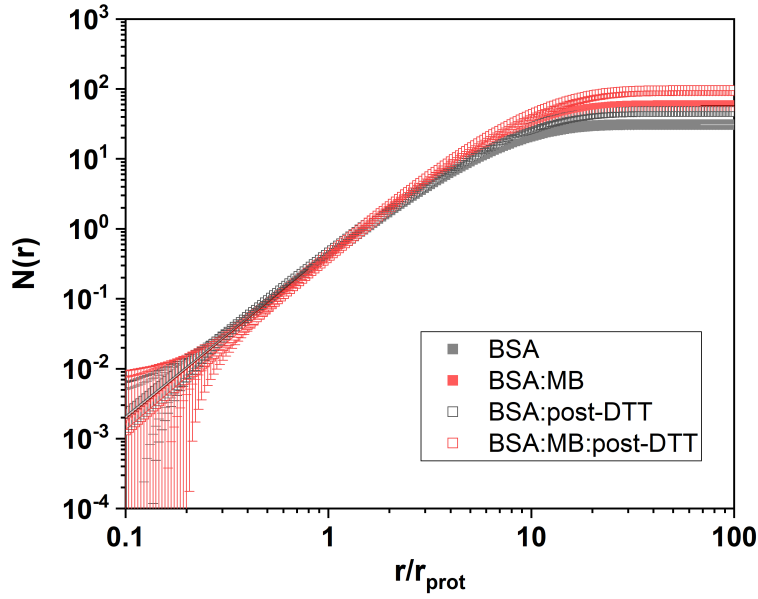


Figure 4.13: The number of proteins in each fractal-like cluster as a function of r , $N(r)$, with respect to the ratio of distance from the centre of the cluster radius (r) and radius of a BSA protein monomer (r_{prot}). $N(r)$ is shown for BSA (grey closed squares), BSA:MBs (pink closed squares), BSA:post-DTT (grey open squares) and BSA:MBs:post-DTT (pink open squares) hydrogels.

the inter-cluster space. The changes between the BSA and BSA:MBs follow the same trend trends in Chapter 3 Section 3.5, with a small decrease observed in the ϕ_c between BSA and BSA:MBs hydrogels ($\phi_c = 13.3 \pm 0.1 \%$ and $12.5 \pm 0.1 \%$). The presence of the DTT in the BSA:post-DTT hydrogels induces a slight decrease in the ϕ_c ($\phi_c = 12.2 \pm 0.2 \%$), however the composite BSA:MBs:post-DTT hydrogel results in the opposite trend where the ϕ_c is the largest at $14.0 \pm 0.1 \%$. Table 4.2 summarises these results.

Table 4.2: Values of the volume fraction of BSA in fractal-like clusters, ϕ_c , the volume fraction of BSA in the inter-cluster region, ϕ_{ic} , for BSA, BSA:MBs, BSA:post-DTT and BSA:MBs:post-DTT hydrogels.

Hydrogel	ϕ_c (%)	ϕ_{ic} (%)
BSA	13.3 ± 0.1	1.82 ± 0.01
BSA:MBs	12.5 ± 0.1	2.48 ± 0.02
BSA:post-DTT	12.2 ± 0.2	1.11 ± 0.01
BSA:MBs:post-DTT	14.0 ± 0.1	1.61 ± 0.02

Figure 4.14B shows that ϕ_{ic} does not vary significantly, with the results summarised

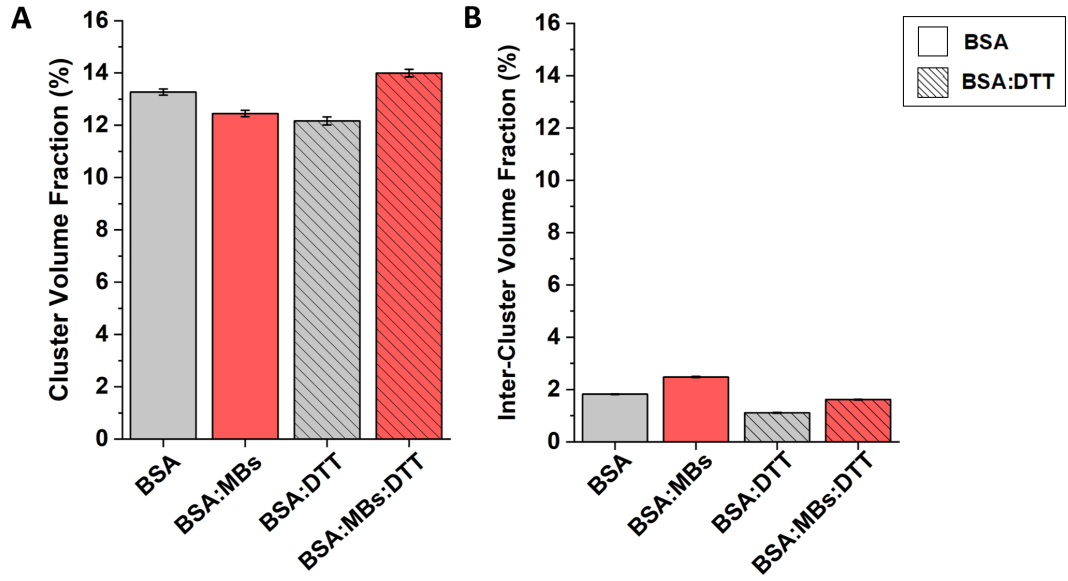


Figure 4.14: A) The volume fraction of BSA in fractal-like clusters, ϕ_c , and B) the volume fraction of BSA in the inter-cluster region, ϕ_{ic} , for BSA (grey), BSA:MBs (pink), BSA:post-DTT (grey striped) and BSA:MBs:post-DTT (pink striped) hydrogels.

in Table 4.2. The presence of MBs increases ϕ_{ic} , while the addition of DTT results in the opposite effect, resulting in a decrease in the protein in the inter-cluster region. Studies by Del Gado *et al.* and Furst *et al.* observe that ϕ_{ic} is more important in terms of influencing in the viscoelastic properties than ϕ_c , for example how the inter-cluster region connects the clusters or the strength of connections between clusters will influence the hydrogels behaviour when force is applied [299, 316, 317]. This implies that small changes in ϕ_{ic} would result in small changes to the G' .

The predicted total number of clusters (n_c) in the different hydrogels can also be extracted from the $N(r)$ curve in Figure 4.13. Figure 4.15 shows n_c for the four hydrogel measured. The BSA:MBs hydrogel showed a significantly lower n_c in comparison to BSA hydrogel, which again is consistent with the results in previous Chapter 3, Section 3.5 (BSA hydrogel: $n_c = 8.10 \pm 0.05 \times 10^{21}$ clusters and BSA:MBs hydrogel: $n_c = 5.03 \pm 0.04 \times 10^{21}$ clusters). Soaking the hydrogels in DTT results in a further reduction in the n_c (BSA:post-DTT hydrogel: $n_c = 3.58 \pm 0.04 \times 10^{21}$ clusters and BSA:MBs:post-DTT hydrogel: $n_c = 1.96 \pm 0.02 \times 10^{21}$ clusters). Interestingly the effect of the DTT and MBs is cumulative for the reduction in n_c , and this correlates with the subtle reduction

in G' observed in the rheological characterisation (Figure 4.6B). This suggests that for BSA hydrogels, the number of clusters controls the bulk, macroscopic hydrogels.

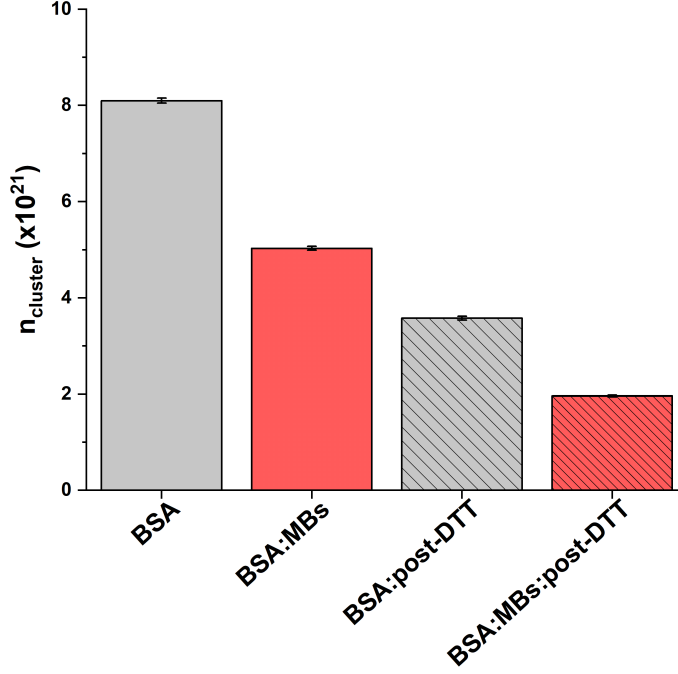


Figure 4.15: Total number of fractal-like clusters, N_{clusters} for BSA (grey), BSA:MBs (pink), BSA:post-DTT (grey striped) and BSA:MBs:post-DTT (pink striped) hydrogels.

4.4 Discussion

In this section the effect of unfolding proteins in cross-linked protein hydrogels was investigated for BSA and BSA:MB hydrogels. The motivation for this investigation into the bulk mechanics and network structure of the BSA:post-DTT and BSA:MB:post-DTT hydrogels, was to understand if a protein hydrogel exposed to a change in the environment that induced protein unfolding in a biomedical application, how this would affect the protein hydrogel.

In the rheological characterisation of the bulk mechanics, the relaxed G'_f was compared for the four hydrogel systems. The MBs resulted in a subtle decrease of ~ 150 Pa in the G'_f between the BSA and BSA:MB hydrogel. The post gelation addition of DTT resulted in a much larger decrease in of ~ 1300 Pa G'_f for the BSA:post-DTT hydrogel

and ~ 1400 Pa for the BSA:MBs:post-DTT hydrogel. The addition of DTT also resulted in an increase in the rate of relaxation in both the BSA:DTT and BSA:MB:DTT hydrogels. These changes observed implied that the unfolding of proteins in a cross-linked BSA hydrogel network has a larger impact the bulk mechanical properties than the presence of MBs in the BSA:MB hydrogel.

SANS allowed for the characterisation of protein network structure in the four hydrogel systems. The fractal dimension of all four hydrogel systems did not experience large changes. The correlation length ξ saw a small increase of $9 \text{ \AA}$ in the BSA:post-DTT hydrogels, and larger increase in ξ with the presence of MBs of 38ξ for BSA:MB hydrogel and 40ξ for the BSA:MB:DTT hydrogel. This implies there is an interaction between the MBs and BSA, that could be linked to the chemistry of the MB shell. The hydrophilicity and flexible chains of low molecular weight PEG molecules such as PEG-2000 (which are present in the MBs used in this study) have been shown, in both theoretical and experimental studies, to reduce serum albumin protein interaction with lipids [294]. Further work is required to understand the interactions between BSA and the lipids in the MB.

To understand the link between the structure of the four BSA hydrogel systems, and the different stiffnesses observed, further analysis was performed to find the volume fractions of proteins in the cluster (ϕ_c) and inter-cluster space (ϕ_{ic}). For all hydrogels measured the ϕ_{ic} was smaller than ϕ_c , implying a larger proportion of proteins was situated in the clusters. The BSA:post-DTT and BSA:MB:post-DTT hydrogels had a lower ϕ_{ic} than the BSA and BSA:MB hydrogels without DTT. ϕ_c also showed very minor changes between the four hydrogel systems. The number of protein clusters in the four hydrogel systems resulted in significant changes. The BSA hydrogel had the highest n_c , and BSA:MB hydrogels resulted in 38% decrease in n_c , this suggests that the presence of MBs in the pre-gel solution reduces the number of clusters that form in the network. The BSA:post-DTT and BSA:MB:post-DTT hydrogels showed further reductions in n_c of 55% and 76%, implying that the unfolding of protein in the network also reduces n_c in the network and is dominant in change n_c compared to the presence of MBs.

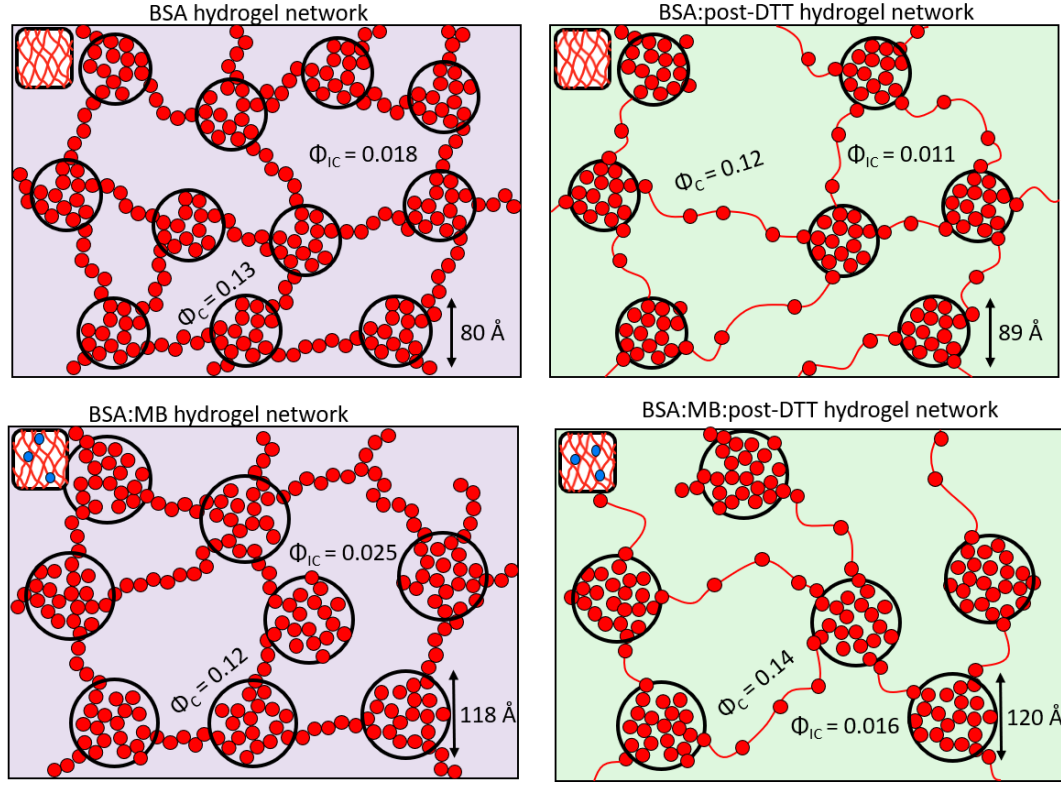


Figure 4.16: Predicted structures from small-angle neutron scattering for BSA (top left), BSA:MBs (top right), BSA:post-DTT (bottom left) and BSA:MBs:post-DTT (bottom right) hydrogels. The black circles represent the fractal-like clusters. For the BSA:post-DTT and BSA:MBs:post-DTT, the red lines between clusters represent unfolded BSA. ϕ_c is the volume fraction of proteins in clusters and ϕ_{ic} is the volume fraction of proteins in the inter-cluster region.

The results from this Chapter and Chapter 4 suggest that there is a correlation between the n_c and G'_f of the protein hydrogel. In classical affine network theory, the number of junction points (μ) and chains between junction points (ν) are proportional to shear modulus G [318]:

$$G' \propto \mu k_B T \quad (4.2)$$

$$\mu = \frac{2\nu}{\psi} \quad (4.3)$$

where k_B is the Boltzmann constant, T is the temperature and ψ is the functionality of the junction. The affine network model could be applied to the system presented here, where the clusters of proteins could be modelled as junction points and the proteins in the inter-cluster space could be modelled as chains between junctions.

Previous studies by Hughes *et al.* showed that unfolding BSA in protein hydrogels at different time points affected the structure and mechanics, with a range in G' of 2.5 - 7.5 kPa observed in the bulk BSA hydrogel [136]. Aufderhorst-Roberts *et al.* demonstrated that by controlling the reaction rate of photo-initiated ditryosine cross-link formation between BSA proteins the mechanical and structural properties could be tuned [123]. By changing the power of the lamp used for photo-initiating cross-link formation, the G' was varied from 1-2.5 kPa. This control over the G' was suggested to be a result of different aggregation regimes that lead to network formation, where slow reaction rates observed at low lamp power were predicted to be reaction rate limited and fast reaction rates at higher lamp powers were in the diffusion limited aggregation regime [319, 320]. These results, in combination with previous studies mentioned here, show that the properties of folded protein hydrogels can be tuned to control the hydrogel properties. A cross-length scale understanding of how to tune BSA hydrogel properties is a desirable feature for biomedical applications, where BSA would be suitable for applications with soft tissues. Further work to understand how these mechanical and structural properties change at 37°C would be informative to the changes that the BSA hydrogel would undergo at body temperature.

Chapter 5

Assessing the Potential of Folded Protein Hydrogels for Biomedical Applications

This chapter explores the suitability of folded protein hydrogels for different biomedical applications, with model protein bovine serum albumin (BSA) for the hydrogel building block. The first part of this chapter is focused on exploring different types of biomedical applications that folded protein hydrogels could be used. Naturally derived biomaterials that can be made reproducibly for cell culture is of interest, due to the similar properties between the biocompatibility, mechanical properties and porosity with the extracellular matrix [94, 321]. Folded protein hydrogels could act as a scaffold for 3D cell culture, where the mechanical and structural properties of the folded protein hydrogel can be controlled via different preparation methods [123, 135, 136, 140]. The viability of cells cultured onto BSA hydrogels for 2D cell culture was therefore assessed to explore the potential application of protein hydrogels for cell culture, before moving on to future studies with 3D cell culture.

When considering using protein hydrogels for drug delivery, the application of the protein hydrogel will determine the size, or shape of the hydrogel that is required [68].

For macroscopic protein hydrogels, the shape and size can be achieved simply by loading the pre-gel solution into moulds of different sizes or shape before initiating cross-link formation. Macroscopic hydrogels for drug delivery can be used as transdermal patches or surgically implanted, but have limitations in their applications due to their size as they can not be injected or ingested. Reducing the size of the protein hydrogel to small hydrogel particles, such as microgels ($>1\ \mu\text{m}$) or nanogels ($>1\ \mu\text{m}$), opens up different entry routes into the body. For example, creating microgels of less than the diameter of a typical needle (1 mm) allows the protein hydrogels to be injected into the body. Microgels of 0.5 - 5 μm in diameter are suitable for inhalation for pulmonary based delivery for optimal aerodynamics to travel through the throat and airways [68, 322]. The option to scale down the bulk protein hydrogel size to a microgel size is therefore vital to expanding the applications that the protein hydrogels could be used for in delivering drugs. This chapter therefore investigates the use of microfluidics for producing microgels, where microfluidics is chosen for its control over fluid flow that allows for size and monodispersity of the microgels[323–325].

The second part of the chapter considers using microbubbles (MBs) embedded within folded protein hydrogels (protein:MB hydrogels) for biomedical applications. Due to their ultrasound (US) responsive properties, using drug-loaded MBs offers a potential method for triggering a burst release of drugs into the surrounding protein hydrogel. For drug delivery, drug-loaded MBs embedded in folded protein could be burst for on-demand drug release with ultrasound, after which the drugs would be free to diffuse out of the surrounding protein hydrogel as depicted in Figure 5.1. Furthermore, bursting the MBs that are embedded in hydrogel network has been shown to create pores in the hydrogel network [215, 216], however this technique has yet to be applied to protein:MB hydrogels. The size of the MBs (1-5 μm) is much larger scale than the inter-cluster space (10 nm) predicted from small angle scattering, therefore this has the potential to trigger an increase in the diffusion of drug molecules out of the hydrogel for delivery. In tissue engineering with hydrogels, a more porous hydrogel can be beneficial for the delivery of nutrients to encourage cell growth in a method that does not decrease the stiffness of the hydrogel [199, 214, 215].

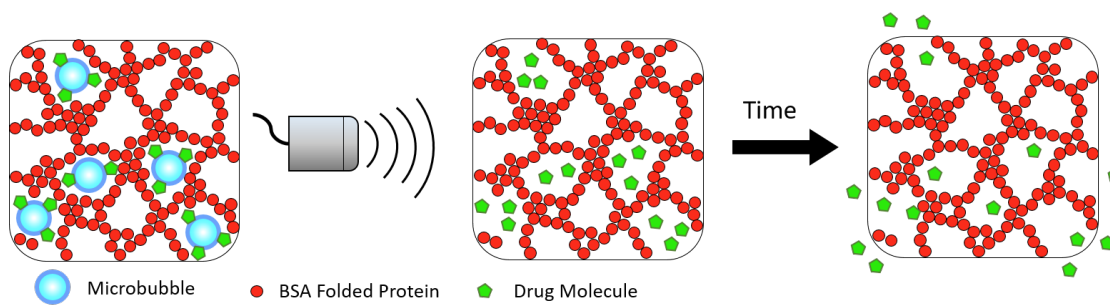


Figure 5.1: Schematic of drug-loaded MBs embedded in folded protein hydrogels. The destruction of MBs from the application of ultrasound releases drugs into the surrounding hydrogel network. After a given period of time the encapsulated drugs leak of the hydrogel.

When considering the use of composite protein:MB hydrogels for future clinical translation, investigating how phospholipid stabilised MBs are affected by being embedded in folded protein hydrogels is important. If the MBs have a short lifetime in protein hydrogels, this reduces the time in which they are useful for in terms of triggering drug release with US from drug-loaded MBs. Short MB lifetime are therefore less suitable for translation into clinic. This study investigates the lifetime of the MBs when embedded in BSA hydrogels to mimic storing the BSA:MB hydrogels in the clinic before use. This can be quantified by how quickly the concentration of MBs decreases over time, as well as how the size of the MBs changes.

5.1 Cell Culture on BSA hydrogels

To evaluate the suitability of BSA protein hydrogels for biomedical applications involving culturing cells, 1×10^5 cells (PANC-1: PSC co-culture in a 1:3 ratio) were seeded on top of the BSA hydrogels, and the cell viability assessed over time. Cell culture and viability assays were conducted by Dr Dela Kpeglo. The viability of PDAC cultures was compared to cells seeded onto a control hydrogel, Cultrex[®] Basement Membrane Extract (BME) hydrogel [326]. BME hydrogel was chosen as a control hydrogel, as it is a commonly used hydrogel scaffold for cell culture, and previous studies have shown success specifically with culturing pancreatic cancer cells on BME hydrogels [326–328]. To assess the viability of the PDAC cultures, an adenosine 5'-triphosphate (ATP) assay

was used on the PDAC cultures to quantify the number of viable cells, by detecting the amount of ATP in the cell culture with luminescence emission. The ATP assay assesses the metabolic activities of cells by detecting their ATP, and therefore the ATP luminescent emission correlates with viability of the cultured cells.

5.1.1 Cell Viability of PDAC Cultures on BSA Hydrogels

The viability of PDAC cultures from different preparation methods of the BSA hydrogel was tested (see Figure 5.2). In the first method investigated (Figure 5.2A), the BSA gels were prepared and sterilised, after which the cells were seeded.

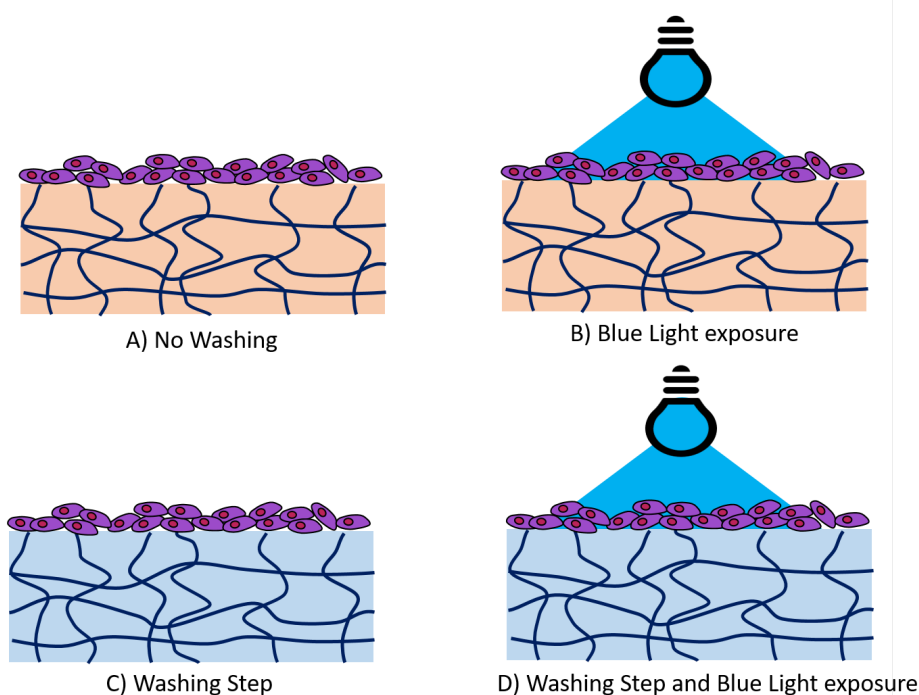


Figure 5.2: Schematic summarising the different experimental scenarios in which PDAC cells (purple) were cultured onto BSA hydrogels. 1) BSA hydrogels with no washing step, 2) BSA hydrogels with blue light exposure after seeding PDAC cultures. 3) BSA hydrogel with washing step before seeding the cells, and 4) BSA hydrogel with washing step before seeding the cells, and blue light exposure after seeding the cells. Not to scale.

Figure 5.3 shows the ATP luminescence emission of the ATP assay performed on the PDAC cultures on day 0 of seeding on to the BSA and BME hydrogels (3 - 4 hours of

culture) and day 2 of culture with the hydrogels. The PDAC cells from the control BME hydrogel had a luminescence of $139,000 \pm 2000$ on day 0 and $130,000 \pm 9000$ on day 2 of culture, with a 6.5% reduction in viability after 2 days. The PDAC cultures from the BSA hydrogel had a luminescence of $118,000 \pm 5000$ on day 0 and $63,000 \pm 2700$ on day 2, with a 46.6% decrease in viability after 2 days. The errors come from the standard error of multiple wells ($n = 12$ wells for PDAC cells grown on BSA hydrogels, and $n = 3$ wells for PDAC cells grown on BME hydrogels) in the well-plate. All PDAC cells from the same passage. The PDAC cultures from the BSA hydrogel showed a large decrease in viability in comparison to the PDAC cultures from the control BME hydrogel, suggesting that this BSA hydrogel was not a good environment for maintaining the viability PDAC cultures. The components required to photo-initiate the protein cross-linking are sodium persulfate (NaPS) and tris(2 2'-bipyridyl)ruthenium(ii) ($\text{Ru}(\text{bpy})_3^{2+}$). Persulfates such as NaPS and ammonium persulfate have been shown to be toxic, predicted to be due to the release of reactive free radicals that can cause damage to the cells [329, 330]. By removing the NaPS and $\text{Ru}(\text{bpy})_3^{2+}$ before seeding the PANC-1 and PSCs cells, an increase viability of the PDAC cultures after 2 days could be observe if the NaPS and $\text{Ru}(\text{bpy})_3^{2+}$ were negatively impacting the PDAC cultures.

In this study, the cells were seeded on top of the hydrogels, however for 3D cell culture the cells would be seeded into the pre-gel solution before cross-link formation. To explore if 3D cell culture is a possible avenue for protein hydrogels with a persulfate based photo-activated cross-linking method, an additional well plate was prepared and the PDAC cells seeded onto the BME and BSA hydrogels. The cells and hydrogels were then exposed to a blue LED light for 5 mins on day 0 (Figure 5.2B), to mimic the set up for initiating the exposure of the blue LED light required for future 3D cell culture with BSA hydrogels. Figure 5.3 shows the ATP luminescence emission for PDAC cultures from the BME and BSA hydrogels, that have been exposed to the blue LED light (BSA:LED and BME:LED hydrogels). The luminescence emission of PDAC cells exposed to blue light was compared for cultures from the BME:LED and BSA:LED hydrogels on day 2 of culture, 48 hours after blue LED light exposure. The luminescence emission of the PDAC culture was $109,000 \pm 6000$ for the BME:LED hydrogels, which

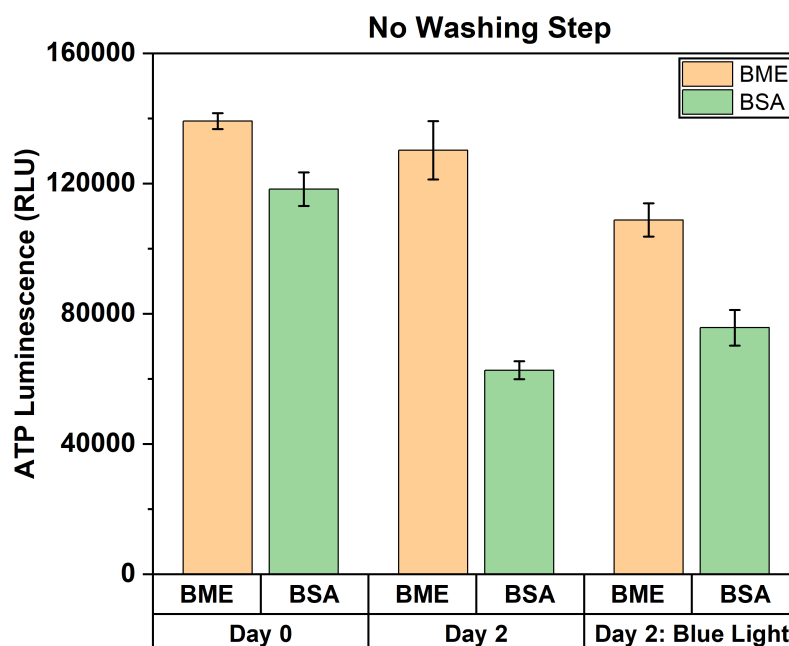


Figure 5.3: The viability of PDAC cultures is tested with an ATP assay for control Cultrex® Basement Membrane Extract hydrogel (BME, orange) and for BSA hydrogels (green). ATP assays were performed on day 0 and day 2 after the PANC-1 and PSCs cells were seeded onto the hydrogels. Additional BME and BSA hydrogels were prepared where the PDAC cultures were exposed to a blue LED light for 5 mins to mimic the photo-activation of folded protein hydrogels.

was lower than the day 0 BME hydrogel luminescence emission by 21.6%. The viability of PDAC cells on the BME hydrogels was lower with exposure to the blue LED light, compared to the PDAC cells with no blue LED light exposure. The luminescence emission of the PDAC culture was $76,000 \pm 6000$ for the BME:LED hydrogels, which was lower than the day 0 luminescence emission by 36.4%. The viability of PDAC cells on the BSA hydrogels was higher with exposure the blue LED light. The blue LED light does not correlate with changes in PDAC viability.

5.1.2 Cell viability with an Additional Washing Step

To improve the viability of the PDAC cultures from the BSA hydrogel, the BSA hydrogel was washed with cell media for 5 mins, 3 times (BSA:Washed hydrogel, Figure

5.2C). This washing step aimed to remove components in the pre-gel solution are not part of the final protein hydrogel network (NaPS and $\text{Ru}(\text{bpy})_3^{2+}$). BME hydrogels were not washed due to the low stiffness (18.98 ± 0.94 , [326]), the washing step could shear the hydrogel. Figure 5.4 showed the ATP luminescence of the PDAC cultures from the BME, BSA:Washed hydrogel, BSA hydrogel exposed to the blue LED light post washing (BSA:Washed:LED hydrogel). The control BME hydrogel had a luminescence emission of $124,000 \pm 3000$ on day 0 and $107,000 \pm 11,000$ on day 2 of culture, showing a 13.7% decrease in the viability of the PDAC culture over 2 days. The PDAC culture from the BSA:Washed hydrogel had a luminescence of $147,000 \pm 9000$ on day 0 and $133,000 \pm 2000$ on day 2 of culture, with a 9.5% decrease in luminescence emission over 2 days. The reduction in luminescence emission over 2 days of the PDAC culture from the BSA:Washed hydrogels was less than in the control BME hydrogel, and percentage decrease in the viability of the PDAC cells was less for the BSA:Washed hydrogel, compared to the BSA hydrogel that was not washed (Figure 5.3). This suggests that the concentration NaPS was successfully reduced in the washed BSA hydrogels before the cells were seeded, and that the BSA:Washed hydrogel is a suitable environment for 2D PDAC cell culture.

The viability of the PDAC cultures exposed to the blue LED light was also compared for BME:LED and BSA:Washed:LED hydrogels (Figure 5.2). The luminescence emission on day 2 of culture for the PDAC cultures from the BME:LED hydrogel was $112,000 \pm 2000$, showing 9.5% decrease in luminescence emission compared to the BME hydrogel on day 0. The luminescence emission on day 2 of culture for the PDAC cultures from the BSA:Washed:LED hydrogel was $121,000 \pm 2000$, showing a 17.7% decrease in viability compared to the BSA:Washed hydrogel on day 0. The decrease in viability for the PDAC cells from the BME:LED hydrogel after 2 days of culturing, was not significantly different to the PDAC cells from the BME hydrogel without blue LED exposure. These results, in combination with the results with unwashed BSA hydrogels, suggests that the blue LED light is not controlling the viability of PDAC cultures, but further repeats are required to confirm this conclusion.

Comparing the viability of the PDAC culture from BSA hydrogel in Figures 5.3 and

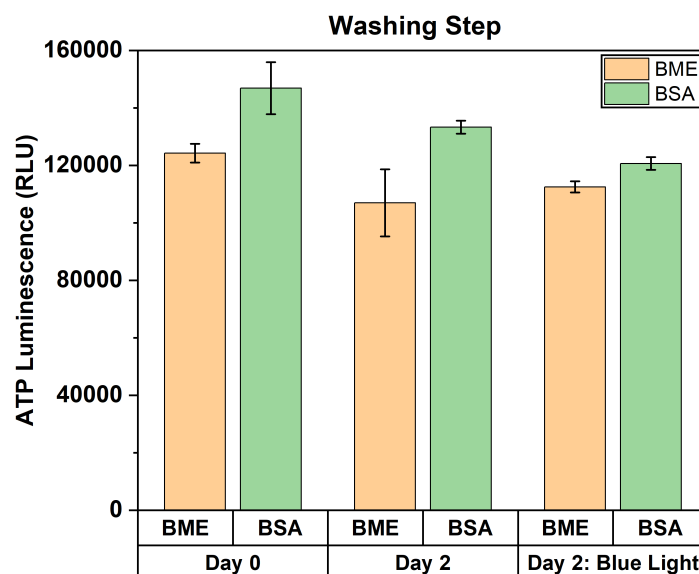


Figure 5.4: The viability of PDAC cultures is tested with an ATP assay for control Cultrex® Basement Membrane Extract hydrogel (BME, orange) and for BSA hydrogels (green). BSA hydrogels were washed prior to seeding on day 0. ATP assays were performed on day 0 and day 2 after the PANC-1 and PSCs cells were seeded onto the hydrogels. Additional BME and BSA hydrogels were prepared where the PDAC cultures were exposed to a blue LED light for 5 mins to mimic the photo-activation of folded protein hydrogels.

5.4 demonstrates that washing out the uncross-linked material is vital for using this cross-linking method of protein hydrogels for biomedical applications. This preliminary study into the viability was only repeated once for both the PDAC cultures on BSA hydrogels with and without the washing step. Further work is therefore required to repeat the viability tests, in addition to testing the cytotoxicity of the BSA hydrogel with a live/dead stain of the PDAC cultures.

5.1.3 Optical Microscopy of PDAC Cells

Bright field microscopic images were taken over a 48 hr period to monitor the PDAC cultures from the BME and BSA:Washed hydrogels from Section 5.1.2. The BSA:Washed hydrogel was washed before the cells were seeded, to remove the pre-gel solution components that were not cross-linked in the protein network. Figure 5.5 shows the images for day 0, 1 and 2 of the PDAC cultures from the control BME hydrogel and BSA:Washed

hydrogels, in a well plate. On day 0, the images shown in the top row of Figure 5.5 of the PDAC cultures on the BME and BSA hydrogels look similar in terms of the homogeneous spread of cells across the well. The PDAC cultures seeded on the BME gels had a small number of denser clusters of PDAC cultures in some of the wells, whereas this was not observed in PDAC cultures from the BSA hydrogels. On day 1, the images in the middle row of Figure 5.5 show that the arrangement of the PDAC cultures dramatic changes in comparison to day 0, for both the BME and the BSA hydrogels. For the BME hydrogels, the PDAC cultures form a small number of structures that their opacity in the microscopic images suggests the cells have structured densely packed clusters. The PDAC cultures on the BSA hydrogels form a much sparser network of many smaller, less dense regions of cells, than on the BME hydrogel. Comparing the changes from day 1 to day 2 (bottom row of Figure 5.5), a smaller change to the density of structures formed by the PDAC cultures was observed than in the first 24 hours of cell culture. The clusters of cells on the BME hydrogels have become significantly tighter and denser by day 2. In the body, PDAC is characterised as a pancreatic cancer that form a stroma, which is a dense area of both cellular and non-cellular components that makes it difficult to target the PDAC cancer cells for treatment [331]. The dense structures observed in cells seeded onto the BME hydrogels are expected of PDAC cultures [326]. Between days 1 and 2, the PDAC cultures on the BSA hydrogels shows a small increase in the density and opacity of some of the cell clusters, and is predicted to continue to become more dense with more days of culturing.

The differences arrangement and structures of the PDAC cultures on the BME and BSA hydrogels observed on day 2 of culture in Figure 5.5 could be due to both physical and chemical differences between the BME and BSA hydrogels. The main components of BME are proteins to mimic the basement membrane, such as laminin, collagen IV, entactin. In the body, these proteins are present in the extracellular matrix (ECM) where they form a supportive structure in the ECM where epithelial and endothelial cells grow [332, 333]. The BSA hydrogel is only composed of one type of protein, serum albumin used in the BSA hydrogels has a function in the body of carrying steroids, fatty acids and thyroid hormones in the blood [281] BME is formed of many fibrous proteins,

whereas BSA is a globular protein. This will result in different structures of the network formed, as fibrous proteins will form a more fibrous network whereas BSA proteins form a connected clustered network (see Section 3.18), which could also affect the migration and morphology of the PDAC cultures. The difference in the PDAC cultures observed could also be due to the differing mechanical properties of the hydrogels used. BME hydrogels have a G' of 18.98 ± 0.94 Pa on day 0 and 17.65 ± 0.88 day 3 [326]. The BSA hydrogels are roughly a 100x stiffer with the G' 1990 ± 110 Pa (see Section 3.3). The stiffness of the pancreas has been determined to be 1.20 ± 0.16 kPa [334], which mechanically is more similar to the stiffness of the BSA hydrogels than the BME hydrogels. The cell to cell communication in more porous, fibrous hydrogels is likely to be higher than in stiffer, globular protein hydrogels. A higher cell-cell communication in a more porous hydrogel will allow for more aggregation [97], and denser structures to form, as observed in the BME hydrogels.

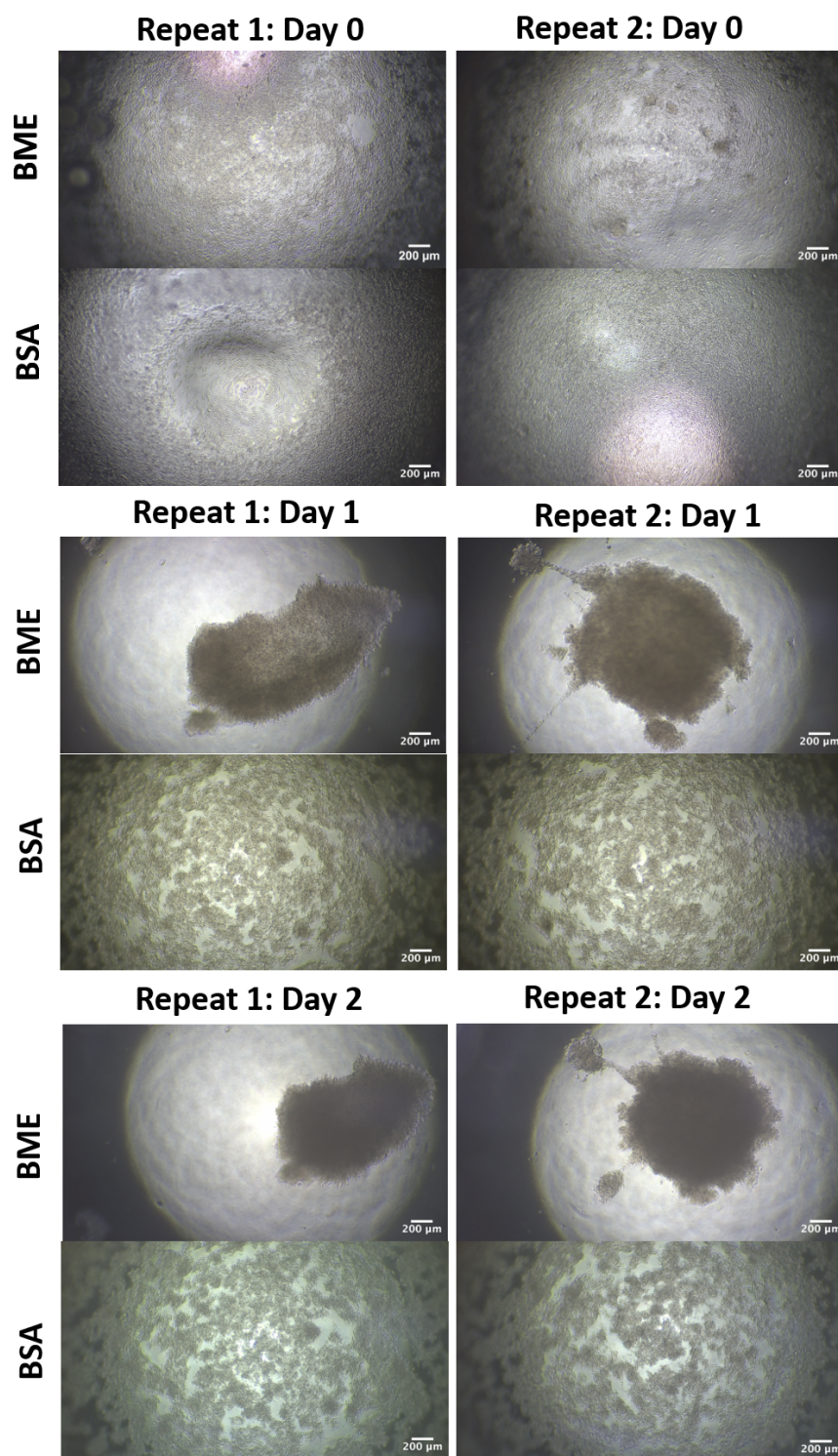


Figure 5.5: Optical microscoscopic images of PDAC cells, compared on day 0 (top), day 1 (middle) and day 2 (bottom) for cells seeded on control BME hydrogels (left) and BSA hydrogels (right). Scale bars: 200 μm .

The stiffness and porosity of the environment that the cells are seeded onto for culturing has been shown to affect the cell behaviour [199, 335]. Cells are more round in shape and spread more on stiffer substrates in comparison to softer substrates. Cell viability and growth is also affected by the substrate stiffness. The metabolic activity of PANC-1 cells on substrates with different stiffness's was compared by Raza et al. [336]. This study compared PANC-1 cells cultured onto PEG-tetra-norbornene (PEG-TNB) hydrogels with two different molecular weights of in a 3 kPa and 6 kPa PEG-TNB hydrogel. The stiffer hydrogels resulted in culturing cells with a higher metabolic activity and different morphology, with more irregular and smaller clusters of cells. This result from Raza et al. was similar to the observations of the PDAC cells in this study also showed that culturing cells on the stiffer BSA hydrogel resulted in smaller clusters of PDAC cells. Lachowski et al [337], show that PSC cells when seeded onto a softer poly-acrylic acid (PAA) hydrogels (1 kPa) are less active, entering a quiescent state, while PSC cells seeded onto stiffer PAA hydrogels (25 kPa) remained active. Both studies linked the stiffness of the matrix to the protein expression from the cells, where both showed vimentin expression was much lower for the softer tissues. A stiffer hydrogel often correlates with in a less porous hydrogel, which could inhibit cell activity. These studies highlight the importance of the matrix stiffness chosen to culture PSC and PANC-1 cells, cell lines used for the PDAC cultures in this study.

An advantage of using a folded protein hydrogel, such as BSA, over the BME hydrogels, is the level of control over the nanoscale, mesoscale and macroscale properties in the BSA hydrogels with the preparation method. The preparation of the BSA hydrogel can be changed to control the structure of the protein networks and change the stiffness of the bulk BSA hydrogel. Further cytotoxicity tests are required to understand how the BSA hydrogel affects cell death, as only cell viability tests have been conducted so far. Monitoring the development of the PDAC cultures on the BSA hydrogels for a longer period of time would give insight if the PDAC cultures form more dense regions, as was observed for the PDAC cultures on the BME hydrogels. This would then determine the suitability of folded protein hydrogels for applications in tissue engineering, by using the protein hydrogel as a scaffold for cell culture, including PDAC cells. However, the

methods of increasing the porosity of the hydrogels can result in lowering the stiffness [199]. Bursting MBs in protein hydrogels for cell culture can also be used as a method to create pores without drastically changing the mechanical properties of protein hydrogels, with the potential for increasing the access of nutrients to cells [199].

5.2 Microgel Production

Microfluidics was investigated as a method to produce droplets containing the BSA pre-gel solution. After the production of the droplets, the droplets were collected and exposed to the blue LED light. This light exposure causes the photo-initiated cross-link formation to begin and therefore results in cross-linked BSA microgels. This section outlines the development of BSA microgel production with different microfluidic devices. Figure 5.6 shows a schematic for the general set-up of the BSA droplet production, where the oil and the BSA pre-gel solution was introduced to the device via syringes and syringe-pumps.

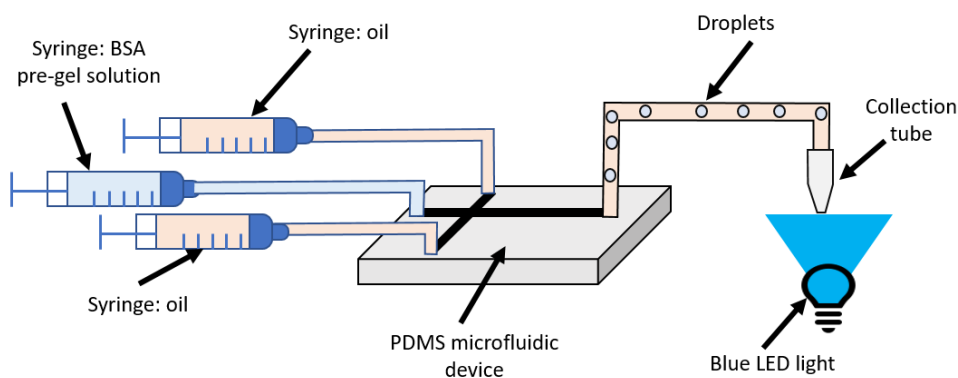


Figure 5.6: Schematic of the microfluidic design for producing BSA microgels, where the direction of fluid flow is left to right. Syringe pumps control the flow rate of the oil and pre-gel solution out of the syringes, and into the microfluidic device. The fluid is collected after exiting the microfluidic device. Not to scale.

5.2.1 Microfluidic Device Design 1 for Droplet Formation

The first device design investigated for droplet production is shown in Figure 5.7. This design involved 3 inlet channels, where 2 continuous phase channels contained grapeseed oil and 1 dispersed channel contained the pre-gel solution. The 3 inlet channels meet at a 50 μm nozzle where the pinch off of the BSA pre-gel solution occurs. The droplets then flow into the outlet channel which had a 580 μm wide expansion. Droplets containing the

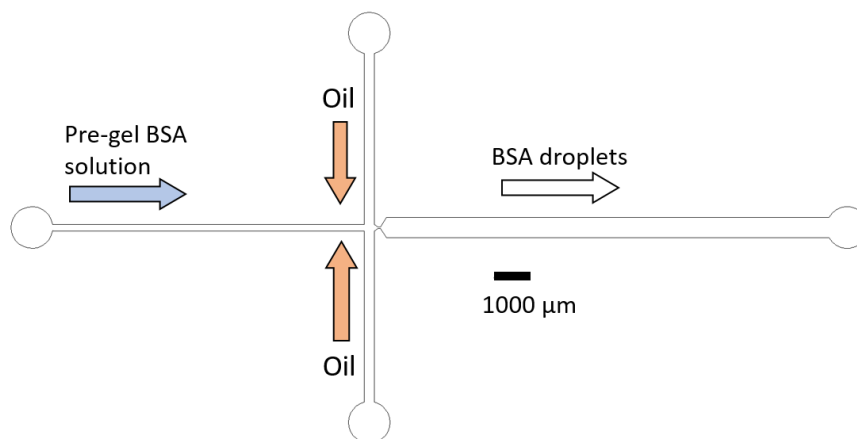


Figure 5.7: AutoCAD design 1 of a microfluidic device for producing BSA microgels. The nozzle width is 50 μm and the expansion in the outlet channel is 580 μm . The channel height was 120 μm . Scale bar: 1000 μm .

BSA pre-gel solution was successfully produced, as shown in Figure 5.8A. The droplets pinch off from the source of the BSA pre-gel solution at the nozzle at flow rates of 0.1 $\mu\text{L min}^{-1}$ for pre-BSA gel channel and 1 $\mu\text{L min}^{-1}$ for the oil channels. Figure 5.8A shows that the droplets do not coalesce in the outlet channel, where the average size of the BSA pre-gel droplets in the microfluidic device was $96 \pm 2 \mu\text{m}$. Figure 5.8B shows the droplets that have cross-linked due to photo-activation. The microgels appear to have coalesced and stuck together before gelation was complete, forming one large aggregate of gel, due to coalescence occurring after formation. Coalescence occurred in the outlet tubing and collection tube, as there was no coalescence observed on chip. An additional difficulty with the droplet formation occasionally occurred, where the BSA pre-gel solution contained large BSA aggregates and caused a build up of material in the nozzle. This materials was difficult to remove, and led to replacing the device with

a new, clean device to continue droplet production.

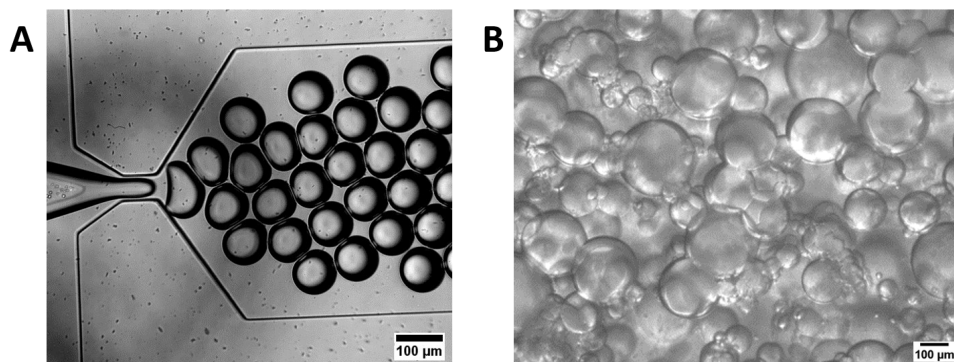


Figure 5.8: A) Optical microscopy image with a high speed camera of the microfluidic droplet formation with design 1, where high shear forces of oil cause the flow of BSA pre-gel solution to be pinched off into droplets. Flow rates were $0.1 \mu\text{L min}^{-1}$ for pre-BSA gel channel and $1 \mu\text{L min}^{-1}$ for the oil channels Scale bar: $1000 \mu\text{m}$. B) Collected BSA pre-gel droplets after the photo-activation of cross-link formation to create microgels. Scale bar: $50 \mu\text{m}$.

5.2.2 Microfluidic Device Design 2 for Droplet Formation

The second design aimed to prevent the BSA pre-gel droplets coalescing before cross-linking. The expansion of the outlet channel after the nozzle was reduced from $580 \mu\text{m}$ to $90 \mu\text{m}$ in order to increase the space between the droplets produced, where the narrowing of channels with laminar flow results in higher flow velocities. The outlet channel reduction aimed to reduce on chip droplet coalescence. To reduce any aggregates building up in the nozzle, BSA pre-gel solution, a filter was introduced after each inlet to trap any large particles before they reach the nozzle. This was implemented to increase the lifetime of the microfluidic device, before it becomes blocked and difficult to use for droplet production. Figure 5.9 shows the schematic of the modified design with a smaller post-nozzle expansion of $90 \mu\text{m}$. The two inlet oil channels were replaced with one oil channel, that splits off into two channels, reducing the number of syringes and syringe pumps required to produce the droplets.

Figure 5.10A shows that the reduction to a $90 \mu\text{m}$ expansion successfully increased the spacing between the BSA pre-gel droplets, therefore preventing them from coalescing

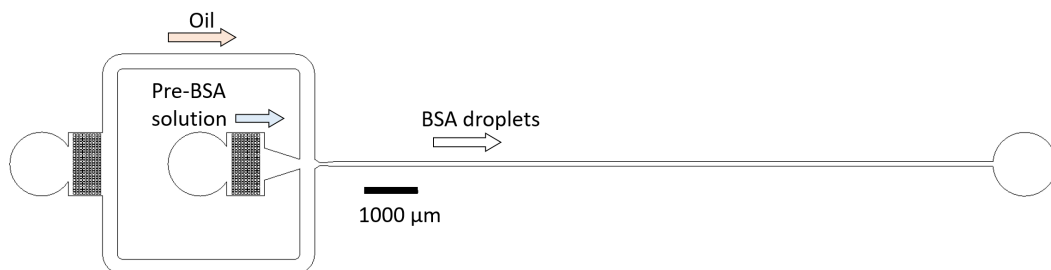


Figure 5.9: AutoCAD microfluidic design 2 for producing BSA microgels. The nozzle width is $50\ \mu\text{m}$, the expansion in the outlet channel is $90\ \mu\text{m}$ and the channel depth was $120\ \mu\text{m}$. A filter after each inlet was introduced to trap large particles. Scale bar: $1000\ \mu\text{m}$.

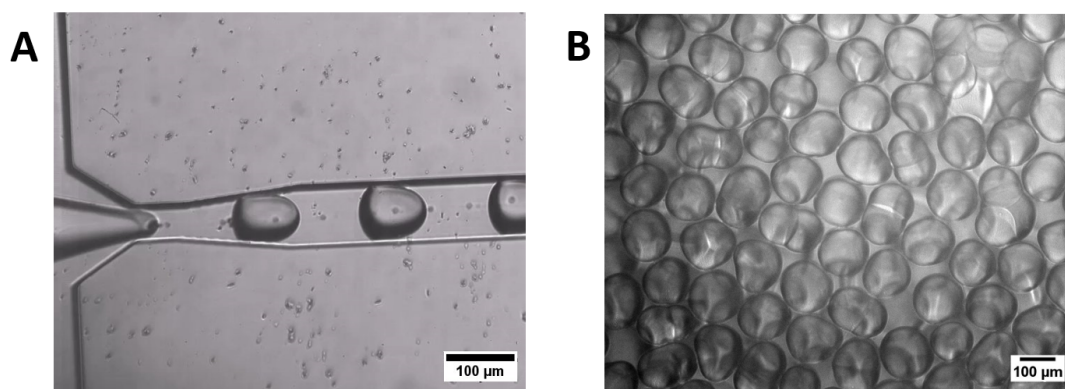


Figure 5.10: A) Optical microscopy image with a high speed camera of the microfluidic droplet formation with design 2, with a $90\ \mu\text{m}$ expansion after the nozzle. Flow rates of $0.1\ \mu\text{L min}^{-1}$ for pre-BSA gel channel and $2\ \mu\text{L min}^{-1}$ for the oil channel. Scale bar: $1000\ \mu\text{m}$. B) Collected BSA droplets, after the photo-activation of cross-link formation to create microgels. Scale bar: $50\ \mu\text{m}$.

on chip. Flow rates of $0.1\ \mu\text{L min}^{-1}$ for pre-BSA gel channel and $2\ \mu\text{L min}^{-1}$ for the oil channels. Figure 5.10B shows the BSA pre-gel droplets after they were collected and exposed the blue LED light to initiate cross-link formation. The collected microgels are more regular in size than the microgels in 5.8B, and formed individual microgels instead of one large aggregate. Some of the microgels that were formed were spherical and $\sim 100\ \mu\text{m}$, while some of the microgels were larger in size $> 150\ \mu\text{m}$ with non-spherical shapes, suggesting that some of pre-gel solution droplets have coalesced before gelation is complete. This demonstrates that BSA microgels can be produced with microfluidics, with the potential to improve the device design, droplet collection method and gelation method. The progression of this work was halted by the COVID19 pandemic and closure

of facilities.

5.2.3 Future Design Modifications for Microgel Productions

This section proposes a potential design modification for the microfluidic device designs and the set up of the gelation, that could prevent the droplets from coalescing before gelation is completed. To further prevent coalescence, the blue LED light could be placed above the microfluidic device to expose the outlet channel to blue light. The cross-linking of the droplets would therefore be initiated before the droplets leave the microfluidic device. The microgels in the outlet channel of microfluidic device design 2 in Figure 5.10 are not touching, suggesting that once cross-linked the microgels will not coalesce. Figure 5.11 demonstrates this potential set-up.

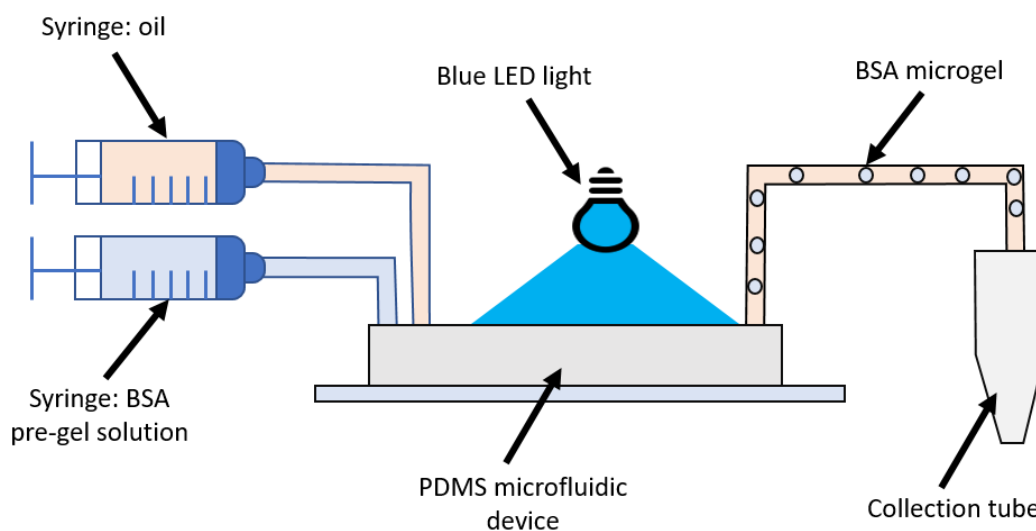


Figure 5.11: Schematic of a possible set up for gelation BSA droplets on chip. Syringe pumps control the flow rate of the oil and BSA pre-gel solution out of the syringes, and into the microfluidic device. The blue LED lamp photo-activates the cross-linking reaction of BSA in the pre-gel solution, while the droplets are still on chip. The fluid is collected after exiting the microfluidic device. Not to scale.

To increase the time that the droplets are on chip for, the length of the outlet channel can be increased. A longer, serpentine shaped channel allows for a longer channel in a small area such that the blue LED light can be staged above the microfluidic device,

illuminating the device and photo-initiating the droplets. Figure 5.12 shows a third device design that includes a longer outlet channel than designs 1 and 2 (Figures 5.7 and 5.9), in a serpentine shape. A balance between long outlet channel for droplet gelation on chip and the increased hydraulic resistance will have to be found. Once microgels can be produced without any prior droplet coalescence, changing the size of the microgels could be trialled. A further modification to allow for the production of smaller droplets could be achieved to reduce the size of the nozzle. The nozzle width used in the previous designs was $50\text{ }\mu\text{m}$, resulting in the production of droplets of $90\text{--}100\text{ }\mu\text{m}$. Reducing the nozzle size could explore how the nozzle size in the microfluidic device can be used to control the resultant microgel size.

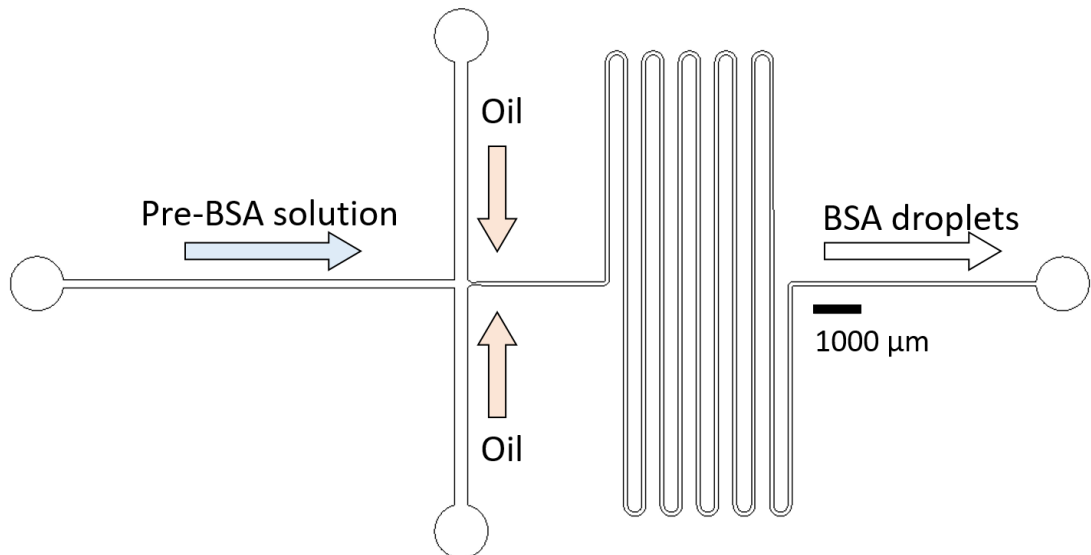


Figure 5.12: AutoCAD design showing design 3, which contains a long outlet channel in a serpentine shape. Scale bar: $1000\text{ }\mu\text{m}$.

5.3 Microscopy of Microbubbles in Buffer and BSA hydrogel

This section demonstrates that microscopy can be used to show the presence of MBs in BSA:MB hydrogels. MBs stored in buffer solution can be characterised with reflection

bright-field microscopy to determine the sample concentration and size of MB population. An example of an optical microscopic image of MBs in buffer solution is shown in Figure 5.13A, where image shows the sample of MBs is diluted 100x for image analysis. Analysis of the size distribution from 15 images of the same sample is shown in Figure 5.13B, showing the sample concentration of $1.69 \pm 0.2 \times 10^{10}$ MB/mL and average diameter of $1.09 \pm 0.24 \mu\text{m}$. This result demonstrates the successful removal of majority of MBs greater than $3 \mu\text{m}$. The concentration of the MB samples produced, with the preparation method used as described in Section 2.2.2, typically produces MBs with a concentration of $0.9 - 2 \times 10^{10}$ MB/mL and an average MB diameter between $1 - 2 \mu\text{m}$.

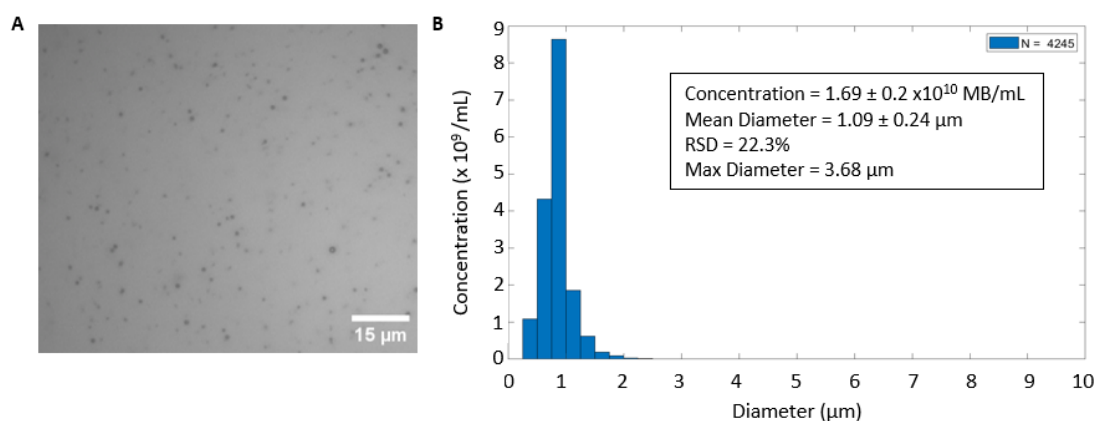


Figure 5.13: A) Example of optical microscope image of MB sample in buffer, diluted 100x for imaging. Scale bar: $15 \mu\text{m}$ B) Size distribution for the sample of MBs, where the relative standard deviation (RSD) and statistics of the sample of MBs was calculated from the analysis of 15 images. Histogram bin width was $0.25 \mu\text{m}$. The total number of MBs counted (N) was 4245.

To demonstrate that MBs were present in the BSA hydrogel, fluorescent lipids (Atto-647n-DOPE) were included in the lipid mix for preparing the MBs. This allowed the MBs to be imaged in both bright field and fluorescence confocal microscopy. Figure 5.14A shows a bright field image of MBs embedded in the BSA hydrogel, where the MBs were diluted to a final concentration of 1×10^9 MB/mL in the pre-gel solution. Figure 5.14B shows a fluorescent image of the same area of the BSA:MB hydrogel with confocal microscopy. Both these images demonstrate that the MBs were embedded in the BSA:MB hydrogel.

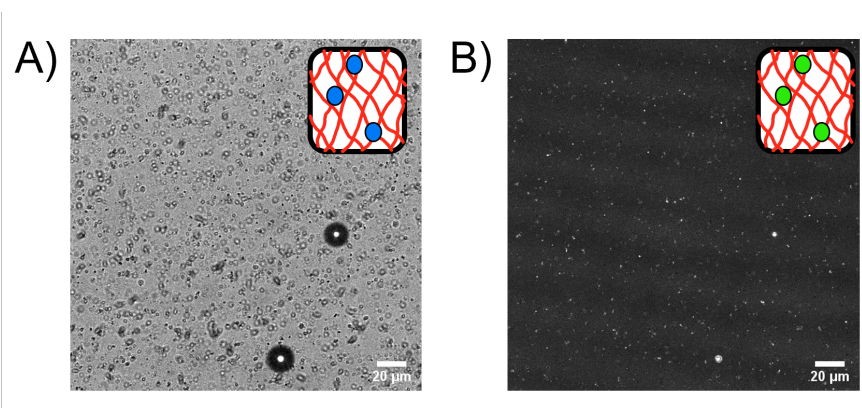


Figure 5.14: A) MBs and B) fluorescent-labelled MBs encapsulated in a BSA hydrogel. Scale bar: 20 μm .

5.3.1 Stability of Microbubbles in Protein Hydrogels

The lifetime of the MBs embedded in the BSA:MB hydrogels was investigated to understand whether the MB stability was affected by embedding the MBs in protein hydrogels. This will give an insight into how long the BSA:MB hydrogel can be stored for prior to use for drug delivery. The stability of MBs was compared for both MBs in solution and the BSA:MB hydrogel stored at 4°C, conditions chosen to mimic the MBs or BSA:MB hydrogel being prepared and stored in a standard fridge before use. Optical microscopy was used to compare the MB stability in buffer solution to MBs in the BSA:MB hydrogel over a 48 hr period. For the MBs stored in buffer solution, the method of taking images and the image analysis for determining the size and concentration of the MB was the same as in Section 5.3. Examples of optical images over the 48 hrs period with examples are shown in Figure 5.15.

Figure 5.16A shows the change in concentration of MBs in solution over time, where 15 images were analysed per time point for three different MB samples. The average concentration of MBs stored in buffer solution at 0 hours was $2.6 \pm 0.2 \times 10^{10}$ MB/mL, and after 48 hours the concentration reduced to $2.9 \pm 1.1 \times 10^9$ MB/mL. The MBs had an 89% reduction in MB population in buffer solution after 48 hrs. The decrease in MBs concentration over 48 hrs can be fitted with an exponential equation to determine the rate of decay:

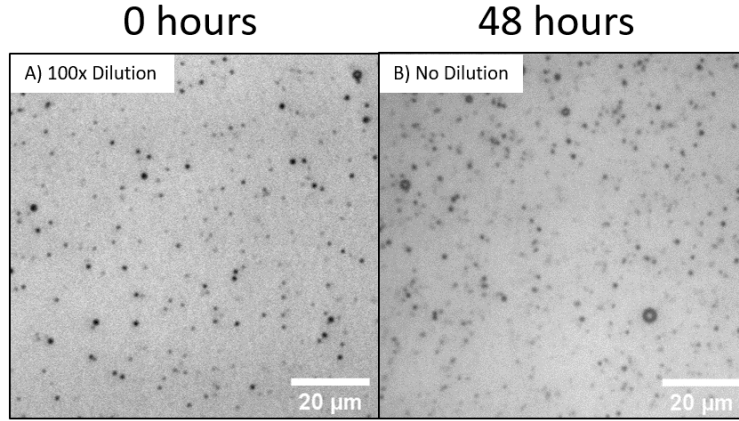


Figure 5.15: Examples of optical microscopic images of MBs in buffer solution A) immediately after preparation at (0 hr), where the MBs are diluted 100x for optimal image analysis and B) 48 hrs after preparation, where the MBs were not diluted for optimal image analysis. Scale bar: 20 μm .

$$C = C_0 \cdot \exp\left(\frac{-t}{\tau_m}\right) \quad (5.1)$$

where C is the concentration of MBs, C_0 is the initial concentration of MBs at time = 0 hrs, and τ_m is the exponential time constant for the concentration of MBs. For the MBs stored in buffer, $\tau_m = 24 \pm 2$ s. The fits are extrapolated in Figure 5.19A to estimate the change in concentration over a 5 day period based off the decay rate for 0 - 48 hrs. The estimated concentration after 5 days for the MBs stored in buffer was 3.95×10^8 MB/mL (97% reduction). Figure 5.16B shows the size of the MBs increases over time, from 1.34 ± 0.03 μm at 0 hrs after production, to 1.51 ± 0.12 μm 48 hrs after production. The increase in size after 48 hrs is due to a more polydisperse sample of microbubbles forming over time, a result that occurs from the coalescence of MBs, where MBs merging together to produce a large MB [221].

For the MBs in the BSA:MB hydrogel, the same reflection microscopy technique and analysis method used for taking quantitative data of MBs in solution was trialed. The resultant microscopy images, showed in Figure 5.13A, have a background of unfocused MBs in different z-heights of the BSA hydrogel and the contrast of the MBs was poorer. The background of unfocused MBs in the BSA:MB hydrogels was not present in the

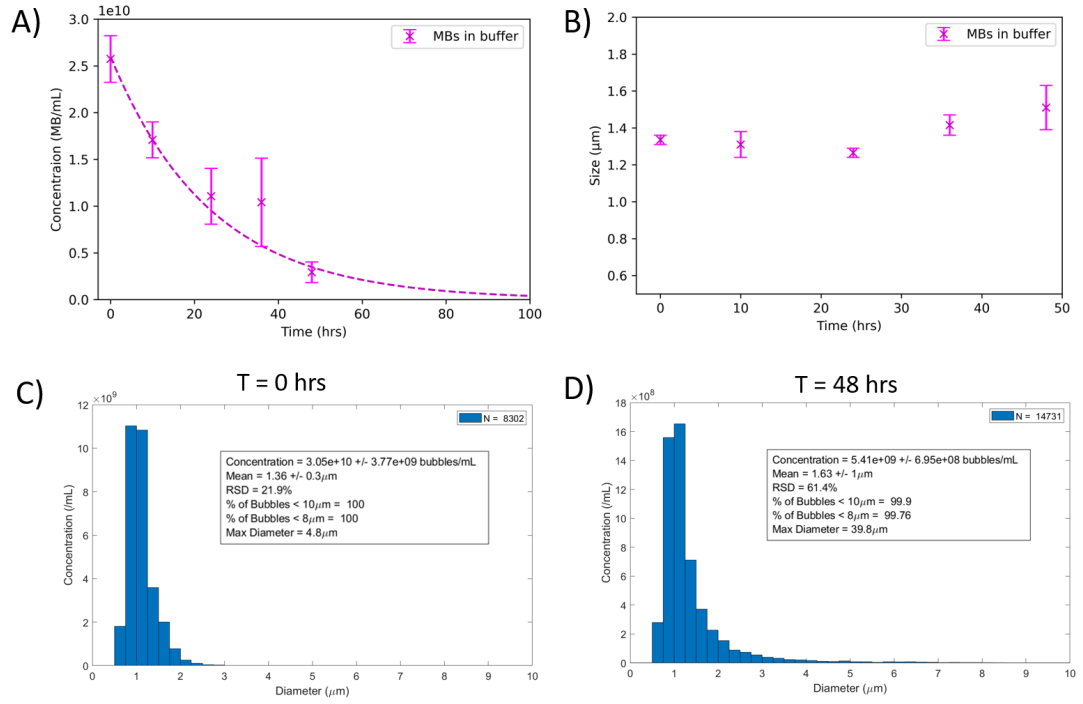


Figure 5.16: A) The concentration of MBs over a 48 hour period for when stored in buffer solution. Dashed lines represent exponential decay fits of the data using Equation 5.1. B) Average size of MBs over 48 hrs. The error bars represent standard error of the repeats.

sample of MBs stored in buffer solution in Figure 5.13A, as the MBs are left to rise to the top of the sample chamber. The MBs in solution were therefore all in the same plane before the images are acquired, and were focused in the optical images. To determine the concentration of the MBs in the BSA:MB hydrogels, the images must first be binarised. This creates an image composed of only black or white pixels, allowing a circle detector algorithm to locate any objects in the binarised image using MosaicSuite [232]. The circle detector algorithm determines information on the number of objects and size of the individual objects. In the BSA:MB hydrogel images, there is a large amount of background to remove due to the unfocused MBs sat in different z-planes in the BSA:MB hydrogel. Furthermore, the contrast of MBs is poor. If the background of unfocused MBs on an image is still present after binarising, the background can be detected and will be treated as an object in the foreground, leading to poor resolution between neighbouring MBs. Figure 5.17B shows that in the binarised image unfocused and unresolved MBs

remain. Figure 5.17C highlights what the circle detector algorithm detects, where the objects have a blue perimeter. This demonstrates how the results of the application of a circle detector algorithm incorrectly determine the MBs with an image of poor contrast, and insufficient background removal. By reducing the threshold for binarising the images, this reduces the number of focused MBs detected and makes the MBs appear smaller, therefore a different method of imaging is preferable.

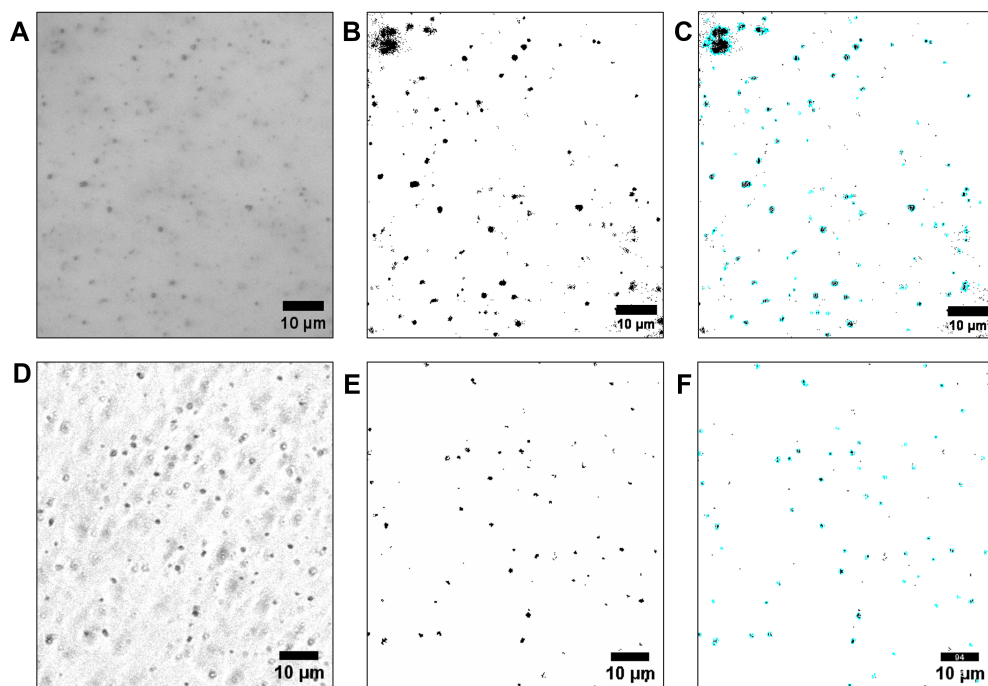


Figure 5.17: Examples of BSA:MB hydrogel images and the analysis steps. A) Reflected bright field optical microscopic image. B) Binarised image of the reflected bright field optical imaged and C) the circle detector algorithm applied, where the detected objects are outlined in blue. D) Confocal optical image, where E) shows the binarised image and F) the circle detector algorithm has been applied. Scale bars are 10 μm .

To determine the stability of MBs embedded in the BSA:MB hydrogel, an alternative method with a confocal microscope was trialled to improve the contrast between the focused and unfocused MBs for more accurate quantitative analysis. Figure 5.17D shows an example of a bright field image, with a laser light source taken with the confocal microscope. The background of unfocused MBs is very different to Figure 5.17A, with an improvement on focusing on MB that are in the plane of view. When taking bright field images using a confocal microscope, differences occur in how the image is created. Firstly, the white light photo detector is in transmission, so the light that passes

through the sample is collected. Secondly, the light source is laser, which reduces the light exposure to a smaller range of planes than with a white light source as in reflection bright-field microscopy. In addition, the confocal microscope allows for the use of higher magnification with a 50x objective and higher resolution images than the nikon 90i used in Figure 5.17A. Combining these features on the confocal microscope for imaging MBs in the BSA:MB hydrogels in Figure 5.17D, showed more contrast between the focused and unfocused MBs. Background removal via binarising the optical image in Figure 5.17E gave an image with less background than in Figure 5.17B. Bright field confocal microscopy was therefore selected as the preferable method for taking quantitative information of the MBs embedded in BSA:MB hydrogels. Figure 5.18A shows examples of the bright-field images with a confocal microscope taken at 0 and 48 hrs, and the removal of the background in the binarised images shown in Figure 5.18B. These images show that the background of unfocused MBs was successfully removed. The size of the MBs after 48 hours in the BSA:MG hydrogel does not show large increases in MBs, unlike the MBs in the buffer solution after 48 hours.

The average concentration of MBs stored in the BSA:MB hydrogel over 48 hrs is shown in Figure 5.19. At 0 hours was $1.22 \pm 0.04 \times 10^{10}$ MB/mL, and after 48 hours the concentration reduced to $7 \pm 1 \times 10^9$ MB/mL, with an 39% reduction in MB population after 48 hrs. To estimate the rate of MB decay in BSA:MB hydrogel, Equation 5.1 was again used to fit the change in concentration over a 5 day period, and this was plotted onto Figure 5.19 (dashed line), predicting the concentration for MBs in the BSA:MB hydrogel was 4.35×10^9 MB/mL (34% reduction).

The MBs stored in buffer solution showed a larger reduction in the concentration than the MBs stored in the BSA:MB hydrogel. Comparing the exponential time constants shows that the concentration decreases at a quicker rate for MBs stored in solution than MBs stored in the BSA hydrogel. This shows that the storing the MBs in the BSA hydrogel increase the lifetime of the MBs, in comparison to MBs stored in the buffer solution. Comparing the 48 hour images in Figures 5.15 and 5.18, there is greater change in the MB size for the sample of MBs stored in the buffer solution, where more large MBs are observed in MBs stored in the buffer solution. To further understand MB stability

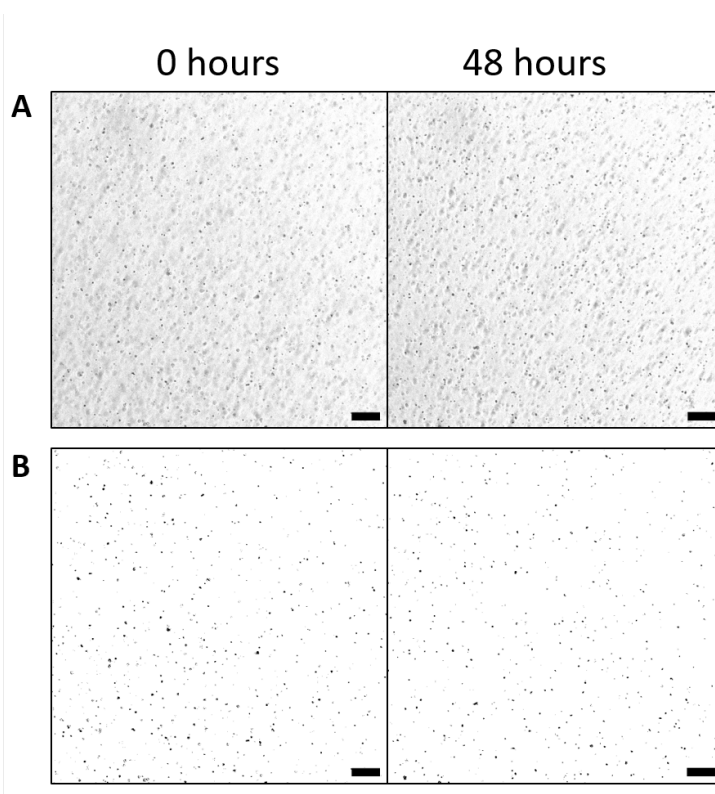


Figure 5.18: A) Examples of optical microscopy MBs in gel over a 48 hour period. B) Binarised images to remove background for analysis of MBs. Scale bar 20 μm .

over time, information on how the size of MBs changes would be of interest. If the sample becomes very polydisperse over time, the acoustic response of the MBs would change. It is therefore important to quantify if the BSA hydrogel impacts the size and concentration of the MBs. However, the resolution limit of the microscope with a 40x microscope was around 600 nm, therefore it was not possible to accurately compare the sizes the MBs in the images that were $\leq 1 \mu\text{m}$ with this technique quantitatively.

The rate of gas dissolution from the MBs is an important property, as the faster the rate of gas dissolution from the MBs, the less stable the population of MBs (see Section 1.3.4). MBs in solution are able to freely diffuse, and are buoyant such that in solution they float towards the top of the solution, where the MBs reach an air interface and the MBs are no longer surrounded by liquid, resulting in the rupturing of unstable MBs. However for MBs in the BSA:MB hydrogel the movement of MBs is limited due being trapped in the BSA hydrogel network. The increased stability of MBs in

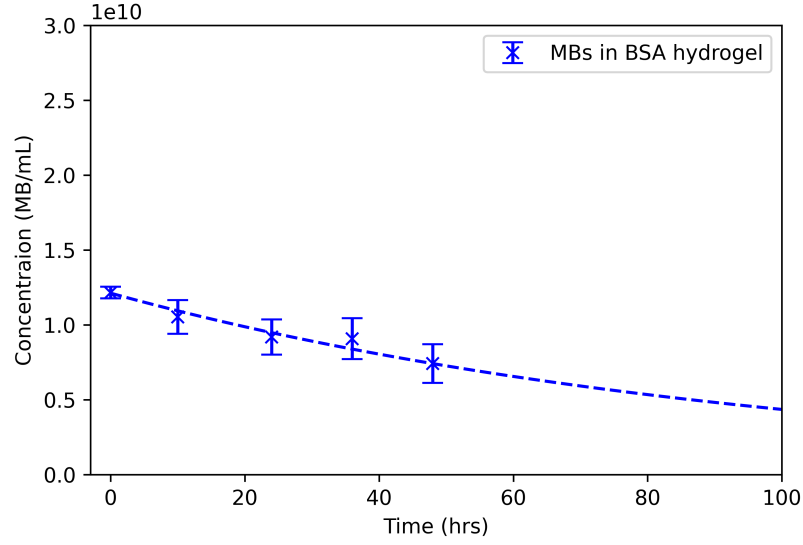


Figure 5.19: The concentration of MBs over a 48 hour period for MBs stored in the BSA hydrogel (blue). The error bars represent standard error of the repeats. Dashed lines represent exponential decay fits of the data using Equation 5.1.

the hydrogels may also be attributed to both the prevention of coalescence, again due to the restricted in position by the surrounding covalently cross-linked network. The increased change in size observed in the MBs in solution (Figure 5.15) over the 48 hrs suggest that the MBs are coalescing more in solution than in the BSA:MB hydrogel (Figure 5.18). By entrapping the MBs in the protein hydrogel, if the MBs are not be able to diffuse significant distances reducing coalescence the MBs will be unable to float, reducing the affect of these factors on the MB lifetime. This storage method could have been improved by filling the head space in the top of the centrifuge tube with heavy gas such as perfluorobutane each time the MBs were pipetted out for dilution and imaging. This has been shown to reduce the rate of gas dissolution in comparison to an air head space.

The Epstein-Plesset equation models the gas dissolution from MBs, and does not take into consideration the buoyancy or coalescence of MBs:

$$-\frac{dR}{dt} = H \frac{1 - f + \frac{2\gamma}{P_a R}}{1 + \frac{4\gamma}{3P_a R}} \left[\frac{D_w}{R} + \left(\frac{D_w}{\pi t} \right)^{1/2} \right] \quad (5.2)$$

where R is the radius of the bubble, P_a is the ambient pressure, γ is the surface tension of bubble, f is a ratio of the concentration of the gas initially dissolved in the system to the concentration of gas at saturation, D_w is the diffusivity of gas in the surrounding medium, and H is the Ostwald coefficient. The predicted stability of MBs based off the Epstein-Plesset and the initial population of MBs in solution at $t = 0$ hrs shown in Figure 5.20.

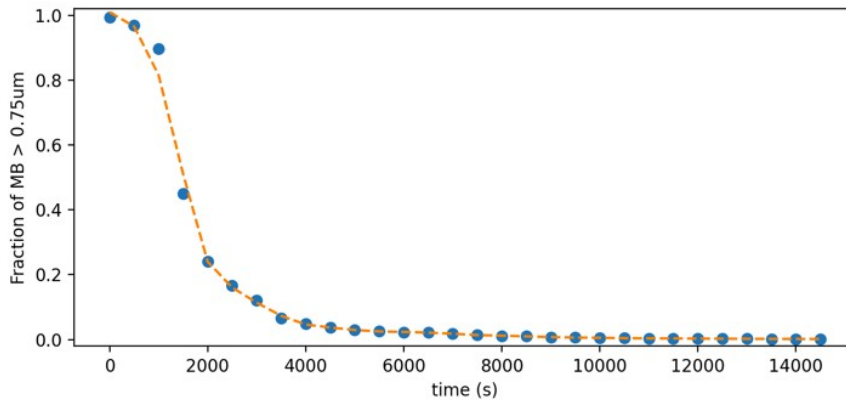


Figure 5.20: The stability of microbubbles (MBs) over time based off the population of MBs reported in Figure 5.16 using the Epstein-Plesset Model. The model was based off a perfluorobutane gas, with a saturation of 1, a shell permeability of $1.25 \times 10^{-5} \text{ ms}^{-1}$ and a surface tension of 0.025 Nm^{-1} . A threshold limit of MBs greater than $0.75 \mu\text{m}$ in diameter were only counted for, as this was the lower limit of the MB detection in the microscopy imaging. Scripting was completed by Prof. Stephen Evans.

If the Epstein-Plesset predicted an increased stability compared to the experimental data, then this would be implied that coalescence and buoyancy are significantly affecting the stability, as the Epstein-Plesset model does not account for these parameters. However, the Epstein-Plesset predicted that MB concentration decreases significantly faster than the experimentally observed MBs in solution and in the BSA:MB hydrogel, where at $t = 6000 \text{ s}$ (or 1.6 hrs) the majority of the MBs are predicted to have ruptured. The longer stability observed in experiments compared to the Epstein-Plesset can be attributed to the shell properties, where the presence of a shell slows down the rate of gas dissolution. The saturation of the gas in the BSA:MB hydrogel is unlikely to be significantly different to the buffer solution, as the hydrogel is $\sim 90\%$ water. Combining this, with the reduces ability of MBs to rise to a gas surface or coalesce, the increased stability in BSA:MB hydrogels is implied to be as a result of increased shell permeability.

5.3.2 Trajectories of Microbubbles in Liquid and in Hydrogels

The increased stability in MBs embedded in a BSA:MB hydrogel network observed in Figure 5.19 could be attributed to the prevention of coalescence and floating of the MBs in the hydrogel network. To quantify the diffusion of the MBs in the BSA:MB hydrogel, 10 trajectories of MBs in the BSA:MB hydrogel was tracked with optical microscopy, where the time step between images was 30 ms. This time step was chosen for a short exposure time to capture the MB movement while maintaining a good quality image for particle tracking analysis. This was compared to MBs in solution to understand the differences MB movement in solution and in BSA hydrogel.

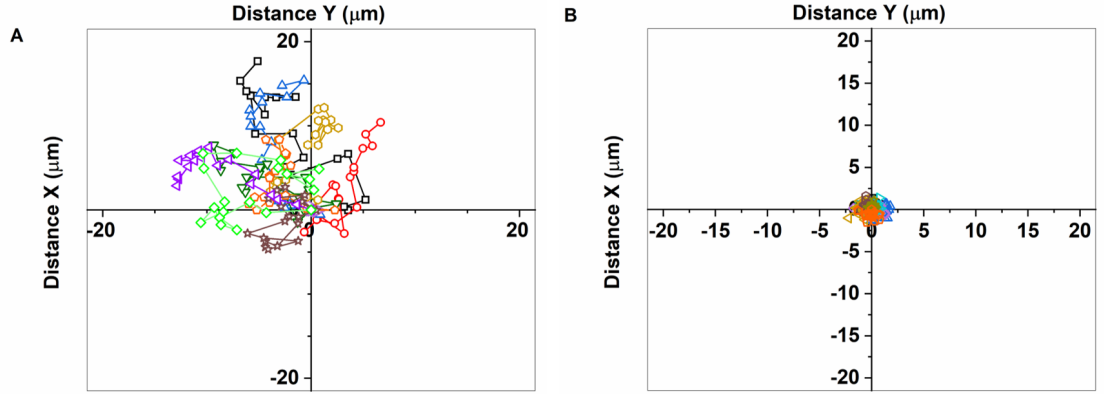


Figure 5.21: The path of 10 trajectories for A) MBs in buffer solution was compared to B) MBs in the BSA hydrogel.

Figure 5.21 shows the path of 20 images taken over 600 ms for 10 trajectories. The mean squared displacement (MSD) could then be calculated with the average displacement of the 10 trajectories. The MSD is then used to determine the diffusion coefficient (D), using the time step between images (t):

$$\langle |x - x_0|^2 \rangle = 2Dt \quad (5.3)$$

Figure 5.21A shows the the trajectories for MBs moving in buffer solution, where the MSD was determined to be $3 \pm 1 \times 10^{-11} \text{ m}^2$ in X direction and $9 \pm 3 \times 10^{-11} \text{ m}^2$ in the

Y direction. The difference in X and Y direction arises due to a drift in the velocity from surface tension of the fluid in the sample chamber. The resultant diffusion coefficients were $2 \pm 1 \times 10^{-11} \text{ m}^2\text{s}^{-1}$ and $8 \pm 3 \times 10^{-11} \text{ m}^2\text{s}^{-1}$ in the X and Y directions. Figure 5.21B shows the trajectories for MBs moving in the BSA:MB hydrogel. The MSD in the X and Y direction are $6 \pm 2 \times 10^{-13} \text{ m}^2$ and $7 \pm 2 \times 10^{-13} \text{ m}^2$, and D in the X and Y direction are $1.1 \pm 0.3 \times 10^{-13} \text{ m}^2\text{s}^{-1}$ and $1.2 \pm 0.4 \times 10^{-13} \text{ m}^2\text{s}^{-1}$. The smaller diffusion coefficient and MSD of the MBs in the BSA:MB hydrogel than in buffer solution demonstrates that the movement of MBs is significantly reduced when the MBs are embedded in the BSA hydrogel. This confirms that the MBs will coalesce significantly less when trapped in the BSA hydrogel network. The MBs will not float to an air interface due to the long timescales required for movement. The reduced movement of the MBs in the BSA hydrogel therefore is concluded to the increased stability observed in in Figure 5.19. With the use of a microscope that allows for a lower exposure time, the diffusion of the MBs in the BSA:MB hydrogel could be measured more accurately.

5.4 Destruction of MBs with Ultrasound

The ability to burst MBs within protein hydrogels is vital for drug delivery and increasing porosity for cell culture. The destruction of MBs within the BSA:MB hydrogels was tested with the application of US. For biomedical applications, the US set-up is must be considered for the safety of the patient as US pulses induce heating to the exposed area. The mechanical index (MI) quantifies the acoustic pressure and applied frequency, where a high MI of over 1.9 will result in unsafe levels of heating to tissue [338]. A MI of less than 0.7 was was trialled first to burst the MBs for clinical safety [339]. Optical images of the BSA:MB hydrogels before and after US application are shown in Figure 5.22A and B. Figure 5.22A shows MBs dispersed in the hydrogel at a concentration of $2.95 \pm 4.21 \times 10^{10} \text{ MB/mL}$. Figure 5.22B shows the BSA:MB hydrogel after the application of US, where the resultant concentration after US was $2.56 \pm 2.76 \times 10^8 \text{ MB/mL}$. The 99.1% reduction in MBs after US exposure demonstrates the successful destruction MBs upon application of US while using a clinically safe mechanical index (MI) of less than 0.7.

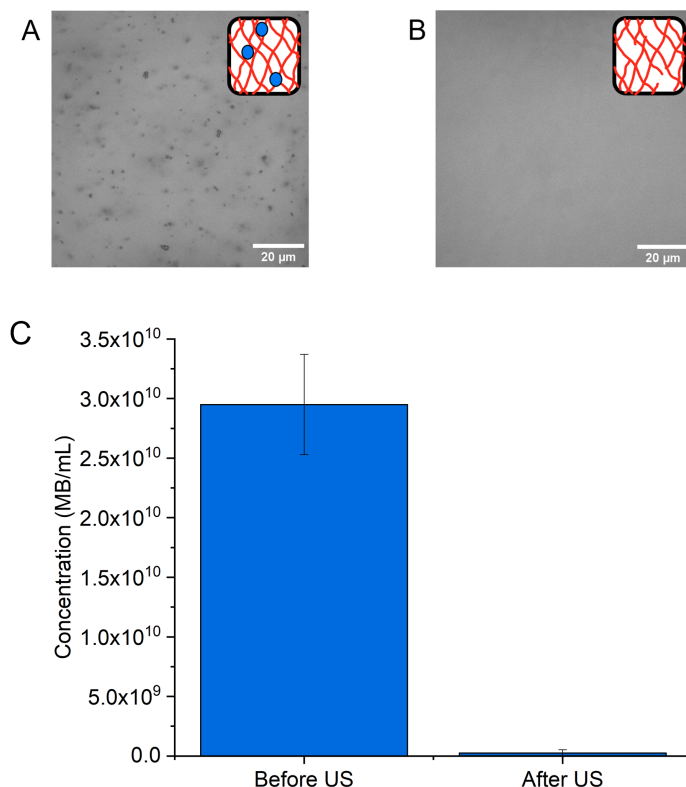


Figure 5.22: Bright field optical images of A) MBs in a BSA hydrogel before ultrasound, and B) after ultrasound application, demonstrating MBs destruction. Scale bars: 20 μm . C) Average concentration of MBs in the BSA:MB hydrogel taken from 15 images, comparing before and after the application of US.

5.5 Conclusion

In this chapter, the BSA folded protein hydrogels were investigated in a number of experiments to assess their potential in different types of biomedical applications. Studies into culturing pancreatic cancer cells onto BSA hydrogels for 2 days demonstrated that the components to initiate the cross-linking of BSA proteins caused a reduction in the viability of 47% of the PDAC cultures. By washing the BSA hydrogel before seeding the cells, the components not cross-linked in the BSA hydrogel (NaPS and $\text{Ru}(\text{bpy})_3^{2+}$) showed a reduction in the viability of 9% of the PDAC cultures, 2 days after seeding the cells. The results suggest that the blue LED light exposure required for 3D cell

culture is not influencing the viability of the PDAC cultures, but further repeats of the study is required to confirm this before progression onto 3D cell culture with the PDAC cultures. Changing the stiffness of BSA hydrogel and creating pores within the BSA hydrogel by bursting embedded MBs with US, would be interesting next steps to explore the dependency of the PDAC cultures on stiffness and porosity.

The BSA hydrogels were also explored for their potential in accessing different drug delivery routes. To do this, control over the size of the BSA hydrogel was explored with the production of BSA hydrogels via microfluidics. This allowed for droplets containing the pre-gel solution to be formed and collected, before the initiating cross-linking reaction such that the droplet becomes a microgel. Further work is required to clean the microgels of the oil that they are produced with.

Using MBs in the BSA hydrogel provides a method for triggered drug delivery, without dramatically changing the mechanical or structural properties of the protein network or the folded fraction of the BSA (see Chapter 3). The inclusion of MBs in folded protein hydrogels can allow for access of the triggered drug release with ultrasound, where drugs can be loaded in the MB shell or in liposomes attached to the MB shell. To understand how the MBs are affected by being embedded in a protein hydrogel network, the stability of the MBs were assessed over time. The rate of decay in the concentration of MBs is quicker for MBs stored in buffer solution, compared to the MBs stored in the protein hydrogel. The size of the MBs is also preserved better in the BSA:MB hydrogel, the MBs in the buffer solution were more polydisperse after the 48 hrs that the MBs were monitored for. The MB stability was improved by embedding the MBs in the BSA hydrogel, such that the coalescence of the MBs is reduced and the flotation of MBs to an air interface is prevented. The MBs in solution and the BSA hydrogel were stored at 4°C to mimic storage before use, the stability measurements need to be investigated at 37°C MBs to understand how long the MBs would be stable for *in vivo*. The MBs were successfully burst with US parameters that are deemed as safe for clinical use, with a mechanical index of less than 0.7 causing the destruction of 99.1% of the MBs.

Chapter 6

Conclusion and Future Work

The results presented in Chapters 3 - 5 of this thesis have investigated folded protein hydrogels, with a focus towards using folded protein hydrogels for biomedical applications. This involved a cross-length scale study from the nanoscale properties of the protein, through to the bulk properties of the protein hydrogel on the impact of phospholipid microbubbles (MBs) embedded in the protein hydrogel network. Furthermore, this work, in combination with previous studies on folded protein hydrogels, has allowed for linking the behaviour across the length scales and predictable behaviours.

6.1 Conclusion

To understand if the cross-linking method used to form the BSA hydrogel network was suitable for biomedical applications, cell viability studies were conducted. The cell viability of PDAC cell cultures seeded onto BSA hydrogels and compared to a control Cultrex[®] Basement Membrane Extract (BME) hydrogel. Although the BSA protein itself was expected to not negatively affect the viability of the cells, the BSA hydrogels contain tris-(bipyridine)ruthenium(ii) ($\text{Ru}(\text{bpy})_3^{2+}$) and sodium persulfate (NaPS) which therefore could be toxic to cells. The results from the cell viability found that the BSA hydrogel resulted in a large decrease in cell viability. The protocol for preparing the

BSA hydrogels for cell culture was modified to include a washing step, with the aim to remove $\text{Ru}(\text{bpy})_3^{2+}$ and NaPS from the BSA hydrogel before seeding the PDAC cells. By including the washing step, the cell viability of cells seeded onto BSA hydrogels improved significantly, implying that $\text{Ru}(\text{bpy})_3^{2+}$ and NaPS was removed. BSA hydrogels could be an interesting biomaterial for cell culture, as they provide the advantage over controlling the nanoscale, mesoscale and macroscale properties. This is not possible with commercially purchased BME hydrogels. Further work is required to study the cell viability over a longer period of time, in addition to cytotoxicity tests. To explore a route for controlling drug release from folded protein hydrogels, the inclusion of microbubbles into BSA hydrogels investigated. If this composite system is successful, it could open up potential avenues for folded protein hydrogels in different biomedical applications. MBs can be drug loaded, and the application of an ultrasound pulse causes the triggered release of drugs from the MBs into the protein hydrogels. Additionally, when MBs are burst in hydrogels, the volume that MBs encompassed no longer has a lipid shell barrier, therefore the MBs leave behind a pore upon destruction. By creating a more porous structured hydrogel, small molecules encapsulated in the hydrogels, such as drugs for therapeutic treatment or nutrients for cell culture, can have increased diffusion.

To first understand if including MBs in folded protein hydrogels would be feasible for drug release, ultrasound was used to burst MBs. The application of a clinically safe ultrasound pulse successfully resulted in a 99.1% reduction in MBs. With further considerations for clinical translation, the lifetime of the MBs would have to be long enough withstand time for storage after production, and after being administered to a patient. The lifetime of the composite BSA:MB hydrogel was studied, while storing the BSA:MB hydrogel at 4°C was investigated. The stability of MBs decreases over time due to gas dissolution through the lipid shell. Further, the buoyancy of the MBs causes the MBs to reach an air interface and the diffusion of MBs results in the coalescence of neighbouring MBs. The lifetime of the MBs in BSA:MBs hydrogels was reported, finding that the use of confocal microscopy with a transmission detector was preferable for determining the concentration of MBs in the BSA:MB hydrogels. The results showed a higher concentration of MBs in the BSA:MB hydrogel compared to the MBs stored

in buffer solution after 48 hours. This was attributed to the prevention of coalescence and buoyancy of the MBs in the BSA hydrogel, due to the MBs being trapped in the protein network. The Epstein-Plesset model predicts the rate of gas dissolution over time, where the rate of gas dissolution correlates with the MB lifetime. Using the Epstein-Plesset model to determine the lifetime of using the size and concentration data of the MBs in solution, the MBs are predicted to be less stable than the observed experimental lifetime observed MBs in the BSA hydrogel. Epstein-Plesset does not factor in buoyancy or coalescence, and under predicts the lifetime of the MBs in the BSA:MB hydrogel. This suggests that buoyancy and coalescence are important factors in the stability of MBs in solution, but have less affect on the MBs in the BSA:MB hydrogel. As the saturation of gas should not differ between MBs in buffer solution and MBs in the BSA hydrogel, this suggests that the cause for the increased stability is related to the shell of the MB. A decrease in shell permeability will reduce gas diffusion from the MB, which could be explained by components in the pre-gel solution adsorbing on to the MB shell. It is possible that the BSA or the $\text{Ru}(\text{bpy})_3^{2+}$ is adsorbing on to the shell due to the presence of the 1,2-distearoyl-sn-glycero-3- phosphoethanolamine-N-[amino (polyethylene glycol)-2000] which has a negatively charged head group, and thus increases the shell permeability. The next stage of this study would be to investigate the stability of MBs in the BSA hydrogel at body temperature, in order to understand the lifetime of the MBs post administration *in vivo*.

The fold of a protein is vital for its functionality, therefore in order to determine the affect of MBs on the fold of the protein circular dichroism (CD) was used. The CD spectra showed that the folded fraction of the secondary structure of BSA in the BSA:MB hydrogel was similar to the folded fraction in BSA hydrogels. This suggests that the biological functionality of the protein hydrogel building block is not affected by the presence of phospholipid MBs. BSA is a model globular protein used in this study, further studies could use different proteins with functions that compliment drug delivery, while maintaining functionality in the presence of MBs.

Previous studies on the so called ‘filler particles’ in a viscoelastic material have shown that the properties of filler particles, such as the volume fraction, size and stiffness, can

influence the mechanical properties of the bulk viscoelastic material [273–277]. For example, the inclusion of some filler particles led to providing mechanical support to the hydrogel network. In some cases the increase in mechanical stiffness observed was to the filler particle significantly interacted with the network of in a hydrogel. Furthermore, changes to the protein and structure of folded protein hydrogels can translate to the bulk properties [123, 136, 245–248]. Therefore, characterising the mechanical properties of the BSA hydrogel encompassed MBs (BSA:MB hydrogel) was essential.

In Chapter 3 the presence of MBs during the cross-linking of BSA resulted in changes to the linear mechanical properties of the hydrogel at high concentrations of MBs. The inclusion of 10^9 MB/mL resulted in a decrease of 300 Pa in the storage modulus (G'). This result demonstrated that MBs can be embedded within protein hydrogels without concerns about weakening the hydrogel stiffness. Previous studies where a decrease in the stiffness of the hydrogel was observed with the inclusion of a filler particle, suggested that the filler particles have mechanically insignificant interactions with the network [275–277]. Specifically for protein hydrogels, it has previously been shown that the alterations to the protein building block can have affects that translate through to the bulk mechanical properties [124, 125, 136]. In this study, CD results demonstrated that the presence of the MBs was not impacting the percentage of α -helices in the secondary structure of the BSA proteins. Classical affine network theory states the shear modulus (G) of a cross-linked network is proportional the number of junction points (μ) [318, 340]:

$$G \propto \mu k_B T \quad (6.1)$$

where k_B is the Boltzmann constant and T is the temperature. The cross-link density (ν), referring to the number of cross-links per unit volume, is proportional to μ and therefore could influence the G :

$$\mu = \frac{2\nu}{\phi} \quad (6.2)$$

where ν is the number of chains between junction points and ϕ is the junction functionality [341]. It has previously been predicted that folded protein hydrogels have a network composed of fractal like-clusters connected by chains of proteins. [119, 136, 342]. The

protein network could therefore be modelled by affine network theory, where the protein clusters are modelled as junction points. Therefore by applying Equations 6.1 and 6.2 the decrease in G' observed in the BSA:MB hydrogels could be attributed to a decrease in the number of cross-links forming. However, quantifying the dityrosine cross-linking efficiency in the BSA and BSA:MB hydrogels was inconclusive in determining whether the cross-linking was influencing the mechanical behaviour.

To further build an understanding of what factors control the mechanical properties of folded protein hydrogels, the next step involved investigating how a cross-linked, folded protein hydrogel would change if the unfolding of the cross-linked protein was triggered. Dithiothreitol (DTT) is a reducing agent that breaks the covalent disulphide bonds in some proteins. Disulphide bonds help maintain the folded structure of proteins, breaking them can result in the protein unfolding [311, 312, 314]. DTT has been shown to induce the unfolding of BSA proteins, by breaking the disulphide bonds holding the fold of the protein together [136]. This study investigated soaking cross-linked BSA and BSA:MB hydrogels in DTT (BSA:post-DTT and BSA:MB:post-DTT hydrogel), in order to induce protein unfolding of cross-linked BSA. In Chapter 4, the rheological characterisation of the mechanical properties showed that the addition of DTT resulted in a 42.4% decrease in the final storage modulus, G'_f between the BSA hydrogel and the BSA:post-DTT hydrogel, and a 42.5% decrease between the BSA:MB hydrogel and the BSA:MB:post-DTT hydrogel. In both the BSA:post-DTT and BSA:MB:post-DTT hydrogels, an increase the exponential decay component in was observed in comparison to the BSA and BSA:MB hydrogels, suggesting the rate of relaxation had increased as a result of BSA unfolding. The addition of DTT dominated over the affect the presence of the MBs had on the viscoelastic properties. As no increase in G' was observed, it implies that there is no significant increase in dityrosine bond formation with the unfolding of the cross-linked protein. This study is important when considering the biomedical applications of chemically cross-linked folded protein hydrogels. For example, if a cross-linked protein hydrogel is exposed to an environment that causes unfolding, then the stiffness of the resultant protein is expected to decrease.

SANS was used to investigate to the structure of the BSA hydrogel network under the

impact of the presence of MBs, in addition to BSA:post-DTT and BSA:MB:post-DTT hydrogels. The SANS results presented in Chapters 3 and 4 showed that the presence of MBs in the BSA hydrogels predicted in a higher correlation length, suggesting the fractal-like protein clusters were larger than the BSA hydrogels in the absence of MBs. The presence of the MBs had a greater affect on the correlation length of the protein clusters than the addition of DTT in the BSA:post-DTT and BSA:MB:post-DTT hydrogels. The changes to the size of the colloidal particle was shown by Zhang *et al.* 2019 did not cause significant changes to the mechanical properties of the bulk colloidal network [299], therefore the increase in correlation length in this study was not expected to influence the G' .

The presence of MBs also showed an increase in the volume fraction of proteins present in the inter-cluster space. These results suggests that the MBs are interacting with the proteins in a way that does not mechanically support the protein network, as an increase in the stiffness of the bulk hydrogel is not observed. The n_c was also investigated for the BSA:post-DTT and BSA:MB:post-DTT hydrogels. The n_c for the BSA:post-DTT hydrogels was lower than the BSA and BSA:MB hydrogels, with a further decrease in n_c observed in the composite BSA:MB:post-DTT hydrogel. This followed the trend reported in Chapter 3, that a decrease in G' of the bulk hydrogel with rheology correlated with a decrease in the n_c with SANS.

To further understand the origin of the decrease in stiffness for BSA:MB hydrogels, small angle neutron scattering (SANS) was used to give insight into the mesoscale structure of the protein network. In this study, the SANS results predicted that the BSA:MB hydrogels had a lower number of fractal-like clusters of proteins (n_c) in the protein network than the BSA hydrogels. The 19% reduction in n_c for BSA:MB hydrogels correlated with the 21% reduction observed in G' . The affine network model states that $G \propto$ the number of junction points. When modelling fractal-like clusters as junction points, the affine network model describes the relationship between the structure and bulk properties observed in this study:

$$G' \propto n_c \tag{6.3}$$

This begins to connect the relationship between the structural properties of the protein network to the bulk mechanical properties when including filler particles into the protein network, with further work required to explore this hypothesis.

Finally, different avenues of administration routes for folded protein hydrogels were considered, by reducing the size of the BSA hydrogel. The production of BSA microgels with microfluidics was explored to decrease the size of the BSA hydrogels to the micron scale. Droplets containing the pre-gel solution were produced on-chip, and collected for photo-initiation off-chip. The resultant microgels were produced on the order of $100\ \mu$. Improvements to the set-up could be further explored to reduce the off chip coalescence, possibly by initiating gelation of the droplets while on-chip. The results demonstrated that microgels can be produced with microfluidics. This opens routes of administration of BSA hydrogels up to include oral, as well as hydrogel patches to the skin surface and implants placed in surgery.

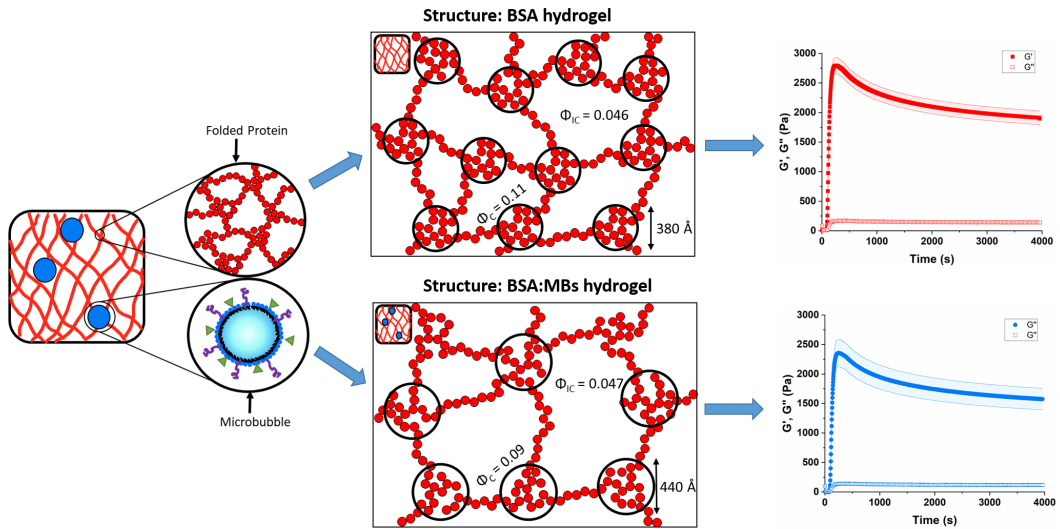


Figure 6.1: Schematic of left) microbubbles in folded protein hydrogels, middle) the predicted mesoscopic structure of BSA and BSA:MB hydrogels from small angle neutron scattering experiments and right) the gelation curves for BSA and BSA:MB hydrogels from rheological characterisation.

6.2 Future Work

Further investigations to link the nanoscale, mesoscale and bulk properties of BSA hydrogels would allow for connections to be made between the length scales, with the end goal of predictive protein hydrogel design and behaviour. The Affine network model states that the density of cross-links influences the shear modulus ($G \propto \mu$) [318], therefore it would be interesting to use an alternative method for quantifying the dityrosine cross-links in protein hydrogels, such as high-performance liquid chromatography (HPLC). This could give further understanding as to whether there are changes occurring in the efficiency of cross-linking in the presence of MBs or DTT.

Continuing the work presented on BSA:MB hydrogel on how mechanics of the BSA:MBs hydrogels post ultrasound differ due to having a more porous structure. This report showed that the structure and mechanical properties of BSA hydrogels are connected. Further studies with changing parameters in the BSA hydrogel that influence the structure and mechanical properties could build a model to predict the structure when changes in the G' are observed. The challenge arises when incorporating changes to the protein building block into a general protein hydrogel model, as this not accounted for in models such as the affine network model and work on colloidal networks from del Gado and Furst [299, 318, 343].

The directions in which that the work presented in Chapter 5 could be taken are diverse. The microgel production with microfluidics requires improvement in order to form smaller, consistently uniform microgels. This could be achieved by changing the microfluidic set-up such that the LED lamp required to initiate the photo-chemical cross-linking could be placed over the outlet channel for the microfluidic device. This would allow for the droplets containing the pre-gel solution to be exposed to the light from the LED lamp while the droplets are on chip. When observing the gelation curve with rheology, the increase in storage modulus peaks around 150 s after photo-initiation, after which network rearrangement dominates the mechanics and the storage modulus decreases. If the length of the outlet channel was increased, then so would the length of time that pre-gel solution droplets are exposed to the LED light while on chip. After

improving the production of BSA microgels to be more uniform, and washing them from the oil used in the microfluidic device, the BSA microgels could be loaded with drugs to allow for different administration routes into the body. Due to the high surface area to volume ratio of microgels, in addition to their small size, microgels are an attractive material for delivering drugs and being encapsulated by cells. Using proteins as the building block for microgels in drug delivery could allow for the combination of a protein that triggers response to help with the efficacy of treatment, for example to trigger an immune response. A study by Abdurakman et al. used alginate microgels with encapsulated MBs as an magnetic resonance imaging (MRI) contrast agent to successfully improve the accuracy of measuring pressure changes in the stomach [344]. As BSA:MB hydrogels only experience small changes in the stiffness of the hydrogel, and improve the stability of MBs, BSA:MB microgels could be an interesting tool for a dual purpose drug delivery and contrast agent system with a long MB lifetime.

The next step in using folded BSA hydrogels for drug delivery would be to monitor the diffusion and release of small molecules from the BSA hydrogel. Monitoring the diffusion of different sized molecules into the BSA hydrogel could provide information on the size of drug molecules suitable for release from folded BSA hydrogels. From previous characterisations of this system, different parameters can be changed in the preparation of BSA hydrogels that lead to different bulk or structural properties, such as presence of DTT in the pre-gel solution or lamp power [123], and in the post-gelation environment, such as soaking the cross-linked hydrogel in DTT or bursting embedded MBs. These could all lead to changes in the diffusion coefficient of small molecules in the BSA hydrogel, offering the potential to tune the diffusion. Understanding how the diffusion can be controlled by the BSA hydrogel preparation method is an exciting opportunity to utilise the BSA hydrogels for tuning release of drugs from the hydrogels. MBs offer an exciting opportunity to load hydrophilic and hydrophobic drugs into the hydrogel. The soaking of the BSA and BSA:MB hydrogels quickly induced a decrease in the stiffness. It would be interesting to explore whether triggering the unfolding of the BSA hydrogels would consequently result in a flux of drug release. Further work is required to understand if the drug release profile from MBs can be improved by

the destruction of drug-loaded MBs in BSA:MB hydrogels. The future of MBs and hydrogels could offer a dual released system with one type of drug in the MB and one type in hydrogel. Many of the considerations of hydrogel properties for drug delivery are also important for culturing cells on hydrogels and tissue engineering. By understanding how to control the diffusion of small molecules in the BSA hydrogels, this control over diffusion could be translated to the diffusion of nutrients for cells in cell culture.

The changes in the bulk mechanics and the structure of the protein network of the BSA:MB hydrogel, in addition to the increased lifetime of the MBs in the BSA:MB hydrogel suggest the MBs are experiencing an interaction with the components of the BSA hydrogel. The interactions between the shell of the MBs and the BSA, $\text{Ru}(\text{bpy})_3^{2+}$ or NaPS requires further work, in order to understand why the MBs have a longer lifetime when encompassed in the BSA:MB hydrogel. Understanding the adsorption of PEGylated and phosphatidylcholine lipids and BSA could be achieved with techniques quartz crystal microbalance with dissipation monitoring (QCMD), which characterised interactions with a surface. This could also inform if the changes to the correlation length of the protein clusters and reduction in stiffness of the protein hydrogel is related to the chemical interactions between the MB lipid shell and the BSA protein.

Appendix A

List of chemicals and experimental apparatus

This section lists the chemicals used in this thesis.

Chemicals

Abil® EM 90	Evonik, Germany
atto-647n-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (atto-647n-DOPE)	Avanti Polar Lipids, USA
Bovine serum albumin, heat shock fraction, protease free, essentially globulin free, pH 7, > 98%	Sigma Aldrich, USA
CellTiter-Glo® 3D reagent	Promega TM , USA
Chloroform	Sigma Aldrich, USA
Cultrex® basement membrane extract (BME) hydrogel	Sigma Aldrich, USA
1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)	Avanti Polar Lipids, USA

1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino (polyethylene glycol)-2000] (DSPE-PEG-2000)	Avanti Polar Lipids, USA
1,2-Dithiothretol, DTT	Formedium, UK
Dulbecco's Modified Eagle Medium (DMEM)	Thermo Fisher Scientific, USA
Deuterium oxide	Sigma Aldrich, USA
Ethanol	Thermo Fisher Scientific, USA
Fetal bovine serum (FBS)	Sigma-Aldrich, USA
GlutaMAX Supplement	Thermo Fisher Scientific, USA
Glycerol	Thermo Fisher Scientific, USA
Hydrochloric acid/HCl	Thermo Fisher Scientific, USA
Isopropanol	Thermo Fisher Scientific, USA
Methanol	Thermo Fisher Scientific, USA
Human pancreatic epithelial ductal adenocarcinoma cells (PANC-1)	European Collection of Authenticated Cell Cultures 87092802, Sigma-Aldrich, USA
Human pancreatic stellate cells (PSC)	ScienCell TM Research Laboratories, Caltag Medsystems Ltd.
Penicillin Streptomycin (PS)	Sigma-Aldrich, USA
Perfluorobutane (C ₄ F ₁₀)	F2 Chemicals, UK
Polydimethylsiloxane (PDMS)	Sylgard 184, Dow Corning USA
poly-L-lysine	ScienCell TM
Silicone oil	Thermo Fisher Scientific, USA
Sodium bicarbonate	Thermo Fisher Scientific, USA
Sodium carbonate anhydrous	Thermo Fisher Scientific, USA
Sodium hydroxide (NaOH)	Sigma-Aldrich, USA
Sodium Persulfate (Na ₂ S ₂ O ₈)	Sigma Aldrich, USA
Sodium phosphate dibasic (Na ₂ HPO ₄)	Sigma Aldrich, USA
Sodium phosphate monobasic (NaH ₂ PO ₄)	Sigma Aldrich, USA

Stellate Cell Medium (SteCM)	ScienCell TM Research Laboratories, Caltag Medsystems Ltd.
Stellate Cell Growth Supplement	ScienCell TM Research Laboratories, Caltag Medsystems Ltd.
SU-8 2075	Dow Corning, USA
Trimethylchlorosilane	Sigma Aldrich, USA
Tris(2,2'-bipyridyl)ruthenium(II) dichloride hexahydrate (C ₃₀ H ₂₄ Cl ₂ N ₆ Ru·6H ₂ O)	Sigma Aldrich, USA
TrypLE Express Enzyme (1×) no phenol red	Thermo Fisher Scientific, USA

Appendix B

Additional Formulae and Derivations

B.1 Derivation of fractal structure factor for fitting SANS data

This following derivation was previously used by Hughes et al. (2021) [136] for analysing small-angle scattering data. To derive the structure factor of a fractal system, the number of monomers (N) of size r_0 is considered.

$$N(r) = \rho_k \left(\frac{r}{r_0} \right)^{D_f} \quad (\text{B.1})$$

where r is the radius of the fractal, ρ_k is the packing fraction which for randomly packed spheres is ≈ 0.639 . Using Equation B.1, the radial number density of a fractal $\rho(r)$ can be determined.

$$\rho(r) = \frac{dN(r)}{dV} = \frac{\rho_k D_f}{4\pi r_0^{D_f}} r^{D_f-3} \quad (\text{B.2})$$

The radial number density can also be described in terms of the radial distribution function, $g(r)$.

$$\rho(r) = \phi_0 g(r) \quad (\text{B.3})$$

where ϕ is the average volume fraction of all the monomers in the system. Substituting in Equation B.1 into Equation B.1 and rearranging leads to:

$$g(r) = \frac{\rho_k D_f}{4\pi\phi r_0^{D_f}} r^{D_f-3} \quad (\text{B.4})$$

Equation B.1 considers fractal systems of infinite size, whereas here we are considering a fractal system of a finite size. In order to model the fractal systems where the with a limit to the size the fractal aggregate can reach

B.2 Derivation of the number of monomers in a finite-sized fractal

The radial number density of a fractal structure can be described in terms of $g(r)$

$$\rho(r) = \phi_0 g(r) \quad (\text{B.5})$$

considering that $\rho(r) = \frac{dN(r)}{dV}$, this can be substituted into Equation B.2 gives

$$dN(r) = \rho(r) dV \quad (\text{B.6})$$

Assuming a radial symmetric fractal object ($dV = 4\pi r^2 dr$):

$$dN(r) = \phi_0 g(r) 4\pi r^2 dr \quad (\text{B.7})$$

$g(r)$ for a fractal system with finite sized fractal aggregates can be substituted in (Equation B.1) giving the following:

$$dN(r) = \frac{\rho_k D_f}{r_0^{D_f}} r^{D_f-1} dr \quad (\text{B.8})$$

To integrate Equation B.2, integration by substitution is used, where $u = r/\xi$. Equation B.2 integrated with respect to r , from 0 the centre of the radius and R is the radius of the fractal cluster.

$$N(r) = \int_0^R \frac{\rho_k D_f}{r_0^{D_f}} r^{D_f-1} dr \quad (\text{B.9})$$

The number of monomers in the fractal cluster in a sphere therefore becomes

$$N(r) = \rho_k D_f \left(\frac{\xi}{r_0} \right)^{D_f} \gamma \left(D_f, \frac{R}{\xi} \right) \quad (\text{B.10})$$

where $\gamma \left(\frac{R}{\xi} \right)$ is the lower incomplete gamma function:

$$\gamma \left(D_f, \frac{R}{\xi} \right) = \int_0^{R/\xi} x^{D_f-1} e^{-x} \quad (\text{B.11})$$

Appendix C

Analysis of Variance Tests

This appendix includes the analysis of variance (ANOVA) tables used to determine if experimental results in Chapters 3, 5, 4 were statistically significant. The ANOVA test was used to determine that the results were statistically significant, where the critical value for the F-distribution (F) is less than F-distribution (F), at a significance level (α) of 0.05

C.1 ANOVA Tests: Chapters 3

C.1.1 Folded fraction of BSA

In Chapter 3, Section 3.2 the remaining folded fraction of BSA after gelation was compared between bovine serum albumin (BSA) hydrogels and BSA hydrogels with embedded microbubbles (BSA:MB). The remaining folded fraction of BSA after gelation of the BSA hydrogel was 81.3 +/- 0.9% and for the BSA:MB hydrogel (10^9 MB/mL), the remaining folded fraction of BSA post-gelation was 79.9 +/- 0.8 %. The ANOVA test, shown in Table C.1 was used to determine that the results were not statistically significant, as $F_C = 7.71$ was greater than $F=0.38$, for $\alpha = 0.05$:

Table C.1: ANOVA test for determining the statistical significance between the results folded fraction of BSA post-gelation for BSA and BSA:MB hydrogels from Chapter 3, Figure 3.4.

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Squares	F
Between Scenarios	3.44	2	1.72	0.38
Error	9.03	2	4.52	
Totals	12.47	5		

C.1.2 Rheology: Gelation Curve

In Chapter 3, Section 3.3 the rheological parameters of BSA and BSA:MB hydrogels. G'_{∞} from Chapter 3, Figure 3.7A, is compared for BSA and BSA:MB hydrogels, 10^7 , 10^8 , and 10^9 MB/mL. To determine which values are statistically significant to the control BSA hydrogel, the ANOVA test was performed with $\alpha = 0.5$ with the results shown in Table C.2. For the BSA:MB hydrogel with 10^7 MB/mL, $F_C=3.46$ and $F=0.91$ therefore the G'_{∞} with inclusion of 10^7 MB/mL is not statistically significant to the BSA hydrogel. For the BSA:MB hydrogel with 10^8 MB/mL, $F_C=5.56$ and $F=1.71$ therefore the change in G'_{∞} with inclusion of 10^8 MB/mL is not statistically significant to the BSA hydrogel. For the BSA:MB hydrogel with 10^9 MB/mL, $F_C=3.35$ and $F=3.61$ therefore the decrease in G'_{∞} with inclusion of 10^9 MB/mL was statistically significant to the control BSA hydrogel.

Table C.2: ANOVA test for determining the statistical significance between the results of G'_{∞} of BSA post-gelation for BSA and BSA:MB hydrogels in Chapter 3, Figure 3.5.

G'_{∞}	BSA:Control	10^7	10^8	10^9
Mean (Pa)	1990	1800	1640	1550
F		0.45	1.71	3.61
F_C		3.47	5.56	3.35

The ANOVA test was applied to the results of the relaxation constant τ in Chapter

3, Figure 3.7B, comparing the control BSA hydrogel to the BSA:MB hydrogels. The results of the ANOVA test in Table C.3 showed that τ for BSA:MBs with 10^7 , 10^8 and 10^9 MB/mL were found not to be significantly different to the control BSA hydrogel.

Table C.3: ANOVA test for determining the statistical significance between the relaxation constant (τ) of BSA post-gelation for BSA and BSA:MB hydrogels.

τ	BSA:Control	10^7	10^8	10^9
Mean (s^{-1})	1150	1130	1120	1160
F		0.11	0.21	0.50
F_C		3.47	5.56	3.35

In Chapter 3, Figure 3.8, the kinetics of gelation, k_{max} and k_{max}/G'_{∞} were compared. The ANOVA test determined that the results for k_{max} (Table C.4) comparing the control BSA hydrogel to BSA:MB hydrogels with 10^7 , 10^8 , 10^9 MB/mL showed no statistical significance. The statistical significance of k_{max}/G'_{∞} was also determined with the ANOVA test, where the results showed no statistical significance, see Table C.5.

Table C.4: ANOVA test for determining the statistical significance between the k_{max} of BSA post-gelation for BSA and BSA:MB hydrogels.

k_{max}	BSA:Control	10^7	10^8	10^9
Mean ($Pa\ s^{-1}$)	45	41	41	36
F		0.68	0.18	0.80
F_C		3.47	5.56	3.35

Table C.5: ANOVA test for determining the statistical significance between the k_{max}/G'_{∞} of BSA post-gelation for the control BSA and BSA:MB hydrogels.

k_{max}/G'_{∞}	BSA:Control	10^7	10^8	10^9
Mean ($\times 10^{-2} s^{-1}$)	0.013	0.022	0.024	0.023
F		0.82	0.58	0.147
F_C		3.47	5.56	3.35

C.1.3 Rheology: Stress-Strain Curve

In Chapter 3, the stress-strain curve in Figure 3.10 was used to compare the behaviour of loading and unloading shear strain. The shear stress at a shear strain of 50% was compared the control BSA and BSA:MB hydrogels. The ANOVA test in Table C.6 determined that the results from control BSA and the BSA:MB hydrogel with 10^9 MB/mL are statistically significant, while the BSA:MBs with 10^7 and 10^8 MB/mL were found to not be statistically significant to control BSA hydrogel.

Table C.6: ANOVA test for determining the statistical significance of the shear stress at a shear strain of 50% between the control BSA and BSA:MB hydrogels.

Shear Stress	BSA:Control	10^7	10^8	10^9
Mean (Pa)	977	939	730	783
F		0.088	1.91	3.84
F_C		3.47	5.56	3.35

Figure 3.10B compares the the G'_∞ from the linear fit of the gelation curve. The control BSA hydrogel and BSA:MB hydrogel with 10^9 MB/mL were statistically significant, while the BSA:MB hydrogels with 10^7 and 10^8 MB/mL were not statistically significant from the control BSA hydrogel.

Table C.7: ANOVA test for determining the statistical significance between the G'_∞ from the linear fit of the stress-strain of BSA post-gelation for the control BSA and BSA:MB hydrogels.

G'_∞ (linear fit)	BSA:Control	10^7	10^8	10^9
Mean (Pa)	1800	1780	1470	1430
F		0.43	1.34	4.04
F_C		3.47	5.56	3.35

The energy dissipated and efficiency of loading and unloading a shear force was compared in Chapter 3, Figure 3.11. The ANOVA test determined that the results of

encompassing 10^7 , 10^8 and 10^9 MB/mL into the BSA hydrogel were not statistically significant compared to the control BSA hydrogel for both the energy dissipated (Table C.9) and the efficiency of loading and unloading (Table C.8).

Table C.8: ANOVA test for determining the statistical significance between energy dissipation of loading and unloading of BSA post-gelation for the control BSA and BSA:MB hydrogels.

Energy Dissipated	BSA:Control	10^7	10^8	10^9
Mean (J/m^3)	18.5	18.4	18.3	14.5
F		0.066	0.405	2
F_C		3.47	5.56	3.35

Table C.9: ANOVA test for determining the statistical significance between efficiency of loading and unloading shear strain of BSA post-gelation for the control BSA and BSA:MB hydrogels.

τ	BSA:Control	10^7	10^8	10^9
Mean (s%)	96.3	96.2	96.5	96.9
F		0.017	0.049	1.13
F_C		3.47	5.56	3.35

C.2 ANOVA Tests: Chapters 5

C.2.1 Rheology: Gelation Curve and Time Sweep

In Chapter 5, Figure 4.7b compares the G'_f for BSA, BSA:MBs, BSA:MBs:post-DTT and BSA:post-DTT hydrogels. To understand the statistical significance of the addition of DTT to BSA and BSA:MB hydrogels the ANOVA test was used. For both the BSA and BSA:MB hydrogels, the addition of DTT post-gelation caused a significant decrease in G' , as shown in Table C.10 and C.10 at $\alpha = 0.05$.

Table C.10: ANOVA test for determining the statistical significance between the BSA and BSA:post-DTT hydrogels.

G'	BSA	BSA:post-DTT
Mean (Pa)	3130	1800
F		18.2
F_C		5.6

Table C.11: ANOVA test for determining the statistical significance between the BSA:MBs and BSA:MBs:post-DTT hydrogels.

G'	BSA:MBs	BSA:MBs:post-DTT
Mean (Pa)	2970	1730
F		25.4
F_C		4.5

The ANOVA test was used to determine if the post-DTT hydrogels were statistically significant in the absence (BSA:post-DTT) and presence of MBs (BSA:MBs:post-DTT). The results in Table C.12 show that the G' of the BSA:post-DTT and BSA:MBs:post-DTT hydrogels are not statistically significant.

Table C.12: ANOVA test for determining the statistical significance between the BSA:post-DTT and BSA:MBs:post-DTT hydrogels.

G'	BSA:post-DTT	BSA:MBs:post-DTT
Mean (Pa)	1800	1730
F		0.47
F_C		4.5

C.2.2 Rheology: Relaxation constant

The relaxation constants of the protein hydrogel post cross-linking, τ_1 and τ_2 , were compared before and after the addition of DTT, for BSA and BSA:MBs hydrogels. The statistical significant was determined for the BSA hydrogel and BSA:post-DTT hydrogel, where the decrease in τ_1 observed with the addition of DTT were statistically significant for $\alpha=0.05$.

Table C.13: ANOVA test for determining the statistical significance between the relaxation constant, τ_1 , for BSA and BSA:post-DTT hydrogels.

G'	BSA	BSA:post-DTT
Mean (Pa)	1125	658
F		49.8
F_C		5.6

The ANOVA test determined the BSA:MBs and BSA:MBs:post-DTT hydrogel, where the decrease in τ_1 observed with the addition of DTT were statistically significant for $\alpha=0.05$ for BSA hydrogels with encompassed MBs.

Table C.14: ANOVA test for determining the statistical significance of τ_1 between the BSA:MBs and BSA:MBs:post-DTT hydrogels.

G'	BSA:MBs	BSA:MB:post-DTT
Mean (Pa)	1040	770
F		46.0
F_C		5.6

The ANOVA test determined the BSA:post-DTT and BSA:MBs:post-DTT hydrogels, where the decrease in τ_1 observed with the addition of DTT were not statistically significant for $\alpha=0.05$ for BSA hydrogels with encompassed MBs.

Table C.15: ANOVA test for determining the statistical significance of τ_1 between the BSA:post-DTT and BSA:MBs:post-DTT hydrogels.

G'	BSA:post-DTT	BSA:MB:post-DTT
Mean (Pa)	660	770
F		0.92
F_C		5.6

The post-DTT hydrogels observed a second relaxation constant, τ_2 . The difference between the τ_2 for BSA:post-DTT and BSA:MBs:post-DTT hydrogel was determined by the ANOVA to test to not be statistically significant.

Table C.16: ANOVA test for determining the statistical significance between the BSA:post-DTT and BSA:MBs:post-DTT hydrogels.

G'	BSA:post-DTT	BSA:MB:post-DTT
Mean (Pa)	7900	8900
F		0.98
F_C		5.6

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