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Chemically crosslinked protein hydrogels for tissue engineering

by

Rossana Boni

Thesis
Submitted to the University of Edinburgh
for the degree of

Doctor of Philosophy

College of Medicine and Veterinary Medicine

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Declarations

This thesis is submitted to the University of Edinburgh in fulfillment of the requirements for the degree of Doctor of Philosophy. I declare that the work presented in this thesis has been composed solely by myself and was carried out in the School of Biological Sciences, the School of Physics and Astronomy, and the UK Centre for Mammalian Synthetic Biology at the University of Edinburgh. The research presented here has not been submitted in whole or in part for any degree at this or any other university. Unless stated otherwise by reference or acknowledgement, the work presented in this thesis is entirely my own.

Rossana Boni, May 2023
Questa tesi è dedicata a mia mamma che ha affrontato e vinto la più grande battaglia della sua vita con coraggio, grazia e risolutezza. E a mio papà, da sempre pilastro incrollabile della nostra famiglia.
Abstract

Tissue engineering aims at replacing or repair organ that have lost function due to injury or disease via the development of appropriate biomaterials. In order to restore function, biomaterials for tissue regeneration must mimic the biophysical properties of the native physiological environment. In the literature several synthetic and natural based biomaterials have been implemented to mimic the physiological environment and restore functionality to injured tissues. However, most biomaterials are based on difficult to modify natural or synthetic components that are limited in their ability to adapt to the native physiological environment due to their intrinsic properties. Recombinant proteins have recently emerged as an interesting alternative to traditional biomaterials because their building blocks can be modified at the DNA level to suit the specific needs of the chosen environment, overcoming the issues linked to the suboptimal ability to chemically modify polymer and natural proteins alike. In particular an engineered approach using functionalised protein hydrogels allows to specify the biophysical properties independently and customise them to suit a particular physiological environment. I have successfully designed and programmed engineered proteins to form covalent molecular networks with defined physical characteristics able to sustain mammalian cell culture without loss of viability. The hydrogel design incorporates the SpyTag (ST) peptide and Spy-Catcher (SC) protein to spontaneously form covalent-crosslinks upon mixing. The genetically encoded chemistry allows to easily incorporate different recombinant proteins in the hydrogels and induce a wide array of controlled biophysical char-
acteristics. By changing the crosslinker protein and ratios of the protein building blocks (ST:SC), I demonstrated that I could alter both the viscoelastic properties and gelation speeds of the resulting hydrogels. Fine tuning the rate of gelation of the biocompatible hydrogels allowed me to adapt the design to biofabrication techniques, such as 3D printing. Finally, I showed that the ST-SC protein hydrogels can be used as a substrate for cell culture, can encapsulate mammalian cells in 3D, and can drive increased mammalian cell attachment. My work demonstrates how protein engineering could be applied to precise medical applications, bridging synthetic biology and tissue engineering.
The main issues facing organ transplantation is the scarcity of available organ donors and the potential for organ rejection. Biomaterials have emerged as a potential solution to organ transplant, where new organs could be grown on external structures and then implanted into a patient in need. In order to develop a successful biomaterial, researchers have to consider the chemical, physical, and mechanical properties of the material itself and their interactions with the human body. The mechanical proprieties of a biomaterial should be as similar as possible to the organ they are aiming to heal, i.e biomaterials should be as strong and as flexible as the native organs. Intuitively, biomaterials must also be biocompatible, hence not toxic to the neighbouring cells or tissues and they should also actively help cells grow and reproduce. Biomaterials can be developed from different sources, such as plastics or plants. Recently, proteins have been exploited to develop biomaterials because their structure can be easily modified to mimic the properties of an organ.

In this project I developed a custom approach to create self-standing nanostructures able to sustain cellular cultures. I used synthetic biology and protein design approaches to construct different types of protein-based nanostructures. These nanostructures are three-dimensional hydrogels that can simulate the physical and biological characteristics of human tissues, inducing cell growth and cell proliferation. I have successfully developed hydrogels with different biophysical characteristics, analysed their strength, flexibility, and speed of formation, demonstrated their biocompatibility with human cells, and adapted their design to 3D printing.

Thanks to new development in protein engineering and synthetic biology, protein hydrogels have the potential to improve quality of life by replacing missing organs or tissues and repair defective functions, thereby eliminating the need for organ donors and avoiding organ rejection issues.
Abbreviations

AFM  Atomic force microscopy
BSA  Bovine serum albumin
DNA  Deoxyribonucleic acid
ECM  Extracellular matrix
ELP  Elastin like polypeptides
GDL  D-glucono-δ-lactone
GelMA  Gelatin methacryloyl
HA  Hyaluronic acid
HRP  Horseradish peroxidase
IPNs  Interpenetrating networks
iPSCs  induced pluripotent stem cells
LCST  Lower critical solution temperature
MBP  Maltose bound protein
MITCH  Mixing-induced two-component hydrogel
MMP  Matrix metalloproteinase
PA Peptide amphiphile
PCL Polycaprolactone
PEG Polyethylene glycol
PEO Polyethylene oxide
PGA Polyglycolic acid
pHEMA Poly-(2-hydroxyethyl methacrylate)
PLGA Polylactic-glycolic acid
PVA Polyvinyl alcohol
PPO Polypropylene oxide
RGD Arginylglycylaspartic acid
SANS Small-angle neutron scattering
SAXS Small-angle X-ray scattering
SC SpyCatcher
SDS-PAGE Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
ST SpyTag
TPR Tetratricopeptide repeat
Chapter 1

Proteins as biomaterials

1.1 Tissue engineering

The field of tissue engineering was first defined by Langer and Vacanti in 1993 in their seminal paper aptly titled ‘Tissue Engineering’ [1]. Tissue engineering combines principles and techniques of cell biology, material science, and engineering to the development of functional substitutes that mimic the structural and physiological nature of the native tissue with the aim to regenerate the functional properties of a defective tissue due to injury or disease [1].

Currently, the clinical gold standard for organ failure or tissue loss is organ transplant, where physicians transplant an organ from one individual to another saving countless of lives. However, the system remains imperfect. According to the NHS, every year close to 7000 people are waiting for a lifesaving organ transplant in the UK alone and more than 400 will die whilst waiting for an organ [2]. The worldwide donor shortage critically limits transplantation, and even if a suitable organ is found within the short timeframe allowed by a patient’s organ failure, there are still substantial issues linked to potential organ rejection where the newly implanted organ is recognised as foreign by the host’s immune systems and thus, rejected [3]. Moreover, patients successfully receiving a lifesaving organ transplant will be forced to take immunosuppressant drugs for the rest of their lives severely reducing their quality of life and increasing the chances of developing infectious diseases [3].
Tissue engineering combines engineering and life sciences to offer potential alternative solutions to organ transplantation [1]. New discoveries in material science and the development of novel smart materials, hence materials able to actively respond to stimuli coming from the surrounding environment, have driven the rapid evolution of the field, reshaping the way medical professionals and engineers alike think about the treatment of injuries and diseases [4]. The paradigm has shifted from relying on extirpative surgical techniques that often left patients with substantial side effect and potential lifelong consequences to the use of new ‘biomaterials’, able to not only regenerate the tissue but also biodegrade within the body without the creation of cytotoxic bioproducts, thereby eliminating the need for a second extirpative surgery to remove the material [5].

Figure 1.1 offers an overview of tissue engineering, detailing the most common biomaterial approaches, various fabrication techniques, such as 3D printing or electrospinning, and the most common characterisation methods widely used for tissue engineering applications, both physical methods, such as spectroscopic techniques aiming at determining the physical properties of the biomaterial and biological methods, including \textit{in vitro} and \textit{in vivo} studies, aiming at determining the material’s biocompatibility.
Figure 1.1: **Overview of tissue engineering** The choice of the most suitable bioengineering approach, the method of production, and the methods of characterisation, both physical and biological, are crucial for the development of an efficacious tissue engineering application suitable for a specific physiological environment. Made with BioRender.com
The term ‘biomaterial’ hints at their double nature, hence their application in the biological and medical field that remains indissolubly linked to the chemical, physical, and mechanical properties of the material itself. Because the ultimate goal of biomaterials is to improve human health by restoring the functions of the natural living tissues and improving one’s quality of life, it is essential to consider the relationship among the properties, functions, and structures of biological materials [5]. Therefore, tissue engineering must ascertain which combination of techniques and approaches is the most suitable to a particular application through a careful and rational approach to the initial biomaterial design.

Initially, tissue engineering aimed at creating biomaterials that simply mimicked the biological and mechanical functions of the extracellular matrix (ECM) found in native tissue. Langer and Vacanti discussed ‘cells placed on or within matrices’, where cells are isolated from the body and positioned onto a matrix fashioned from natural materials such as collagen or synthetic polymers, that allows permeation of nutrients and waste but prevents an immunogenic reaction from destroying the transplant [1]. The solution proposed by Langer and Vacanti is still the core aim of most tissue engineering platforms, but nowadays the field has evolved and newly developed biomaterials should actively encourage a similar cellular reorganisation to that of the original tissue. In particular, two pioneering discoveries have opened new possibilities in the field. First, Takahashi and Yamanaka’s groundbreaking discovery of induced pluripotent stem cells (iPSCs), i.e. the ability to recreate undifferentiated cells from differentiated cells, such as adult fibroblasts, via four factors, Oct3/4, Sox2, c-Myc, and Klf4, and subsequently control iPSC differentiation in another cell type [6] brought forward the idea that patients’ own cells could be redifferentiated into healthy cells able to replace defective ones. Second, the discovery that the behaviour and differentiation of cells is influenced by the environment that they are seeded on [7] has spurred the development of new structures able to closely mimic the native environments of cells and control their fate.
Given the complex nature of the human body, it is unsurprising that there are significant differences between the mechanical properties and structures of different organs, [8]. For example, the stiffness range in living tissues spans from 11 Pa of intestinal mucus to 20 GPa of bone, 1.2. Because the primary aim of this thesis is to develop a novel protein based biomaterial aimed at liver regeneration, it is important to notice that the stiffness of liver is reported close to 10 kPa.

Figure 1.2: The elastic moduli (E) of different tissues on a logarithmic scale. Tissues are organized by increasing crescent moduli. The weakest reported tissue is the brain, with a reported stiffness of 1 kPa, whilst the strongest tissue is the bone with 20 GPa. The stiffness of liver, the target organ of this project, is reported close to 10 kPa. Figure reproduced from Guimarães et al. [8].
Tissue engineering must consider not only the macroscale properties of a tissue, but also the mechanics and structures measured at the nano and microscale, often caused by heterogeneities of biological tissues. For example, in the lung the pleurae is considerably stiffer than the parenchyma, making the mechanics of the individual components more relevant for tissue engineering compared to the bulk tissue properties [8]. However, if a layered tissue like the bone is subjected to tensile deformation, the behaviour of the bulk material is dictated by the overall mechanical modulus [8]. Therefore, depending on the aims of the final application, tissue engineering needs to balance focusing on mimicking bulk mechanics, crucial to ensure in vivo stability, with mirroring nanoscale properties, essential for regulating cell behaviour.

Moreover, the complexity and the heterogeneity of human organs makes the analysis of their mechanical properties even more difficult, due to differences in their response in static or dynamic conditions. For example, static compressive tests will measure the elastic response of the bone’s mineral component, but only dynamic analysis will be able to elucidate the viscous response of collagen, that allows bones to absorb shock [8]. Shear rheology is the most common method of analysis of the dynamic properties of an organ, and as such, it is a technique widely used in tissue engineering. Chapter 4 of this thesis defines and describes rheological methods of analysis in detail.

New advances in the field of tissue engineering have clearly established that approaches to engineer biological tissues must take into account the mechanics, both static and dynamic, of native tissues, as well as the final application of the engineered matrix.

As previously mentioned, this thesis focusses on the development of a novel protein based biomaterial aimed at liver regeneration. Liver regeneration is not trivial because its architecture is quite complex as the organ is made up a various different cell types, including stellar cells, Kupffer cells, and endothelial cells, primarily hepatocytes [9]. These different cellular components allow the liver to carry out its secretory, metabolic, and detoxifying functions, such as protein synthesis, erythropoiesis, and lipid metabolism [9]. Liver disorders are primarily caused by
alcoholism, hepatitis, or cancer, and, currently, liver transplantation is the gold standard for patients suffering from liver diseases [9]. Other interim approaches, such as hemodialysis, failed to improve patient survival whilst severely diminishing their quality of life [9].

A unique and interesting feature of the liver is that the organ is able to self regenerate even after significant damage thanks to the great replicative properties of hepatocytes [10]. Because of their replicative capacities, hepatocytes transplantation on suitable tissue engineered matrices are often studied as an alternative to liver transplantation to regain liver function. However, one of the main challenges in liver tissue engineering is maintaining the phenotype and function of hepatocytes in culture for more than a week, as these cells are very sensitive to their microenvironment and tend to differentiate into other types of cells, such as fibroblasts, when their biological environment is not carefully maintained [9]. Therefore, the application of tissue engineered matrices to culture hepatocytes for liver regeneration requires a careful and balanced design that takes into account multiple factors of the engineered substrate, including pore size, topography, surface chemistry, and stiffness.

Taking into account the specific requirements of an engineered application combined with new scientific discoveries, the new ideal scenario in tissue engineering is based on 1) developing a suitable matrix with specific biochemical and physical cues; 2) harvesting fibroblasts from a patient with organ failure and rein-duce cell pluripotency; seed patient derived stem cells onto the matrix; 3) cells will differentiate into the tissue of interest thanks to the specific biochemical and physical cues engineered in the matrix and the tissue or organ can be reimplanted into the patient, Fig. 1.3. This ideal solution proposed by tissue engineering would not only overcome issues related to the global shortage of donors, as donors would no longer be needed, but also eliminate the possibilities of organ rejection because the new tissue would be grown from autologous cells.
Research is still far away from the ideal scenario proposed here, but recent advances in customisable biomaterials are moving towards making this ideal scenario a reality. Among the major challenges now facing tissue engineering is the need for more complex functionality as well as a thorough understanding of biomaterials and their interactions with the physiological environment for a successful translation into the clinical setting. Understanding the biomaterial-environment relationship is not trivial, as the complexity of the physiological environment arises from a network of nonlinear interactions cooperating at different hierarchical levels and at different time scales, that regulate the behaviour of the system to adapt to external perturbations preserving homeostasis [12]. The complexity of the physiological environment is also further altered by local pathologies that add specific features to the system inducing significant changes. As our understanding of the complex physiology of the human body increases, so should the complexity of the matching biomaterial aimed at tissue regeneration. Therefore, there is a clear need to devise new ways for biomaterials to adapt to the crucial interactions relevant to the specificity of the targeted physiological environment and critically adapt to its physical and biological parameters.
Regardless of their final application, successful biomaterials should act as functional scaffolds, where seeded cells can attach, proliferate, and differentiate. Essentially, there are two approaches that tissue engineering applies to create suitable scaffolds: organ decellularization and fabrication of scaffolds using new adaptable non-native materials. Briefly, in organ decellularization physical and chemical methods are combined to lyse the organ’s cells and wash them away, leaving a biological scaffold composed of native ECM that can be seeded with new cells and reimplanted into the patient [13]. The rationale behind organ decellularization is based on using native mammalian ECM rather than foreign materials that could mimic it, hence providing the cells with their exact microenvironment that, in turn, affects migration, proliferation, and differentiation. However, the success of this method relies upon the complete removal of the cell remnants which could cause an immunogenic response in vivo. Moreover, most decellularization methods result in disruption of the ECM architecture as well as loss of surface structure and composition [14]. Due to these clear disadvantages and the suboptimal use of harsh physical and chemical methods, the application of organ decellularization is tissue engineering has been limited.

The second approach exploited by tissue engineering is the fabrication of suitable scaffolds using new synthetic or natural materials. Research surrounding the discovery and characterisation of new materials that can offer a 3D support to seeded cells and mimic the native ECM has vastly increased in the last 20 years, leading to the creation and optimisation of hydrogel scaffolds, crosslinked hydrophilic gel-like structures that can repair or regrow damaged organs and hold great potential for tissue engineering [5,15].
1.1.1 Hydrogels

In general, hydrogels are defined as 3-dimensional networks of physically or chemically crosslinked macromolecules that can swell in water and produce an elastic structure, Fig. 1.4 [15]. Hydrogels have received exceptional attention in the last 50 years due to their wide range of applications. After Wichterle and Lim developed the first biocompatible hydrogels in 1960 [16], hydrogels applications have spun absorbents [17], agriculture [18], drug delivery [19], pharmaceuticals [20], and biosensors [21], just to name a few. Of note, each of the aforementioned publications per application is a detailed review highlighting hundreds of different specific hydrogels used in the respective fields. Therefore, hydrogels are an important class of biomaterials incredibly well characterised and used in the literature for most applications where smart materials are involved. However, it is clear that it would be impossible for a hydrogel to simultaneously fill all the requirements needed for a specific application. For example, adsorbent hydrogels should have high absorption rate, low re-wetting, and little to no residual monomers, as opposed to hydrogels optimised for drug delivery that should prioritise porosity and stimuli-responsiveness to unload encapsulated drugs at the right moment. Thus, there is no such thing as the perfect hydrogel, but rather the best optimised hydrogel for the application at hand.

First, hydrogels optimised for tissue engineering should be biocompatible, hence able to exist within the body without eliciting an immunogenic reaction or cause apoptosis in the neighbouring cells. Second, hydrogels for tissue engineering should focus on both classical physical parameters (i.e. mechanical stiffness and degradation) as well as biological cues (i.e. cell adhesion and proliferation). The mechanical properties of a hydrogel should mimic the properties of the target tissue, hence the tissue that should be regenerated, whilst the rate of hydrogel degradation should match the rate of regeneration of the targeted organ, Fig. 1.4. The paradigm behind hydrogels optimised for tissue engineering is based on the development of a specific platform, with specific physical and biological properties, where patient-derived stem cells are seeded and differentiated based on the biophysical cues offered by the hydrogel. Therefore, the choice of material, synthetic or natural polymers or recombinant proteins, as well as the crosslinking mechanism will influence both
the physical and the biological properties of the resulting hydrogels and, as such, they should be selected with care and rationality.

Two crucial parameters that are almost always explored when investigating hydrogels for tissue engineering applications are the hydrogel’s stiffness, usually monitored via rheology, and its biocompatibility, usually monitored by incubation of the hydrogels with a specific cell line in vitro and by implantation in vivo. Briefly, rheology measures the elastic or storage ($G'$) and viscous or loss ($G''$) moduli of the hydrogels, where if $G' > G''$ the system is a viscoelastic solid rather than a liquid, $G'$ is proportional to the stiffness of the hydrogels, and the critical strain is the shear at which the hydrogel breaks [22]. An accurate and comprehensive explanation of rheological behaviours and measurements is detailed in Chapter 4 of this thesis.

Figure 1.4: Hydrogels optimised for tissue engineering. Top: Hydrogels are 3-dimensional networks of physically or chemically crosslinked macromolecules that can swell in water. Created with BioRender.com. Bottom: Hydrogels optimised for tissue engineering should mimic the properties of the target tissue, A) soft hydrogel optimised for brain tissue engineering, B) stiff hydrogel optimised for chondrogenesis, and C) rigid hydrogel optimised for bone tissue engineering. Figure reproduced from Dalby et al. [23].
Hydrogels for tissue engineering can be subdivided based on the material that they are composed of: synthetic polymers or natural polymers, polymer-protein hybrid networks, or protein hydrogels, Fig. 1.5. The vast majority of hydrogels used for tissue engineering applications are based on synthetic polymers, such as the most common polyethylene glycol (PEG), polyglycolic acid (PGA) and its variation poly-lactic-glycolic acid (PLGA), or polyvinyl alcohol (PVA). These materials have strong mechanical properties, they can be processed using a variety of different techniques, including 3D printing, electrospinning, and melt-electrowriting, and they are biocompatible [24]. However, they are inert, therefore they do not possess biochemical cues that enhance cell attachment, proliferation, and differentiation. Conversely, naturally occurring polymers such as cellulose or chitin, have excellent biocompatibility and high swelling capacity but weaker mechanical properties, often below 10 Pa, that hinder their biofabrication [24]. Therefore, in the literature balanced and careful blending of natural and synthetic polymers is often exploited to overcome the limitations of each material. Moreover, the addition of arginylglycylaspartic acid (RGD), the minimal recognition sequence required for cell attachment, is the most common peptide motif incorporated in tissue engineering applications to enhance cellular attachment in both natural and synthetic hydrogels [25].

However, both synthetic and natural polymers are still bound by their properties, meaning that whilst techniques for functionalisation and chemical modification of polymer-based hydrogels are common, the materials are still indissolubly linked to their original structure and backbone, which is unmodifiable. Given the complexity of the human body and the high level of specificity required by a physiological environment, often further altered by a local pathology, tissue engineering research has started to move from synthetic hydrogels towards the development of protein-based hydrogels, Fig. 1.5. That is because recent advances in protein engineering have allowed for the programming of specific building blocks to form functional biomaterials with customisable biophysical properties, thus creating hydrogels with a higher level of customisation and tailorablebility when compared to natural or synthetic polymers [26, 27].
Herein, I will give an overview of the most commonly used materials for tissue engineering, spanning from synthetic to natural polymers, towards the development of protein-based hydrogels.

Figure 1.5: **Tissue engineering hydrogel classified based on the material they are composed of.** A) hydrogels composed of synthetic polymers are strong but inert, reproduced from Boni et al. [28]. B) protein polymer hybrid hydrogels combine the strength of synthetic polymers with the bioactivity of natural polymers or proteins, adapted from Grove et al. [29]. C) protein hydrogels form functional biomaterials with customisable biophysical properties, adapted from Banta et al. [30].

### 1.1.2 Hydrogels composed of synthetic polymers

Given the plethora of literature available on hydrogels composed of synthetic polymers for tissue engineering applications, I will focus on recent advances aiming at liver regeneration, the target organ of this project.

The most common synthetic polymer used in tissue engineering is polyethylene glycol (PEG). PEG is a biodegradable synthetic polymer composed of ethylene oxide (EO) units well suited to tissue engineering due to its hydrophilic properties, biocompatibility, and non-immunogenicity. Early work by Pishko and co-workers suggested that the viability and the protein production of hepatocytes entrapped in hydrogels can be modulated by PEG [31]. For example, the authors showed that while PEG composition has no effect on hepatocytes cell viability, increased amounts of PEG were correlated with a decrease in protein production by the cells, likely due to transfer limitations in hydrogels with higher PEG compositions. The authors also incorporated the RGD peptide in the PEG hydrogels by
incubating acryloyl-PEG-NHS (N-hydroxysuccinimide) with the peptide solution at room temperature and showed that hydrogel surface modification via RGD resulted in increased albumin production and decreased fibronectin production. The authors hypothesised that cells incubated in the RGD containing hydrogels detected the presence of RGD and diverted cellular resources to the production of albumin rather than remodelling the ECM environment, downregulating fibronectin [31]. Along the same lines, Underhill et al. used low molecular weight PEG based hydrogels to maintain good viability of mouse embryonic liver cells whilst retaining expression of albumin and alcohol dehydrogenase, crucial markers of liver functionality [32]. More recently, Baharvand and colleagues developed injectable extracellular vesicles (EVs) derived from mesenchymal stem cells encapsulated in PEG for hepatic regeneration [33]. The controlled biodegradation of the PEG hydrogels in vivo via cleavage of the bonds between the PEG macromeres allowed for the sustained release of the EVs over four weeks after intraperitoneal infusion. According to the authors, the prolonged availability of the EV in the fibrotic liver environment thanks to the PEG slow degradation was responsible for the increased therapeutic effect and reversion of fibrosis observed.

Another class of polymer extensively used in liver tissue engineering is polylactic acid (PLA), polyglycolic acid (PGA), and their co-polymer polylactic-glycolic acid (PLGA) due to their hydrophilicity and biodegradability. However, concerns regarding the use of these materials in the human body are linked to frequent and unplanned degradation in vivo due to hydrolysis weakening their mechanical properties and causing immunogenic reactions [28]. Nevertheless, Jiang et al. successfully developed electrospun PLA nanofibers coated with collagen for primary hepatocytes growth. After 15 days in culture, the hepatocytes exhibited high cell adhesion and strong albumin secretion as well as urea synthesis, hallmarks of hepatocytes function [34]. Similarly, Pollock and co-workers showed that human derived hepatocytes cultured on biodegradable PLA matrices inside a flow bioreactor have great survival and proliferation rates and the system could offer a viable option for hepatocytes transplantation [35].
Polycaprolactone (PCL) is an FDA approved biodegradable polymer with strong mechanical properties and excellent processability widely used in biomedical applications. The Hay lab at the University of Edinburgh has pioneered the use of subcutaneous PCL implants supporting PSCs 3D spheroids for liver regeneration [36]. Thanks to the strong mechanical properties of PCL, the implants were fabricated via electrospinning, loaded with stem cell-derived 3D hepatocytes aggregates, and implanted in vivo. The subcutaneous implants of 3D hepatocytes were shown to be well tolerated and vascularised as well as supporting failing liver function. Hay and Callanan also developed a hybrid electrospun PCL-ECM scaffold with excellent mechanical properties (3.58 MPa) able to sustain HepG2 cell attachment and viability in vitro as well as support gene expression of both liver and ECM function genes [37]. Recently, another group has validated the use of electrospun PCL for liver tissue regeneration. Semnani et al. developed PCL scaffolds functionalised with galactosylation of chitosan [38]. The hybrid scaffold combined galactose containing chitosan, that increased liver cell function and improved cell survival, with PCL which conferred strong mechanical properties to the hydrogel. Galactose increased the hydrophilicity, contact angle, and degradation rate of the hydrogels as well increasing viability of HepG2.

Polyvinyl alcohol (PVA) is another commonly used polymer in tissue engineering that can yield strong biocompatible hydrogel via freezing-thawing without the need for an additional crosslinker. PVA functionalised with gelatin, incorporating the RGD motif, scaffolds were developed by Sethuraman and co-workers and loaded with PLGA nanoparticles containing hepatocytes growth factors [39]. After 28 days in culture, the hepatocytes seeded onto the scaffolds exhibited good cell adhesion and spheroid formation with higher levels of albumin and urea secreted. Similarly, Forte et al. developed PVA-phytagel (PHY) composite hydrogels to mimic the viscoelastic behaviour of liver tissue [40]. The mechanical properties of the hydrogel were modulated by varying the concentration of each hydrogel component and the authors found that increasing the concentration for both PVA and PHY increased the ability of the hydrogel to sustain stress, but at higher concentrations of PVA compared to PHY the material became stiffer but more brittle. By further tuning the composite hydrogel properties, the authors developed a PVA based artificial material able to accurately mimic the viscoelastic properties of liver.
Poloxamers, also known by their trade name Pluronic, are a class of temperature dependant synthetic block copolymers based on hydrophilic polyethylene oxide (PEO) chain and a hydrophobic polypropylene oxide core (PPO) arranged in an ABA triblock structure (PEO-PPO-PEO). Pluronic are particularly useful in tissue engineering as they remain liquid at room temperature but undergo a sol-gel transition at near physiological temperature and pH. Fu et al. exploited the thermosenstivity of Pluronic hydrogels to develop a drug loaded hydrogel delivery system for the treatment of liver cancer ascites [41]. As expected, the hydrogel presented thermoreponsive rheological properties with $G'$ close to 10,000 Pa above 28 °C. The hydrogel also showed remarkable ability to sustain the delivery of antitumour drugs in vitro that translated into the reduced volume of ascites in vivo. Along the same lines, Wang et al. developed thermoreversible Pluronic based nanocarriers for drug delivery against liver cancer that exhibited similar drug loading and release to Fu et al. [42].

Gelatin methacryloyl (GelMA) hydrogels have been widely used in tissue engineering due to their suitable biological properties and tuneable physical characteristics. In particular, as a derivative of gelatin, GelMA retains some essential properties of the native collagen ECM, namely the RGD cell attaching motif as well as target sequences for metalloproteinases (MMPs) that encourage cell remodelling [43]. However, gelatin is more soluble and presents less antigenicity and less structural variations due to different sources compared to collagen thanks to the hydrolysis process that denatures the tertiary structure of collagen [44]. The addition of methacryloyl substituent groups to gelatin allows GelMA to photocrosslink in mild physiological conditions. Depending on the designed microstructures, the mechanical properties of GelMa hydrogels range from < 1 to 800 kPa, making this material suitable for a wide variety of tissue engineering applications. GelMa is also particularly versatile and can be combined with numerous other materials, such as electroconductive carbon nanotubes or gold nanoparticles, giving rise to conductive networks and matrices with increased stiffness for bone regeneration [44]. Fast curing GelMa based hydrogels were used by Liu et al. to develop and maintain a cell culture microenvironment covering the cellular micropatterning of C3A and 3T3 cells [45]. GelMa hydrogels were successful in maintaining the precise arrange-
ment of the cells, but the authors found that with increasing GelMA concentration, cell viability gradually decreased. Thanks to its tuneable mechanical properties, GelMa was also combined with liver decellularized ECM and hepatocytes to form a cell laden bioink, i.e. a hydrogel developed for 3D printing [46]. The composite hydrogel was found to have a smaller $G'$ compared to native liver tissue but excellent cell viability in vitro. The hydrogels laden with cells were also successfully 3D printed and the cylindrical cavity inserted into the 3D printed design was found to increase the circulation of cell medium and improve cell metabolism.

Poly-(2-hydroxyethyl methacrylate) (pHEMA) materials were the first to be used by Witchterle for soft contact lenses in 1960 thanks to their hydrophilic, biocompatible, and transparent properties [16]. Nowadays pHEMA hydrogels have found widespread use for biomedical applications spanning from their original ophthalmic use to wound healing, bone tissue regeneration, and drug delivery systems [47]. In liver tissue engineering, pHEMA-bisacrylamide (BAA) hydrogels were optimised by systematically varying the scaffold’s components in view of mimicking the microenvironment of liver cells [48]. The authors developed scaffolds with mechanical properties mimicking healthy liver ($G' = 2.9$ kPa) by keeping the ratio of pHEMA:BAA close to 2:1 and hydrogels mimicking more rigid unhealthy cirrhotic liver ($G' = 18-20$ kPa) by significantly increasing the amount of pHEMA compared to BAA. Evaluation of hepatic cell viability and cell functionality mirrored the expected behaviour on each matrix, with the cells seeded on ‘healthy’ liver scaffolds showing increased metabolic activity and cell attachment compared to the cells seeded onto the fibrotic liver scaffold.

In summary, the use of synthetic polymers in tissue engineering is advantageous because of their mechanical strength and availability combined with their compatibility with numerous fabrication techniques and potential for scale-up, Fig. 1.6. However, concerns remain linked to the potential of toxic residual monomers as well as the presence of immunogenic degradation products [28]. Moreover, whilst synthetic polymers are indeed biocompatible, they are inert scaffolds, therefore they offer a stable support to seeded cells but they do not provide biochemical cues that enhance cell attachment, proliferation or differentiation as they lack functional groups for cell interaction, Fig. 1.6 [24]. Thus, synthetic polymers are
predominantly used to add or increase the mechanical properties of scaffolds and subsequently blended with bioactive natural polymers or proteins to develop functionalised hybrid scaffolds.

Figure 1.6: **Schematics of synthetic polymer based hydrogels.** Hydrogels composed of synthetic polymers present strong mechanical properties but they are inert scaffolds and they do not provide biochemical cues that enhance cell attachment, proliferation or differentiation. Created with BioRender.com.
1.1.3 Hydrogels composed of natural polymers

The use of natural polymers in tissue engineering is advantageous due to their enhanced biocompatibility and cell attachment features combined with natural biodegradation kinetics and tuneable properties. Moreover, natural polymers are analogous, if not identical like collagen or keratin, to proteins already present in the human body, therefore risks linked to immunogenicity and cytotoxicity upon implantation are minimised [28]. Natural polymers used in liver tissue engineering have different origins, including ECM components such as collagen, polymers from marine life, alginate, from crustaceans, chitosan, or from insects, spider silk. Once again, I will focus on recent advances detailing natural polymer hydrogels aiming at liver regeneration.

Matrigel is perhaps the most common commercially available matrix used for cell attachment and differentiation. Matrigel is an extract from mouse Engelbreth-Holm-Swarm mouse sarcoma, containing ECM derived proteins such as collagen IV, laminin, perlacan, and growth factors [49]. Matrigel components reconstitute into a solid at 37 °C and, as such, Matrigel has been used in more than 12,000 publications for a wealth of studies involving embryonic, normal, stem, or malignant cells [49]. The average modulus of Matrigel was investigated via atomic force microscopy (AFM) in an aqueous, temperature controlled environment and reported to be approximately 450 Pa [50]. However, the modulus of Matrigel varies significantly in the literature, potentially due to discrepancies in how the modulus is measured and due to the tendency of Matrigel to soften below 37 °C [50]. Therefore, accurate control over the temperature at which the modulus of Matrigel is measured is crucial to obtain a precise measurement. In addition, due to the complex nature of Matrigel, formed by more than 1800 unique proteins, its mechanical properties are mostly heterogeneous and local regions of hydrogels often exhibit elastic moduli several times higher than the average modulus of the sample [51]. Matrigel is also particularly unsuitable for clinical use or for organoids culture due to its murine tumour origin and its very high (up to 50%) batch to batch variation. Because of these downsides, research is slowly moving away from Matrigel to find a suitable replacement usable in clinical settings [51]. Nonetheless, to date, Matrigel is still considered the benchmark against which most other hydrogel materials are
In liver tissue engineering, Matrigel was used to increase the viability, metabolism, and functionality of precise liver slices encapsulated in microfluidics chambers designed as an in vitro model of liver metabolism. The authors showed that embedding liver slices in Matrigel prevented the slices from coming into contact with the polycarbonate surface of the microfluidics device, thereby increasing the viability of the cells over 72 hours [52]. Similarly, Matrigel was used as the support structure for liver organoids for more than 12 months [53]. Liver stem cells seeded onto the Matrigel substrate were able to differentiate into hepatic organoids and display hepatocytes function in vitro.

Collagen is a key component of the constitutional framework of most vertebrates and, as such, it is the most versatile and used material in tissue engineering. In particular, humans have 28 collagen like proteins with the most common being collagen type I, fibrillar collagen, which is the main constitutive component of connective tissues, proving structural support to bones, skin, tendons, cartilage and nerves [54]. Skardal and co-workers developed a collagen I based bioink for 3D printing and tested matrix organisation, printing proprieties, and cell biocompatibility and functionality [55]. The authors modulated the rheological properties of the bionik by varying the ratio between collagen I and hyaluronic acid (HA), showing that an increase in collagen led to a small increase in the elastic modulus, with G’ going from 2,000 Pa in 2:1 collagen I:HA to 3,500 Pa in 3:1 collagen I:HA. They also quantified hepatic stellate cell viability and showed overall good cell viability (> 80%) but more pronounced cell shape changes in the formulation containing an increase in collagen compared to HA, suggesting that collagen is primarily responsible for cell attachment and spreading. Finally, liver constructs containing primary human hepatocytes and liver stellate cells were successfully bioprinted and their functionality was maintained for two weeks, indicating the suitability of the bioink for liver tissue regeneration [54]. The combination of collagen and HA was also used to develop an artificial liver model to study alcoholic liver disease [54]. The elastic modulus of the collagen/HA hydrogel was in line with the one reported by Skardal and co-workers, 2,500 Pa, and the good proliferation and spreading of HepG2 cells supported the use of these 3D hydrogels as an engineered liver model. The model was then validated in vivo for the study of alcoholic fatty liver.
Alginate is a naturally occurring polymer typically derived from brown seaweed characterised by biocompatibility, ease of functionalisation, low cost, and mild gelation based on the addition of divalent cations, usually Ca$^{2+}$ [56]. These qualities have made alginate very attractive to tissue engineering and applications based on alginate scaffolds span wound healing, drug delivery, and bone tissue engineering. However, a critical issue in the use of alginate for biomedical applications is its reported toxicity due to the potential presence of impurities, such as heavy metals, endotoxins, and polyphenolic compounds linked to its marine origin [56]. Therefore, alginate has to undergo thorough purification before use to minimise immunogenic or inflammatory responses in vivo. Filippi et al. developed a high throughput screening platform to encapsulate hepatocytes in alginate scaffolds shaped as microdisks and test the encapsulation conditions [57]. The authors used a novel approach based on in situ crosslinking of the alginate directly in the cell culture wells via internal gelation using CaCO$_3$ and D-glucono-δ-lactone (GDL). This method yielded homogenous structures compared to the more traditional gelation method mediated by ion diffusion in the polymer network, where the uneven distribution of Ca$^{2+}$ leads to heterogeneous structures. However, the authors reported overall less functionality of the hepatocytes embedded in the microdisks produced by internal gelation compared to the those embedded in beads gelated via ion diffusion. This was ascribed to the lower oxygenation gradient of the microdisks due to their smaller size. Indeed, increased oxygenation on alginate fibres was shown to be crucial in the maintenance of hepatocytes viability. Seki et al. developed hepatic microorganoids on alginate hydrogel microfibres that were able to mimic in vivo hepatic cord structures and allowed long term (>90 days) preservation of hepatic functions and hepatocytes survival under high oxygenation [58].

Chitosan is a linear polysaccharide derived from the chemical deacetylation of chitin, the most abundant structural polysaccharide found in crustaceans. Chitosan is not only biocompatible, non-toxic and biodegradable, but it also presents antibacterial, antifungal, and antitumour activity [59]. However, the mechanical proprieties of chitosan are suboptimal, therefore combination of chitosan with other polymers, or the introduction of chemical crosslinks is commonly exploited to increase its stiffness [59]. Photocrosslinkable chitosan-based hydrogel sponges were used as effective haemostatic agents to prevent bleeding in rats’ livers and they were
proven to be comparable to the clinical gold standard, TachoComb, a fibrin coated collagen fleece commercialised as a topical haemostatic agent [60]. Interesting work by Taya et al. introduced phenolic groups in chitosan, thus obtaining hydrogels via peroxidase catalysed crosslinking (HRP with H₂O₂ as a substrate). The authors showed that the gelation kinetics of the chitosan hydrogels were dependant on the content of phenolic groups with fast gelation kinetics observed with increased HRP and decreasing H₂O₂ concentrations [61]. Similarly, the authors showed that incorporating galactose groups in the chitosan hydrogels increased their mechanical properties, but considerable reduction of the phenolic groups led to a decrease in the mechanical strength of the resultant gels regardless of the number of galactose groups substituted. HepG2 cells seeded onto the hydrogels showed good cell viability and mitochondrial activity but higher spheroid formation with higher galactose concentrations.

Cellulose is a linear polysaccharide composed of anhydrous glucose units and it is the primary structural component of green plant cell walls. Cellulose based biomaterials have attracted attention for tissue engineering applications especially due to their sustainability, availability, and renewability, given that cellulose can be extracted from waste biomass processing [62]. Cellulose nanofibril hydrogels were investigated to serve as suitable scaffolds for liver organoids. The hydrogel exhibited weak mechanical properties (G’ = 84 Pa) but great resistance to deformation (critical strain at 100%), and self-healing behaviour where G’ recovered quickly when the strain was lowered. However, the authors showed a decrease in metabolic activity as well as poor morphology of the liver organoids grown onto the cellulose hydrogels. This was ascribed to its weak mechanical properties and a potential mismatch in degradation rate [63]. Yliperttula and Urtti also investigated nanofibrillar cellulose hydrogels for liver tissue engineering. They also reported very weak mechanical properties of the gel (G’ = 10 Pa) but good self-healing and shear thinning properties that prompted the authors to use this system as an injectable application. The spontaneously formed gel after injection provided sufficient mechanical support for HepG2 and HepaRG cell growth and differentiation [64]. Yliperttula and Urtti increased the biocompatibility of their cellulose hydrogels by adding hyaluronan-gelatin (HG) to the scaffolds. The cellulose/HG hydrogels showed improved cell morphology, expression and localisation of hep-
atic markers, but, unfortunately, no rheological results were reported [65].

α-keratin is a protein naturally found in all vertebrates, particularly in human hair and horns, nails, claws and wool of mammals. The harder β-keratins are only found in reptiles and bird feathers. Keratin is characterised by a high amount of cysteine, required for the disulphide bridges that confer strength and rigidity to the material [66]. In tissue engineering, keratin has found widespread applications particularly in skin wound healing given its natural presence on the outer layer of skin. For example, injectable keratin-based hydrogels were used as wound dressing materials in liver injury models [67]. The authors showed that the mechanical properties and the gelation kinetics of the hydrogels were strongly pH dependant, where at higher pH values the G’ decreased (G’ = 1500 Pa at pH 3.5 and G’ = 150 Pa at pH 7.2). Due to the cytocompatibility requirements, the authors used the physiological 7.2 pH hydrogels for further in vitro and in vivo studies, showing good haemostatic ability in liver injury models and good drug delivery efficiency. The Van Dyke group has also used renewable keratin protein hydrogels for their haemostatic properties. They harvested keratin from local barber shops, extracted keratin using established protocols, and validated their product by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The keratin hydrogels performed well as haemostatic gels, by adhering on the surface of the wound and absorbing blood in a rabbit model of liver injury [68]. The Van Dyke group built upon their previous results by developing keratin based hydrogels, K31 and K81, that spontaneously formed viscous hydrogels with porous structures upon rehydration [69]. They reported low mechanical properties of the hydrogels (G’ = 2x10^2 Pa), but excellent cell adhesion, mediated by β1 and β3 integrins, and platelet activation in a porcine liver injury model.

Silk is a fibrous protein produced by silkworms (Bombyx Mori) and spiders in nature with unique properties suitable to tissue engineering. In particular, silk shows great mechanical strength, excellent biocompatibility, minimal immunogenicity, limited bacterial adhesion, and controllable biodegradability [28, 70]. Kaplan’s extensive research on silk has found recent applications in liver tissue engineering. Pure silk fibroin extracted from Bombyx Mori was used to develop silk/collagen composite sponges to support the long-term culture of primary human
hepatocytes [71]. Silk was combined with collagen using three different methods, passive collagen absorption onto the silk scaffold (1), lyophilized silk autoclaved directly in a collagen solution (2), and collagen containing silk scaffold autoclaved once more in a collagen solution (3). The hepatocytes cultures were successfully maintained for 1 month but the scaffolds showed different levels of cell adhesion and metabolism. In particular, the third scaffold showed increased functionality, albumin production, and enzyme activity due to the increased levels of collagen that supported hepatocytes cell attachment. Ghosh and co-workers combined Bombyx Mori silk with decellurised liver (DCL) to produce a bioink suitable for 3D printing and hepatic differentiation [72]. The reported G’ was somewhat dependant on the ratio between DCL and silk, with an increase in DCL leading to less dependence on frequency of the hydrogels, indicative of increased stability. However, all silk-DCL hydrogels presented essentially the same weak viscoelastic properties with G’ = 10\(^1\) Pa. The addition of covalent crosslinking via the tyrosinase enzyme led to a significant increase in the storage modulus with G’ = 10\(^4\). The Silk/DCL scaffolds were successfully 3D printed and maintained the differentiation and function of hepatic cells, whilst elevating the regulation of liver tissue specific genes and protein expression when compared to 2D culture models. An interesting take on silk hydrogels was developed by Lu et al. who created ferrimagnetic silk fibroin hydrogels by embedding hydrophilic iron oxide nanocubes (IONCs), responsible for ferrimagnetism, into an injectable silk hydrogel [73]. The authors showed excellent self-healing properties of the hydrogels, crucial for injectable applications, that were not altered by the addition of IONCs at low concentrations, and acceptable mechanical properties (G’ close to 10\(^3\) Pa). Thanks to the excellent biocompatibility of silk that masked the ferrimagnetic nanocubes, the hydrogels showed comparable cell viability to control. The authors validated their hydrogels in vivo by injecting them into rabbit hepatocellular carcinomas via an ultrasound guided intervention that exploited their ferrimagnetic nature, resulting in effective ablation of deep located tumours.

Other natural polymers, such as fibrin and gelatin often in combination, have been successfully applied in liver tissue engineering as hydrogels able to sustain the culture of liver organoids or as haemostatic agents in haemorrhaging livers [74–77].
In summary, hydrogels based on natural polymers are particularly advantageous due to their biocompatibility, abundance, and presence of biological cues, such as cell adhesion sequences, that can guide cell reorganisation, Fig. 1.7 [24]. However, natural polymers are often characterised by weak mechanical properties and batch-to-batch variability due to their complex chemical structures and thermal sensitivity, and by poor processing using biofabrication techniques [28]. Therefore, tissue engineering often exploits combinations of natural polymers with synthetic ones to confer stiffness to natural hydrogels.

Nevertheless, both natural and synthetic polymers are limited by their chemical composition. Functionalisation techniques and rational blending between synthetic and natural polymers does create suitable scaffolds, but it is impossible to genetically modify the structure of either polymer or alter their backbones to further increase the complexity and adaptability of the resulting materials. Given the complexity of human physiology, a new class of biomaterials based on chemically synthesised peptides and recombinant protein hydrogels has emerged [27]. Thanks to the increased control over the protein building blocks offered by protein engineering, this new class of materials is poised to develop highly tuneable hydrogels that combine the biological cues of natural polymers with the flexible mechanical properties of synthetic polymers, thereby increasing the sophistication and versatility of biomaterials [26, 27].
Figure 1.7: **Schematics of natural polymer based hydrogels.** Hydrogels composed of natural polymers have biological cues, such as cell adhesion sequences, that guide cellular reorganisation, but they often present weak mechanical properties. Created with BioRender.com.
1.1.4 Protein hydrogels

The aim of this project was to develop fully functional 100% recombinant protein hydrogels. This is because the macroscopic properties of the hydrogels that have to mimic the physical and biological properties of the native tissue, can be encoded into the microscopic building blocks of the proteins, inducing a high level of customisation and tailorability in the hydrogels that would not be necessarily possible using naturally occurring or synthetic polymers. By changing the protein sequence used and the crosslinking methods exploited to create protein-based hydrogels, researchers are able to tune the properties of the resulting networks, increasing stimuli-responsiveness, controlling pore size, modulating degradation, and enhancing biocompatibility [78]. Of note, mammalian cells encapsulated in hydrogels secrete matrix metalloproteinases (MMPs) over time as they get used to a new microenvironment. MMPs can degrade peptide/protein-based hydrogels and weaken their mechanical stability [79]. However, MMPs secretion can also be used an advantage by rationally designing and genetically encoding for MMP cleavage sites inside the hydrogels, thus modulating the degradation of the hydrogels in vitro and in vivo.

In general, folded protein hydrogels are mechanically robust and their ability to withstand an applied force resulting in elastic properties is crucial to multiple physiological processes. For example, the ability of major components of the ECM to extend and contract allows for better cell migration and adhesion [80]. Amongst several other techniques, atomic force microscopy (AFM) has been used to measure the forces that occur upon folding and refolding of single protein domains [81]. Briefly, there are two methods of operations used to stretch proteins on an AFM, force-extension mode and force-clamp mode. In AFM force-extension, a single protein is stretched between the tip of a flexible cantilever and a flat surface at a constant velocity, causing the cantilever to bend. However, at a certain protein dependant force, one of the domains of the proteins unfolds, causing the cantilever to fall, determining the mechanical stability and mechanical strength of the protein. Conversely, in AFM force-clamp mode, a single protein is held at a constant stretching force and the cantilever is kept at a constant deflection for a fixed period of time, allowing to measure the unfolding process as a measure of time [80]. AFM
has shown that many proteins generate, sense, transmit, or are subjected to mechanical forces during their normal functioning and that different proteins display widely different mechanical stability [82].

Of particular interest for folded protein hydrogels is the ability of AFM experiments to investigate the strength of a ’mechanical clamp’, a combination of elements of the secondary structure localized at the break point of a protein, that represent the main mechanical barrier to stretching [82]. Mechanical clamps are at the base of a protein’s mechanostability and their widespread presence in proteins derived from bacteria, including E.coli, suggest that this functional motif is necessary to maintain the appropriate mechanostability and structural integrity in vivo [82, 83]. Clearly, in order to confer the appropriate stability and strength to the resulting folded protein hydrogels, it is crucial to ascertain the mechanical strength of the single proteins involved into the formation of the matrix.

In recent years, AFM force spectroscopy experiments on protein folding and investigation of the stability of mechanical clamps have provided careful insight into the folding pathways and mechanical stability of single proteins, revealing hidden complexities and enriching our understanding of the intricate force interplay in protein hydrogels.

Protein hydrogels can be primarily subdivided into physically entangled systems and permanently crosslinked hydrogels, Fig. 1.8. In physical hydrogels, the protein chains entangle, typically via hydrogen, ionic or hydrophobic bonds, or crystalline formation and create transient junctions, resulting in overall weaker hydrogels and reversibility of the bonds. Conversely, in chemically crosslinked hydrogels the covalent bonds are not reversible and the hydrogels normally present stronger mechanical properties [22].
Figure 1.8: **Physically entangled VS chemically crosslinked hydrogels.** In physical hydrogels the protein chains entangle via hydrogen, ionic or hydrophobic bonds and create transient and reversible junctions. In chemically crosslinked hydrogels the covalent bonds are not reversible and the hydrogels present stronger mechanical properties. Created with BioRender.com.
1.1.4.1 Physically entangled systems

Molecular self-assembly is the association of molecules into defined structures via non covalent intermolecular interaction such as hydrogen bonding, coulombic, van der Waals, and electrostatic interactions. These structures are not the result of random aggregation, but rather specific control over the nanostructure and hierarchical order [84]. Physically entangled systems based on the self-assembly of peptides allow for the efficient production of materials with tailored properties, thus molecular self-assembly has found widespread application in tissue engineering [85]. In particular, hydrogels based on self-assembled peptides are primarily used due the reversibility of their non-covalent bonds in response to changes in the environments, such as pH, temperature or salts, and their consequent ability to re-heal after shear. However, it should be noted that most self-assembled peptides are chemically synthesised, thus the process is expensive and hard to scale-up [86]. Moreover, chemical synthesis generates substantial petroleum based waste, and as such, it is not very sustainable [86]. Herein, I will give a brief overview of the most common self-assembly peptides used to create biocompatible hydrogels.

Small peptide hydrogelators

Short peptides of low molecular weight characterised by less than 10 amino acids, have been investigated for their ability to self-assemble and form biocompatible hydrogels. Fluorenylmethoxycarbonyl (Fmoc) and naphtalene (Nap) based hydrogelators are the most commonly used groups in tissue engineering.

Fmoc – protected dipeptides self-assemble into strong supramolecular nanofibers networks via hydrogen bonding and $\pi-\pi$ interactions in aqueous environments [87]. The strong mechanical properties of Fmoc hydrogels were correlated to the $\beta$-sheet secondary structure of the peptide nanotubes, stabilised by the $\pi-\pi$ interactions between the aromatic rings [88]. Hydrogels composed of self-assembled Fmoc peptides have found widespread application in the literature, due to their biocompatibility, ease of chemical modification, and strong mechanical properties. Seminal work by Ulijn et al. and Gazit et al. simultaneously reported the use of an aromatic phenylalanine–phenylalanine dipeptide, FF, bearing the Fmoc group, Fmoc-FF, to form supramolecular hydrogels used as scaffolds for cell culture [88, 89]. Ulijn et al. analysed a library of seven Fmoc-dipeptides forming at
pH < 4 and found that the gelation pH of the Fmoc-peptides could be fine-tuned by mixing different peptides or by adding a basic Fmoc-amino acid, lysine, into the mixture, thus creating Fmoc based hydrogels stable at physiological pH 7. Gels that were stable at physiological pH were then successfully used as 2D and 3D scaffolds for the support of chondrocytes. Of note, in their analysis, Uljin and co-workers mention that apparently more stable gels are formed when mixing different peptides (Gly-Gly and Phe-Phe) at 50:50 compared to either pure peptide materials, suggesting the presence of a highly cooperative network [89]. Gazit et al. quantified the stiffness of Fmoc hydrogels by carrying out rheological experiments on the materials. They found that the stiffness of the hydrogels increased with increase in peptide concentration ($10^3 < G' < 10^5$ Pa) and that the hydrogels were strong and rigid. They correlated this mechanical strength to the $\beta$-sheet structure and the aromatic nature of the Fmoc-FF peptide. They also demonstrated the potential of Fmoc hydrogels as a drug delivery platform, by encapsulating fluorescent molecules in the hydrogels and monitoring their slow release into its aqueous environment [88].

Following on from Ulijn et al. and Gazit et al., several other research groups made use of Fmoc – dipeptides functionalised with the adhesion motif RGD to form supramolecular hydrogels. For example, Schwartz et al. improved hepatocytes survival and functionality on Fmoc-RGD hydrogels [90], Zhuo et al. further highlighted the potential applications of Fmoc-RGD hydrogels as implantable drug delivery systems in ophthalmology [91], and de Cienfuegos et al. combined Fmoc-RGD peptides with fibrin improving the mechanical properties and biocompatibility of the composite hydrogel [92].

Similar to Fmoc dipeptides, Xu and co-workers reported a new class of molecular Nap-based hydrogelators, formed by conjugates between dipeptides and naphthalene, a common fragment in clinically approved drug molecules, that gave rise to self-standing hydrogels in water at very low concentrations at pH ≈ 2 [93]. Xu et al. proposed that the supramolecular arrangements of Nap-based hydrogels rely on hydrogen bonding between dipeptides and $\pi-\pi$ stacking of the naphthyl groups, which leads to the 3D supramolecular nanofibers confining water and forming hydrogels. The research group analysed the stability of their Nap-based hydrogels, determining that steric hindrances caused by large side chains cause the for-
formation of very weak gels and further increases in steric congestion hinders their formation completely. They also quantified the stiffness of the Nap hydrogels, comparable to that of average Fmoc hydrogels (\(G' = 5,000\) Pa for their best hydrogelator). The authors also quantified the biocompatibility of the hydrogels and showed excellent cell viability (nearly 100%) in the presence of Nap hydrogels. However, it is unclear how they offset the acid environment in which the hydrogels are formed [93].

Adams et al. moved forward the understanding of Nap-based hydrogels by controlling the kinetics of gelation and analysing their effects on the resulting mechanical properties of the materials [94]. The authors used glucono-\(\delta\)-lactone (GdL) to control the final pH of the Nap hydrogels, which in turn regulated the kinetics of self-assembly. They probed the mechanical properties of the hydrogels formed with different concentrations of with GdL using rheology. Remarkably, they found that despite the different kinetics pathways in which the networks are formed, the final plateau values of \(G'\) and \(G''\) are very similar, suggesting that the final hydrogels all have similar mechanical properties. However, the impact of the kinetics of gel formation is visible in the amplitude sweeps where the breakdown in the hydrogels structure can be correlated to the volume of GdL. In particular, the hydrogels formed with less GdL at higher final pH (2.94 mg/mL and 4.46 mg/mL and pH = 4 and 4.5 respectively) were able to sustain less deformation breaking at 1% strain, whilst the hydrogels prepared using higher concentrations of GdL and lower final pH (14.42 mg/mL and pH = 3.1) were more stable under external forces, with critical strain at 5%. Given that the final properties of the hydrogels were very similar, but the kinetics of assembly had a clear effect on the ability of the networks to withstand strain, the authors proposed that the crosslinks between the fibres are different, rather than the fibres themselves. This was consistent with the findings of Pochan and Schneider that demonstrated how control over the gelation trigger (temperature in this case) of a de novo tree-stranded \(\beta\)-sheet hydrogel, TSS1, led to changes in the network morphology and temperature dependant reactions [95].
In tissue engineering, Nap based hydrogelators have been used to enhance the therapeutic efficiency of mesenchymal stem cells in ischemia [96], and as 3D scaffolds for cell culture, where they improved cell viability compared to Matrigel, the current gold standard in hydrogels [97].

An interesting physical hydrogel system named MITCH (Mixing-Induced Two-Component Hydrogel) based on two short recombinant protein polymers C7 and P9, CC43 WW domain and the proline rich PPxY ligand respectively, has been explored for tissue engineering applications. Beyond the critical concentration, MITCH chains begin to entangle and form a semi dilute entangled solution which transitions into a hydrogel. The networks structure of MITCH is transient and is not only responsive to changes in temperature, pH, and ionic strength, but the authors also showed how changes in the molecular composition of the building blocks, namely the crosslinking density between C7 and P9, affect the bulk properties of the resulting hydrogel [98]. MITCH was also shown to be an excellent candidate for injectable applications as it improved retention of adipose derived stem cells in mouse after subcutaneous injection [99]. However, the reported mechanical properties of MITCH were very weak (≈30 Pa), and MITCH conjugation with a PEG crosslinker was not successful in increasing the reported storage modulus [100].

**Hydrogels based on α-helix forming peptides**

The coiled-coil is one of the major structural motifs for proteins, normally involving a seven residue heptad unit, abcdefg, where positions a and d are generally occupied by hydrophobic residues and e and g are often charged residues. The hydrophobic interaction between a and d and the electrostatic interactions between e and g are primarily responsible for the stability of the coiled-coil structure. Coiled-coil protein folding domains have been used as physical crosslinks for protein hydrogels as they are based on two or more stable α-helices looping around each other to form a superhelix.

One of the most common hydrogelation approaches driven by coiled-coil formation is based on triblock proteins ABA consisting of short leucine zippers end blocks (A) flanking a middle random coil block (B). Seminal work by Tirrell and
co-workers showed that self-assembly of leucin zippers flanking a water-soluble polyelectrolyte domain led to the formation of a transient hydrogel network, that was reversed to solution at high pHs or temperatures, due to the denaturation of the leucine zippers [101]. Shen and Tirrell extensively characterised hydrogels based on leucine zipper domains. They showed control over the hydrogel properties via the formation of disulphide bonds that stabilised the protein hydrogels near physiological pH, broadening the scope of these materials in tissue engineering [102]. Moreover, whilst investigating the dynamic properties of leucine zipper hydrogels, they found that ABA hydrogels have a strong tendency to form looped configurations to avoid stretching the middle block, but changes in pH or ionic strength that expand the middle block increased the formation of bridges throughout the networks, which led to an increase in the storage modulus of the hydrogels and a longer relaxation time [103, 104].

Following on from Tirrell, Kopeček et al. also showed a similar coiled-coil ABA system where two terminal coiled-coil domains flanked a central water soluble random coil segment (B) [105]. Using microrheology, Kopeček et al. showed that increasing the concentration of the hydrogelators led to the transition of the system from purely viscous materials to elastic solids. Interestingly, hydrogel formation was not only dependant on protein concentration but it was also correlated well with the oligomerization state of the coiled-coil domains. Therefore, in the same concentration range, the polymer showing evidence of association from a dimer to a tetramer required lower concentration to form an elastic solid compared to the polymer showing primarily monomer to dimer association. Similarly to Tirrell, Kopeček also demonstrated the reversibility of gelation via the addition or removal of guanidine hydrochloride that denaturated the coiled-coil domains [105, 106].

More recent work has explored the uses of leucine zippers for tissue engineering. For example, George and co-workers developed self-assembly leucine zipper hydrogels with calcium binding motifs for the regeneration of dentine and bone [107], and Rodríguez-Cabello et al. developed an elastin-like block flanked by leucine zippers able to sustain cell culture [108].
The Regan group has also developed helix based hydrogels, using the 34 amino acid tetratricopeptide repeat (TPR) domains concatenated into arrays and crosslinked with their corresponding peptide attached to a 4-arm-PEG crosslinker. A single TPR unit adopts a helix-turn-helix structure and when repeated in tandem TPR forms a superhelical structure. The TPR based hydrogels formed spontaneously without the need for chemical crosslinking agents via multivalent peptide ligands. The hydrogels exhibited weak mechanical properties ($G' = 270$ Pa) but excellent stimuli-responsiveness, as the junctions forming the networks were both pH and ionic strength dependant allowing for reversible gelation [29]. The authors also explored the role of the concentration and the ratio of the components on the kinetics of gelation, showing that the rate of gelation is strongly dependant on the concentration [109]. Interestingly, when exploring the influences of the ratio of the components on the kinetics of gelation they found that when the TPR:PEG are equimolar 1:1, the hydrogels form slowly and when the PEG ratio is increased 1:2, hydrogels form significantly faster. However, when the TPR component is present in excess compared to the PEG crosslinker 2:1, a hydrogel does not form. This was ascribed to an excess in peptide binding sites available where the majority of the protein-peptide interactions will not lead to productive crosslinking and the formation of a percolating network. Finally, the authors also showed remarkable biocompatibility of the hydrogels and great potential of the system as drug delivery vehicles thanks to the successful encapsulation and release of an anticancer compound [109].

**Hydrogels based on $\beta$-sheet forming peptides**

$\beta$-sheet is a structural motif of peptides containing alternating hydrophobic and hydrophilic residues present in natural proteins such as silk fibroin that has been explored to design self-assembled peptide based hydrogels. $\beta$-sheet self-assembly is driven by the interactions of the hydrophobic residues, repulsed by aqueous media, and the hydrogen bonds forming between the polar residues and the water molecules [110].

$\beta$-hairpin peptides have been designed to assemble in physically crosslinked hydrogels. Pochan and Scheider showed that the addition of salts to the twenty amino acid peptide MAX1 allows the peptide to transition from random coil for-
formation to \( \beta \)-hairpin structures and self-assemble into a rigid fibrillar networks (\( G' > 1500 \) Pa) [111]. The self-assembly kinetics can be fine-tuned by varying peptide concentration, ionic strength, and temperature. Pochan and Scheider also confirmed both the non-cytotoxicity of their hydrogels using NIH 3T3 cells, their inherent antibacterial activity, and their ability to self-heal [111, 112]. A variation of MAX1, named LNK1, was designed to pack specifically into a lock and key structure by replacing four valine residues with the bulky non-natural 2-naphthylalanine amino acid at the N and C termini flanking a single alanine residue [113]. This lock and key structure was built specifically to hinder branching of the fibrils during assembly, which in turn led to the inability of LNK1 to recover after shearing, highlighting how molecular design can control and modulate the rheological properties of hydrogel networks. Variants of MAX1, KVW10 and EVW10, also showed remarkable self-healing properties after 200% shear strain, suggesting great potential for injectable applications, but their biocompatibility has not been confirmed yet [114]. In tissue engineering, \( \beta \)-hairpin hydrogels have found widespread use as platform for drug discovery thanks to their ability to be injected under shear flow and heal into a hydrogel [115].

RADA16 (RADARADARADARADA) and its commercially available version, Puramatrix, is composed of 16 alternating hydrophobic and hydrophilic amino acids able to self-assemble into nanofibers and form hydrogels. RADA hydrogelation is based on ionic interactions between aspartic and arginine residues, hydrophobic interactions between the alanine residues, and hydrogen bonding associated with the \( \beta \)-sheet type structure [116]. Other common \( \beta \)-sheet derived hydrogels, such as KLD12 and EAK16, have similar self-assembly principles into supramolecular nanostructures and they have been used extensively in tissue engineering. For example, Puramatrix has been shown to sustain cell culture of hepatic cells [116], human foetal Schwann cells [117], and dental pulp stem cells [118], KLD12 based hydrogels has been particularly successful for chondrogenesis and cartilage repair [119–123], and EAK16 hydrogels enabled antibody deposition in vivo [124] and drove increased osteogenic cellular attachment [125].
FEK16 and K24 have also been investigated for their ability to form β-sheet fibrillar gel in aqueous solution, but their biocompatibility is yet to be proven in vitro or in vivo, despite the research groups suggesting the uses of these materials in tissue engineering [126, 127].

Hydrogels based on peptide amphiphiles

Peptide amphiphiles (PAs) are typically composed of a hydrophilic peptide sequence covalently bonded to a hydrophobic alkyl chain. PAs can self-assemble into supramolecular nanostructures thanks to hydrophobic interactions between the aliphatic carbon tails and by hydrogen bonding of the peptide moiety that directs nanofibers β-sheet assembly [110].

Stupp et al. first characterised and reported the biocompatibility of PAs hydrogels by demonstrating that a solid gel was formed in vivo in rats after injection of a PA solution mixed with neural cells [128]. Stupp et al. also demonstrated how variations in the β-sheet sequence of the PAs is an effective method to control the stiffened of the resulting hydrogels. The authors found that substituting valine resides with alanine whilst keeping the peptide length constant, led to a dramatic change in the mechanical properties of the resulting hydrogels, where the G’ of VVVVAA was twice that of VVAAAA [129]. It is important to notice that Val has a stronger likelihood to form β-sheet whilst Ala promotes α-helix formation, thus the increased mechanical strength showed by the Val hydrogels is unsurprising. Moreover, they also showed a decrease in G’ when ala residues were present closer to the hydrophobic core (AAAVVV) compared to the periphery regions (VVVAAAA), thus demonstrating that the hydrogen bonds closer to the hydrophobic core of the fibril are more important than the hydrogen bonds closer to the hydrophilic regions [129].

Further work by the Stupp group on the mechanics of PA nanofibers gels showed how the gelation kinetics, stiffness, and strain recovery change based on the triggering gelling agent, either HCl or CaCl₂ [130]. The authors showed that final mechanical properties of the PA hydrogels formed via HCl or CaCl₂ are very similar, but their behaviour under strain is drastically different, insofar as the CaCl₂-PA hydrogels could sustain up to five times more strain than the HCl-PA hydrogels. This is quite remarkable as the two different self-assembly triggers lead to different
gel formation interactions, where the calcium ions form electrostatic bridges between molecules and changes in pH neutralise the charges of the acidic residues. Interestingly, they also showed that CaCl$_2$-PA hydrogels formed slower (approximately 1.5 hours before they reached the same modulus as HCl-PA hydrogels), potentially due to slower diffusion of the calcium ions throughout the hydrogel network, and they are unable to recover as quickly as the HCl-PA hydrogels once plastic deformation has occurred [130]. Overall, the authors showed very interesting mechanical changes where the G’ values of the hydrogels were essentially the same, but strain resistance and speed of gelation were remarkably different, showing how simple changes in hydrogel formation can lead to tuneable properties. Further work confirmed the successful use of PA hydrogels as injectable applications primarily in neural tissue engineering [131–133].

**Hydrogels based on elastin-like polypeptides**

Elastin is a naturally occurring protein in the human body, particularly important in tissues such as blood vessels and skin, where elasticity is crucial for the correct function of the organ. Derived from human tropoelastin, elastin like polypeptides (ELPs) contain the characteristic pentapeptide repeat unit VPGXG, where X is any residue but proline that does not allow helix formation at the fourth position [134]. ELPs retain the core properties of naturally occurring elastin and, due to their innate biocompatibility and cell attachment features, have found widespread applications as hydrogels [134]. ELPs are reversible thermally responsive polypeptides that gel by lower critical solution temperature (LCST) phase behaviour, therefore ELPs are highly soluble in aqueous solution below the transition temperature, T$_t$, and they aggregate into coacervates when heated above T$_t$ [135]. The choice of the amino acid residue X in the VPGXG pentapeptide is crucial to determine the inverse temperature transition properties of the ELPs, namely hydrophobic residues reduce the transition temperature whilst hydrophilic residues increase it [136].

The LCST behaviour of ELPs has been successfully exploited to create injectable formulations where a defect must be filled. Early work by Chilkoti and co-workers paved the way for genetically engineered ELPs for the reparation of cartilage. The authors characterised the effects of temperature induced phase transition on the shear properties of ELPs solutions, showing that ELPs that coacervates at
35°C had rheological properties similar to collagen. Moreover, they also confirmed the biocompatibility of ELPs coacervates by successfully incubating chondrocytes in ELPs solutions [137]. More recent work by the Chilkoti group has built upon their previous findings and combined four structured polyaniline helices into three disordered ELPs inducing the hierarchal assembly of porous viscoelastic networks with improved the mechanical properties (G' = 12.2 kPa compared to only ELPs G' = 23 Pa). These hybrid networks remained reversible, but their aggregation and dissolution temperature were altered based on the ratio between ordered and disordered domains in the initial peptide design. The materials were also injected in vivo and showed little to no immunogenic reaction over time and increase vascularisation, indicative of tissue growth and wound healing [138].

However, the mechanical properties of ELPs coacervates are often too weak (20-100 Pa) to successfully mimic the viscoelastic properties of native tissue, therefore attempts were taken to increase the rheological properties of ELPs via physical crosslinking [139]. The first example of physically crosslinked ELP hydrogels was demonstrated by Urry and co-workers that used radiation to crosslink ELPs into hydrogels. The authors demonstrated that γ-radiation can successfully stiffen the hydrogels and that as the dose of radiation increases, the modulus of the hydrogels increases, indicating an increase in crosslinking density [140]. For example, hydrogels irradiated with 15 Mrad presented G' = 50 kPa, whilst hydrogels irradiated with 30 Mrad had G' = 200 kPa [140]. However, as most hydrogels are developed for biomedical applications, irradiation is not suitable as a physical crosslinking strategy. More recent work has shown that arresting the phase separation of concentrated solutions of ELPs can yield extremely stiff networks with shear moduli close to $10^5$ Pa. The authors found out that ‘plastic’ ELPs (XPAG)n above 20 wt % in water form a translucent stiff hydrogel by reversible thermoresponsive gelation and they demonstrated that the stiff gelation is due to hydrophobic chain collapse above the transition temperature and the critical concentration 15 wt % [141]. Alternatively, further work by the Chilkoti group created genetically engineered ELPs containing a large hydrophobic block and a hydrophilic terminus that were crosslinked via metal ion coordination by adding zinc ions [142]. First the diblock polypeptide was thermally triggered to self-assemble and then reversibly crosslinked via metal ions into hydrogels. These hydrogels presented increased mechanical properties
(G’ = 10^5 Pa), remarkable self-healing properties, and they were reversible via the addition of a strong metal chelator. However, there are concerns regarding the biocompatibility of these hydrogels as zinc ions do present a level of toxicity. Of note, self-healing materials are materials able to recover, fully or partially, their original set of properties after they have been damaged by excessive external forces, meaning that self-healing materials can regain their original shape and mechanical properties after a cycle of loading and unloading of external stress. Usually, physically crosslinked hydrogels present a degree of self-healing as the noncovalent bonds can reversibly undergo repeated cleavage and reformation under mild conditions [143]. Self-healing materials have attracted widespread interest in tissue engineering because their ability can be exploited to expand the lifetime of the hydrogel and increase the reliability of the materials [144].

Another common application in the literature is the incorporation of residue repeats from silk into ELP, creating silk-elastin-like polypeptides (SELPs) as the silk blocks are able to crystallise into β-sheets and increase the mechanical strength of the resulting material. Moreover, by rational design of the ratio between silk and ELP building blocks, the material’s structure, strength, and biodegradability can be fine-tuned. Kaplan and co-workers have published extensively in the field and developed tuneable biocompatible SELPs able to self-assemble into nanoparticles under mild conditions and encapsulate drug or other biologically active domains [145–147].

However, the most common method to enhance the mechanical properties of ELP hydrogel is by introducing covalent crosslinking into the system, thus creating a chemically crosslinked hydrogel rather than a physically crosslinked one.

1.1.4.2 Chemically crosslinked hydrogels

Chemically crosslinked hydrogels generally present stronger mechanical properties compared to physically crosslinked ones due to their non reversible bonds and, as such, they have found widespread applications in tissue engineering [22]. Hydrogels can be chemically crosslinked via photopolymerization, enzymatic crosslinking, and other methods. However, it has to be noted that a crucial down-
side of chemically crosslinked hydrogels is the presence of a crosslinking agent, that is often cytotoxic. Herein, I will give a brief overview of the most commonly used chemically crosslinked methods for hydrogels suitable for tissue engineering.

**Hydrogels based on photopolymerisation**

Photopolymerisation is based on a light source that triggers gel formation. Photopolymerisation is generally applied on materials containing a methacrylate group that allows photoinitiation thanks to the presence of a double bond that makes the compound highly sensitive to UV or LED radiation with consequent induction of chain polymerisation [22]. Photopolymerisation is highly advantageous as rational design can allow for region selectivity of crosslinking under mild conditions and temperatures. Moreover, changes in the concentration of the photoinitiator, the wavelength and the power of the light, the distance of the light to the sample, and the exposure time, can induce a wide variety of controllable properties in the resulting hydrogels. However, the main drawback of photocrosslinking in tissue engineering is the obvious need for a photoinitiator and prolonged exposure to irradiation, often incompatible with cells. Moreover, prolonged exposure to light could cause local temperature rise, increasing the cytotoxicity of the material.

Most commonly, the mechanisms of photocrosslinking via blue LED are based on the presence of tyrosine (Tyr) residues in the proteins that induces crosslinking between reactive species. For example, the Dougan group used bovine serum albumin (BSA) based hydrogels with tyrosine crosslinking residues as a model to investigate network architecture and the mechanics of protein hydrogels [148] as well as Tyr induced crosslinking of maltose bound protein (MBP) to tune the mechanisms of entanglement and the macromolecular mechanics of protein hydrogels [149, 150]. Other common crosslinking residues are phenylalanine (Phe) and histidine (His).

However, there are still concerns linked to the potential cytotoxicity of these crosslinking strategies and substantial work has been carried out to improve the biocompatibility of photocrosslinking. For example, Camci-Unal and co-workers developed BSA – glycidyl methacrylate (GMA) based hydrogels chemically photocrosslinked using the biocompatible photoinitiator Irgacure 2959 that produces
free radicals upon irradiation with UV light, initiating photopolymerisation. Un-
surprisingly, the authors showed that an increase in GMA induces stronger hydro-
gels due to the increased number of crosslinks and consequently smaller porosity. 
However, as expected the higher levels of GMA also reduced cell viability and 
the smaller porosity caused significant limitations in cell migration and elongation 
within the scaffold [151]. Interesting further work exploited the use of riboflavin 
(Rib) or vitamin B2, as a biocompatible photocrosslinker for BSA hydrogels. Rib 
has absorption bands near the UV and the visible light region and present essential 
roles in tissue regeneration. However, Rib alone cannot initiate crosslinking, but it 
needs the presence of an electron donor co-initiator, such as an amine that generates 
the free radicals needed for gelation. Rusu et al. made use of this biocompatible 
photoinitiator by combining Rib and L-arginine, acting as an amine. The extent of 
crosslinking was dependant on the concentration of Rib, which in turn regulated 
porosity, thermal stability, and swelling. The authors demonstrated the increased 
biocompatibility of this BSA-Rib system after oral administration in mice [152]. 

Photopolymerisation is often combined with physical thermal polymerisa-
tion creating a partially reversible dual crosslinked hydrogel. For example, Park 
and co-workers recently improved the mechanical properties of a thermally respon-
sive methylcellulose based bioink via tyramine based photocrosslinking. The dual 
crosslinking system allowed for the tailoring of the extrusion properties based on 
both temperature and visible light and their rheological properties were fined tuned 
by tailoring the concentration of the photoinitiator and the duration of irradiation. 
The biocompatibility of the system was retained by incorporating photosensitive vi-
tamin B2 derivatives during crosslinking, similarly to Rusu et al., and by crosslink-
ing the system under visible light to prevent UV induced cytotoxicity [153].
Hydrogels based on enzymatic crosslinking

In general, enzymatic crosslinking uses transglutaminases or peroxidases based enzymes due to their mild reaction environment and crosslinking efficiency [154]. Transglutaminases catalyse the formation of a covalent bond between the γ-carboxamide group of peptide-bound glutamine and the free amine groups of protein or peptide-bound lysine whilst the most common peroxidase, horseradish peroxidase (HRP), catalyses the phenol and aniline derivatives conjugation using \( \text{H}_2\text{O}_2 \) as a substrate [154].

Recent work on transglutaminase mediated covalent crosslinking of Bambara protein isolate demonstrated the formation of protein hydrogels (G’ = 10^3) when the enzyme was added and no gel was observed in the absence of the enzyme [155]. As expected, the enzyme also played a crucial role in tuning hydrogel’s porosity and textural properties. Other groups have used HRP to enzymatically crosslink tuneable silk-based hydrogels and demonstrated how increasing \( \text{H}_2\text{O}_2 \) elevated the crosslinking density and in turn induced stronger mechanical properties, but at the expense of lowering the biocompatibility of the hydrogels [156,157]. To overcome the toxicity of \( \text{H}_2\text{O}_2 \), Oliviera et al. developed HRP-crosslinked protein hydrogels using calcium peroxide \( \text{CaO}_2 \) as a substrate. However, the authors noticed an increase in cell death particularly in the hydrogels crosslinked with \( \text{CaO}_2 \) compared to the ones crosslinked with \( \text{H}_2\text{O}_2 \), ascribed to the presence of calcium ions in the hydrogels and the hydrogel’s transition to a crystalline \( \beta \)-sheet conformation, that has been linked to reduced cell viability and apoptosis [158].

Hydrogels based on other crosslinking methods

Chemically crosslinked hydrogels can be synthetised via ‘click’ chemistry, formation of a Schiff base, and via Michael addition.

Broadly, click chemistry involves cycloaddition reactions, such as the selective Diels-Alder reaction between a diene and a substituted alkene, a dienophile, or the more common copper catalysed alkyne-azide cycloaddition reaction [22]. Click chemistry reactions are characterised by high efficiency and good hydrolytic stability, crucial properties for the facile encapsulation of cells in hydrogels, thus interest around click chemistry reactions in tissue engineering has grown [159].
Recently, Heilshorn et al. have developed rapid Diels-Alder crosslinked PEG hydrogels by substituting the commonly used diene furan with the electron rich cyclic diene fulvene [160]. The authors showed a 10 - fold increase in the gelation kinetics of the PEG hydrogels with the fulvene based hydrogels reaching complete gelation in 20 minutes rather than the 10 hours needed by the furan based hydrogels. The improved gelation kinetics allowed for the encapsulation of mesenchymal stem cells with improved cells distribution throughout the hydrogels compared to furan crosslinked hydrogels where the authors observed significant cell sedimentation. However, it has to be noted that the G’ reported for all the Diels-Alder hydrogels was 100 Pa, hindering the use of these hydrogels for more robust applications in tissue engineering. Successful attempts to increase the storage modulus of hydrogels using Diels-Alder reactions have been carried out. For example, Kimura et al. increased the crosslinking density of PLA and PEG hydrogels using the chain-extending Diels-Alder reaction between the furanyl terminal of PEG coupling with 1,8-bis(maleimido)diethylene glycol (BMG). The authors reported an increase in G’ from 3 kPa to 11 kPa in the presence of the coupling agent BMG. However, this system still showed partial gel-to-sol transition with increasing temperature, limiting its applicability with cells [161].

Another ‘click’ chemistry reaction is based on the copper catalysed alkyne-azide cycloaddition reaction. Similarly to the Diels-Alder reaction, this reaction is also characterised by fast gelation kinetics and high yields. Recently, Heise et al. developed chemically crosslinked polypeptide interpenetrating networks (IPNs) based on the copper-catalysed alkyne–azide cycloaddition of azide-terminated PEG crosslinkers and alkyne functional polypeptides [162]. The authors reported good mechanical properties of the IPNs hydrogels that increased with the increased number of PEG arms from bis-PEG-N\(_3\) with G’ = 522 kPa to tris-PEG-N\(_3\) with G’ = 8674 kPa. Interestingly, the authors reported that when the PEG crosslinker was increased to tetrakis-PEG-N\(_3\), the hydrogels’ modulus unexpectedly reduced (G’ = 173 kPa). This phenomenon was ascribed to the increased distance between the crosslinking junctions due to the higher molecular weight of PEG. Moreover, the authors added a second interpenetrating network by orthogonally crosslinking bis-PEG-N\(_3\) with varying molar ratios of pentaerythritol tetraacrylate. Interestingly,
they found that the mechanical properties of the hydrogels improved with increased acrylate concentrations up to thiol:acrylate 1:2 when the hydrogel modulus was reduced from 3677 kPa to 604 kPa. This reduction was attributed to the excess acrylate moieties yielding a heterogenous structure. Finally, the authors also demonstrated the biocompatibility of their IPNs, but they found reduced metabolic activity and increased cells death of mesenchymal stem cells grown on the IPNs compared to control, albeit not statistically significant [162].

Most issues related to the alkyne-azide cycloaddition reaction are due to the use of the copper catalyst, prone to oxidation that reduces efficiency and also limits the biocompatibility of the reaction in tissue engineering [22]. Seminal work by DeForest and Anseth has developed cytocompatible click-based hydrogels formed by copper-free alkyne-azide reactions [163]. The research by DeForest and Anseth has opened up the use of copper free alkyne azine reaction in tissue engineering with most groups substituting the copper catalyst to improve biocompatibility and efficiency. For example, Huebsch et al. exploited a copper free azide alkyne cycloaddition to bioconjugate RGD peptides to alginate based hydrogels. The reaction was remarkably efficient at pH 6 and the addition of RGD peptides increased cell viability, spreading, and differentiation compared to the alginate only hydrogels [164]. Similarly, Mandenious and co-workers developed modular hyaluronan acid-PEG base hydrogels crosslinked via a bioorthogonal copper free click chemistry for liver regeneration [165]. The authors showed good mechanical properties of the hydrogels (\(G' = 10^3\) Pa) dependant on the ratio between the materials with an excess of N3 moieties hindering the formation of a hydrogel (\(G' < G''\)) but an excess of cyclooctyne-modified hyaluronan acid (HA-BCN) strengthening the resulting hydrogel. The copper free chemistry also increased the biocompatibility of the hydrogels as HepG2 cells were able to migrate in 3D throughout the gel and maintain good viability after 13 days in culture.

Hydrogels can also be crosslinked through the formation of a Schiff base typically formed between amine and aldehyde groups or through Michael addition, between a Michael donor, an enolate or other nucleophile, and a Michal acceptor, usually a \(\alpha,\beta\)-unsaturated carbonyl [22].
Th reactive double bond linking carbon and hydrogen atoms of the Schiff base is a covalent bond particularly suited to the fabrication of hydrogels for tissue engineering due to its high efficiency in physiological environments and its pH reversibility that confers self-healing, shape memory, and stimuli responsiveness to the hydrogels [166]. The self-healing properties made possible by the double bond of the Schiff base have attracted numerous applications for injectable hydrogels functionalised with antimicrobial peptides for wound healing, where the ability to cover an irregular wound is crucial. For example, Wu and co-workers have recently developed chitosan based hydrogels functionalised by the antimicrobial peptide AEKA and crosslinked by Schiff base [167]. The hydrogels exhibited remarkable self-healing properties attributable to the Schiff base reactive crosslinking where the G’ value dropped rapidly above the critical strain (500% strain), but when the strain was lowered (1% strain) the G’ recovered and G’ > G’”, with G’ = 10^3 Pa. The hydrogels also exhibited antibacterial properties against a broad spectrum of bacteria and excel cell viability (> 90%) after 72 hours. Similarly, Zhang et al. developed sodium alginate/hyaluronic acid based hydrogels functionalised with sanguinarine, an alkaloid with broad spectrum antimicrobial activity, via Schiff base crosslinking [168]. The hydrogels presented good mechanical properties (G’ = 10^3 Pa), albeit somewhat dependant on frequency indicative of potential instability in the crosslinking, remarkable antibacterial activity and good cell viability in vitro.

Hydrogels synthesised using Michael addition have also found applications as injectable hydrogels thanks to their excellent shear thinning behaviour. For example, Yang et al. developed ibuprofen – KYIGSRK conjugated hydrogels for repairing spinal cord injury [169]. The rational design was based on the in situ conjugation between the acrylate groups of PEGDA and the amino groups of lysine/ibuprofen – KYIGSRK via Michael’s addition where the ibuprofen – KYIGSRK peptide was designed to have the non-steroidal anti-inflammatory activity of ibuprofen, the two lysine K were needed for hydrogel functionalisation via Michael addition, and YIGSR was added to promote cell adhesion and axonal outgrowth. The hydrogels presented good mechanical properties (G’ = 10^3 Pa), where higher concentrations led to faster gelation kinetics, shear thinning behaviour, and excellent recovery after shear, confirming their suitability for injectable applications. The hydrogels promoted primary dorsal root ganglion neurons adhesion and proliferation.
and were able to fill irregular cavities in vivo by injection promoting nerve regeneration and inhibiting inflammation [169]. Similarly, Eelkema and co-workers developed injectable covalently crosslinked dextran based hydrogel via Michael addition loaded with the peptide antigen SIINFEKL able to activate specific T cells [170]. As expected, the hydrogels presented good shear thinning behaviour, rapid self-healing properties, and good cell biocompatibility with dendritic cells. However, is has to be noted that the hydrogels presented very weak mechanical properties, $G' < 10$ Pa, hindering their use in more robust tissue engineering applications.

The chemical crosslinked methods listed so far have excellent advantages, such as ease of use, functionalisation, excellent efficiency, and good mechanical properties. However, most are characterised by a similar disadvantage: the presence of a crosslinking agent, or substrate, that reduces the biocompatibility of the reaction for tissue engineering applications. SpyTag-SpyCatcher is a genetically encodable covalently crosslinking method that does not require the addition of an external crosslinking agent and it is stable in physiological conditions (i.e. $37$ °C and pH 7), rendering this system particularly suitable for tissue engineering applications. Moreover, because SpyTag-SpyCatcher can be genetically encoded into a protein of interest, it can be expressed recombinantly using suitable organisms, such as E. coli, thereby avoiding the chemical synthesis of peptides and increasing the sustainability of this tissue engineering approach [27]. SpyTag-SpyCatcher offers the possibility to bridge tissue engineering with synthetic biology, the research field exploiting genetic tools to engineer living cells and organisms, to redesign living systems and develop new responsive materials with dynamic and programmable functionalities [27].
1.1.4.3 SpyTag- SpyCatcher

SpyTag-SpyCatcher was discovered by Howarth and co-workers by splitting the second immunoglobulin-like collagen adhesin domain, CnaB2, of *Streptococcus pyogenes* into a peptide, 13 amino acids - SpyTag, and protein fragment, 138 amino acids - SpyCatcher [171]. The reactive unprotonated lysine of SpyCatcher nucleophilically attacks the carbonyl carbon of the aspartic acid in SpyTag, catalysed by the neighbouring glutamic acid, reconstituting the two partners and undergoing covalent reaction, Fig. 1.9.

![Reaction scheme of SpyTag-SpyCatcher](image)

Figure 1.9: Reaction scheme of SpyTag-SpyCatcher. The reactive unprotonated lysine (K31) of SpyCatcher nucleophilically attacks the carbonyl carbon of the aspartic acid (D117) in SpyTag, catalysed by the neighbouring glutamic acid, forming an isopeptide bond with H2O as the leaving group.

SpyTag-SpyCatcher is highly efficient, with the second order rate constant determined to be $1.4 \times 10^3 \pm 43 \text{ M}^{-1} \text{s}^{-1}$. Howarth and co-workers tested the dependence of the reaction over a variety of different conditions, such as pHs, buffers, detergents, and temperatures, including 37 °C, and determined that there was no substantial differences in the reaction in all condition trialled. SpyTag was also determined to be reactive at the N-terminal, internal, and C-terminal sites and the covalent interaction between SpyTag-SpyCatcher was established to be more than 20 times stronger than antibody-antigen or streptavidin-biotin interactions [171]. In particular, the strongest noncovalent interaction based on streptavidin-biotin can be broken in seconds by molecular motors [172] or in milliseconds by shear forces [173], limiting its applications in cellular systems. However, molecular motors are not able to break the covalent bond formed by SpyTag-SpyCatcher, thereby allowing the use of this system to target specific proteins and cell types [171].
Because both SpyTag and SpyCatcher are composed of the 20 amino acids common to all organisms, they can be genetically fused to any protein of interest and expressed in different organisms, allowing scientists to control and follow how the new protein behaves [171]. Moreover, because SpyTag-SpyCatcher has a high tolerance to its surroundings and requires no artificial amino acids or added cofactors, this reaction can be adapted to a wide range of situations inside and outside of the cell. In addition, because of their high specificity, SpyTag and SpyCatcher react with each other but not the many other proteins present, highlighting their suitability to applications involving human cells. Finally, SpyTag-SpyCatcher are particularly suitable to develop modular constructs, as both SpyTag and SpyCatcher can be inserted either at the N-terminal, internal, C-terminal sites or in all three sites simultaneously of a protein of interest, allowing the creation of multiple complex geometries and protein-based structures with multivalent points [171]. Several SpyTags or SpyCatchers can also be linked together via peptide-based linker, i.e. glycine rich linkers, creating controllable modular arrays suitable to various synthetic biology and tissue engineering applications.

A second and third generation of SpyTag-SpyCatcher, named SpyTag002/SpyCatcher002 [174] and SpyTag003/SpyCatcher003 [175] respectively, were engineered to react faster than the original pair whilst maintaining the high specificity of the reaction. In particular, the second generation SpyTag002/SpyCatcher002 was engineered to react 12 times faster than the original pair with a rate constant of 2.0 x 10^4 ± 0.2 M⁻¹ s⁻¹ [174], whilst SpyTag003/SpyCatcher003 reacts up to 400 fold faster than the original pair with a rate constant of 5.5 x 10^5 ± 0.6 M⁻¹ s⁻¹ [175]. The second and third generations of SpyTag/SpyCatcher were engineered to overcome the limitation of the original pair that has relatively slow reaction at cellular expression levels and whose rate of reaction is below the 10⁵ to 10⁶ M⁻¹ s⁻¹ considered to be the onset of diffusion-controlled protein–protein interactions [174, 175].

Given the stability of SpyTag-SpyCatcher over a variety of different conditions and its genetically encodable approach, this system has found widespread applications in the literature, from vaccine production [176, 177], to enzyme cyclization [178], protein attachment onto surfaces [179], creation of complex macromolecular topologies [180], and uranyl decontamination from seawaters [181].
Of particular interest for tissue engineering is the stability of SpyTag-SpyCatcher at 37 °C, human body temperature, and its reported high specificity in the presence of bacterial and mammalian cell environments. This has naturally led to the successful use of this complex to form hydrogels with specific biological and physical characteristics. First, SpyTag-SpyCatcher has been used extensively to functionalise polymer-protein hybrid hydrogels. Clark and co-workers developed a hybrid hydrogel formed by mixing SpyTag-γPFD-Cys with maleimide-activated 4-arm PEG as a stable platform for the incorporation of functional proteins [182], and Minamihata et al. used a streptavidin-SpyCatcher protein crosslinked with thiol-PEG to develop hybrid hydrogels functionalised via biotinylated RGD and a fibroblast growth factor – SpyTag [183]. Moreover, SpyTag-SpyCatcher has also been used to functionalise and assemble fully protein hydrogels. For example, Li et al. used GRG₅RG₄R – SpyCatcher as ‘blank slate’ hydrogels functionalised via SpyTagged protein ligands [184], whilst Sun et al. covalently assembled the C-terminal adenosylcobalamin binding domain (CarH₅C) of the CarH photoreceptor protein to form photoresponsive hydrogels using SpyTag-SpyCatcher chemistry [185].

Of considerable importance for this project is the ability of SpyTag-SpyCatcher to not only functionalise protein hydrogels, but also create hydrogels by spontaneous covalent crosslinking. Tirrell et al. successfully developed SpyTag-SpyCatcher protein hydrogels using ELPs as the crosslinker ST-ELP-ST-ELP-ST [186], whilst Li et al. used the folded globular domains GB1 and FnIII and as their building blocks [187]. In both cases, gelation of the protein hydrogels occurred spontaneously at room temperature via the covalent crosslinking of SpyTag and SpyCatcher, the hydrogels were stable and did not show signs of degradation in aqueous environments, and were able to encapsulate mammalian cell culture in 3D without loss of cell viability. Tirrell et. al also showed maintenance of stem cell pluripotency in the hydrogels [186], whilst Li et. al demonstrated the potential of the hydrogels as drug delivery systems [187].
Given the aforementioned successful findings, this project aimed at using SpyTag-SpyCatcher technology to create and optimise protein hydrogels via spontaneous covalent crosslinking for tissue engineering applications, Fig. 1.10. The main advantages of the SpyTag-SpyCatcher system in tissue engineering is the lack of a potentially cytotoxic crosslinking agent and the ability to genetically encode it in virtually any protein sequence. Therefore, the sequence encoding for the SpyTag peptide or the SpyCatcher protein can be added to a protein of interest whilst maintaining the crosslinking ability of SpyTag-SpyCatcher. These features allowed me to develop hydrogels with tailored properties, where by changing the length of the protein building blocks, I was able to induce different physical characteristics in the resulting hydrogels that would not have been possible using only synthetic or naturally occurring polymers. Moreover, because all the proteins used in this project were expressed recombinantly in *E. coli*, the method exploited here were more sustainable compared to the chemically synthesised peptides detailed in the previous sections.

Figure 1.10: **Protein hydrogel created using SpyTag-SpyCatcher.** This project aimed at using SpyTag-SpyCatcher technology to create and optimise protein hydrogels via spontaneous covalent crosslinking for tissue engineering. Created with BioRender.com.
Chapter 2

Hydrogel formation and stability

The text, figures, and experiments in this and following chapters (sections: 2.2.1, 3.2.1, 4.2.2.1, 4.2.2.2, 4.2.3.1, 4.2.3.2, 5.2.2, 5.2.3, 5.2.5) were collected in one manuscript published by the ‘Journal of Structural Biology’ after peer review:

“Chemically Crosslinked Protein Hydrogels from Repetitive Protein Arrays”
Rossana Boni, Elizabeth Blackburn, Dirk-Jan Kleinjan, Mantas Jonaitis, Flora Hewitt-Harris, Megan Murdoch, Susan Rosser, David C. Hay, and Lynne Regan.

In order to provide a structure suited to this thesis, the sections have been split in their respective overarching topics and chapters.

2.1 The crosslinkers

The work carried out in this thesis is based on the physical and biological characterisation of protein hydrogels designed using the SpyTag-SpyCatcher system. In this preliminary results chapter, I will give a quick overview over SpyTag-SpyCatcher hydrogel formation, swelling abilities, and stability in cell culture media. The preliminary experiments described here paved the way for the thorough physical and biological characterisation detailed in the following results chapters of this thesis. Herein, SpyTag-SpyCatcher will be referred to as ST-SC.
A disadvantage of using ST-SC protein hydrogels compared to their polymer counterpart or other crosslinking methods is the low mechanical properties. In both Tirrell et al. and Li et al., the reported stiffness of the hydrogels was quite low, ≈ 100 Pa, suggesting that the biomaterial would only be suitable for very soft tissue applications, such as the fibroblasts used by both groups [186, 187]. Therefore, we set out to use a protein whose reported mechanical properties were strong and tuneable as the crosslinker for our protein hydrogels.

*S. aureus* surface protein G, SasG, (PDB ID: 3TIQ) is a well characterised mechanically strong, elongated, stiff, rod-shaped protein formed from tandem repeats of ‘EG’ consisting of two structurally related domains: E (50 residues) and G5 (78 residues), formed from single-layer triple-stranded β-sheets [188]. The crystal structure of stable SasG (GEG) revealed a highly extended topology akin to a cylinder with 20 Å in diameter and 170 Å in length where the head-to-tail arrangements of the β-sheets is responsible for the elongation and the interdigitation between the residues involved in the domain interfaces gives the protein rigidity [188]. Further small-angle X-ray scattering (SAXS) and atomic force microscopy (AFM) analysis determined that SasG is an extended monomeric molecule behaving as a series of overlapping cooperative units in solution with remarkable mechanical strength and mechano-stability [189]. Moreover, it was readily possible to genetically engineer proteins having up to 7 EG repeating units following the GEG monomer, and thus change the length of the protein, whilst keeping the core stiffness [189]. Given the remarkable mechanical strength and repetitive structure of SasG (GEG), the protein was chosen as the main crosslinker in this project. Alongside SasG, a longer version of the protein, named SasGlong, having 3 EG domains following the core SasG monomer (GEG+ 3x(EG)) was engineered and used as the second crosslinker to broaden the properties of the resulting hydrogels. It was decided not to increase the length of SasG further because an increase in protein length was correlated with a remarkable decrease in protein expression and, given the yields necessary to create the protein hydrogels, it would have been too time consuming to produce enough quantities of longer versions of SasG [190].
The mechanical strength of SasG was investigated at length by Brockwell et al. via AFM [83]. Briefly, Brockwell et al. systematically substituted aminoacids at specific locations within all three GEG regions of SasG, showing how a single substitution can have profound effects on its mechanical strength. The authors identified that the force bearing ability of SasG is linked to mechanical clamps at the N- and C- terminal of the protein as well as the side-chain interactions between the β strands and that disrupting the hydrogen bonding and sidechain packing interactions in the mechanical clamps induces a change in the mechanical phenotype, by decreasing the unfolding force or altering the transition state of the protein. The origin of this great mechanical strength is unknown, but the authors speculated that it might be due to the function of the protein in nature as SasG allows the bacterial cell to sustain attachment under shear force [83]. Because of the reported strong SasG mechanics that confer stability and strength to the protein [83], its modular nature that allowed the creation of proteins with different lengths, and its ease of expression in E. coli [188], SasG was chosen as the primary crosslinker for this project.

The two SasG proteins were engineered to carry two SpyTag (ST) motifs, one at each end of the chain, ST-SasG-ST and ST-SasGlong-ST respectively, Fig. 2.1. The constructs as described are 17 nm in length for ST-SasG-ST and 51.5 nm for ST-SasGlong-ST [191]. The ST crosslinkers were combined with three different constructs named SC3, SC4 and SC5 (∼ 5 nm for each SpyCatcher unit). The repetitive constructs carry three, four, or five respectively SpyCatcher (SC) units linked together by two comparable flexible glycine-rich linker (GGS)\textsubscript{2}RS for SC3 and SC4, and (GGS)\textsubscript{4}GSG for SC5, Fig. 2.1. SC arrays contained multiple SC repeats to allow the binding of multiple ST onto the same SC array, ensuring the formation of crosslinked hydrogels. Moreover, using SC arrays with changing lengths allowed to induce different properties in the resulting hydrogels as an increased number of ST would be able to bind to a longer array.

Both ST crosslinkers, SC3, and SC4 were cloned by previous members of the Regan Lab [191], whilst SC5 was cloned by current Regan Lab member Fokhrul Islam. Our SAXS analysis on the SC arrays showed that the structure of SCs is consistent with a species that contains folded elements with flexible linkers. Moreover,
we showed that the SC arrays are predominantly extended structures, with no indication of stacked association between SC units in the array [192].

Mixing of the ST crosslinkers with the SC arrays led to the recreation of a covalent bond between ST and SC and consequent interchain crosslinking driving the formation of a percolating network culminating in the development of a self-standing hydrogel, Fig. 2.1. In this project, hydrogels composed of either ST-SasG-ST or ST-SasGlong-ST combined with SC3, SC4 or SC5 were created and their physical and biological properties were tested and investigated with a view of using them for tissue engineering applications. Of note, SC5 is only partially used in this project as Fokhrul cloned the construct in 2022.

Throughout this project, it was assumed that the proteins used to create the hydrogel would remain folded. Supporting evidence of the folded nature of the proteins can be found in Chapter 4 where the properties of the hydrogels substantially changed when the constituting proteins were unfolded via urea. However, no specific experiment aiming at determining whether the proteins remained folded was carried out. Two techniques, circular dichroism (CD) and Fourier-transform infrared spectroscopy (FTIR) could be used to confirm that both SasG and SC arrays remain folded in the hydrogels, and they could be used to calculate the population of folded protein in the cross-linked hydrogel. Specifically, CD is an excellent method of determining the secondary structure of proteins via changes in the optical transition of the amides in their polypeptide backbones [193]. Because different structural elements have characteristic CD spectra, CD can be used to estimate the structure of proteins and monitor conformational changes due to temperature, mutations, heat, denaturants or binding interactions [193]. Similarly, changes in the vibration frequencies of a protein’s FTIR spectra allows to identify changes in its secondary structure, such as aggregation or misfolding [194].
Figure 2.1: **Components of the SpyTag/SpyCatchers hydrogels.** (A) precursors components of the hydrogel networks. Top: SpyTag/SpyCatcher system. Triple (SC3), quadruple (SC4), and quintuple (SC5) SpyCatcher arrays (pink crowns) linked together by two comparable glycine rich linkers (green), (GGS)$_2$RS for SC3 and SC4 and (GGS)$_4$GSG for SC5; the two ST-crosslinker-ST proteins (ST as yellow arrows and crosslinkers as blue rods). SasG composed of the core monomer GEG (PDB: 3TIQ), and the longer version SasGlong (GEG + 3x(EG)). (B) schematic representation of the crosslinking between SpyTags and SpyCatches leading to the formation of a covalently crosslinked hydrogel. Triple (SC3) SpyCatches domains in pink linked together by the green glycine rich flexible linker, ST-SasG-ST crosslinker in yellow (SpyTags) and blue (SasG). Image adapted from Boni et al. [192].
2.2 The hydrogels

Following protein purification and freeze drying as described in the Materials and Methods, the freeze-dried protein powders were easily resuspended in water at the desired molar concentration, 1, 2, 3, 3.5, 4 mM for ST-SasG-ST and ST-SasGlong-ST and 1 mM for SC3, SC4, and SC5. Once the millimolar stocks were prepared, the ST-SC protein hydrogels were created by simply manually mixing together an SC array with an ST crosslinker at room temperature in a microcentrifuge tube, inducing spontaneous gelation of the components.

To first ascertain whether a hydrogel had formed, I carried out the commonly used inversion test [23], i.e. I simply inverted the microcentrifuge tube containing the ST-SC mixture and verified that the hydrogel remained at the bottom of the tube, rather than running down at the top, indicative of the presence of a liquid rather than a solid, Fig 2.2. Hydrogels formed when the protein concentration was above the 1 mM threshold. Experiments with lower protein concentration (µM) were carried out, but the mixture remained liquid, Fig. 2.3. This is consistent with the work of Tirrell et al. and Li et al. that created their hydrogels by mixing millimolar concentrations of ST and SC [186, 187].
Figure 2.2: Inversion test carried out on the ST-SC protein hydrogels at millimolar concentrations. A) ST-SasG-ST at 2, 3, 3.5, and 4 mM combined with SC3 at 1 mM. B) ST-SasG-ST at 2, 3, 3.5, and 4 mM combined with SC4 at 1 mM. C) ST-SasGlond-ST at 2, 3, 3.5, and 4 mM combined with SC3 at 1 mM. D) ST-SasGlond-ST at 2, 3, 3.5, and 4 mM combined with SC4 at 1 mM.

Figure 2.3: Inversion test carried out on the ST-SC protein hydrogels at micromolar concentrations. SC3 at 10 µM combined with ST-SasG-ST or ST-SasGlond-ST at (A) 20 µM, (B) 30 µM, or (C) 40 µM. The mixture remained liquid.
2.2.1 Stability in water, swelling properties, and stability in cell culture medium

I set out to investigate the stability of the ST-SC protein hydrogels by testing their stability in water, and their consequential swelling properties, and their stability in hepatocyte cell culture medium, kindly donated by the Hay lab. It was crucial to determine if the newly formed hydrogels were stable in aqueous environments and in the presence of cell culture medium, given that the project aimed at using the ST-SC hydrogels for tissue engineering applications and, specifically, liver regeneration.

First, I simply created the ST-SC protein hydrogels via spontaneous gelation, as described before. After gelation was complete, I added 1 mL of ultrapure water to each Eppendorf tube and incubated overnight at room temperature. The next day, the hydrogels did not show visible signs of erosion or degradation, Fig. 2.4. Further, I determined the hydrogel’s swelling abilities. As before, after gelation was complete, I added 1 mL of ultrapure water to each Eppendorf tube containing the hydrogel and incubated overnight at room temperature. I removed excess water by blotting using lint free paper and weighed the hydrogels (Ww). Subsequently, I freeze dried the hydrogels and weighed them again (W0). The percentage of water intake was calculated using: \((Ww - W0) * 100 / W0\) [70]. The water content of the hydrogels was calculated to be \(\approx 50\%\) after 24 hours, with no significant differences between ST-SasG-ST (2 or 4 mM) or ST-SasGlong-ST (2 or 4 mM) combined with SC3 (1 mM) or SC4 (1 mM), Fig. 2.5.
Figure 2.4: Example of the stability of the ST-SC protein hydrogels in water. A) ST-SasG-ST + SC3 in 1 mL of ultrapure water. B) ST-SasGlong-ST + SC3 in 1 mL of ultrapure water. After 24 hours, the hydrogels were stable and did not show visible signs of degradation.
Figure 2.5: **Percentage of water intake of the ST-SC protein hydrogels.** The water content of the hydrogels was calculated to be ≈ 50% after 24 hours, specifically at 2 mM ST: 45.21 ± 0.15% for ST-SasG-ST + SC3, 55.89 ± 1.15% for ST-SasG-ST + SC4, 52.54 ± 4.37 % for ST-SasGlong-ST + SC3, and 57.14 ± 3.95 % for ST-SasGlong-ST + SC4; at 4 mM ST: 50.15 ± 3.13 % for ST-SasG-ST + SC3, 57.08 ± 4.81 % for ST-SasG-ST + SC4, 48.45 ± 1.42 % for ST-SasGlong-ST + SC3, and 56.18 ± 5.75 % for ST-SasGlong-ST + SC4. Data is presented as ± SD with N = 3.
Finally, I set out to determine the stability of the ST-SC hydrogel in cell culture medium. In particular, I wanted to determine if hydrogel formation could happen in cell culture medium. First, I tested the ST-SC reaction on an SDS-PAGE gel. Briefly, I mixed together 10 µL of ST-GFP at 50 µM with 10 µL of SC4 at 50 µM, incubated at room temperature for an hour, and stopped the reaction by adding 5 µL of gel loading buffer (GLB) containing 1,4-dithiothreitol (DTT), that reduces the disulfide bridges and unfolds proteins. Then, I run the reaction on a SDS-PAGE gel to verify the presence of product bands, indicative of the successful reaction between ST and SC, Fig. 2.6. Further, I created the ST-SC hydrogels as described before (2 mM ST and 1 mM SC) in basal medium (William’s E) and Hepatocytes Selection Medium (HSM - William’s E fully reconstituted), kindly donated by the Hay lab. The hydrogels formed as expected in the cell culture medium indicating the suitability of the ST-SC protein hydrogels for tissue engineering and liver regeneration, Fig. 2.7.

Figure 2.6: Test of the ST-SC reaction on an SDS-page gel in water, basal medium, (William’s E), and Hepatocytes Selection Medium (HSM - William’s E fully reconstituted). At 0 minutes, the reaction is stopped immediately using GLB after mixing and the components are visible (ST-GFP at 31 kDa and SC4 at 54 kDa), whilst when the reaction is carried out for 60 minutes, the components are consumed and new product bands are visible (black boxes). The large protein present in the HSM medium is foetal bovine serum (FBS), a typical constituent of cell culture media.
Figure 2.7: ST-SC hydrogels can form in basal medium (William’s E) and Hepatocytes Selection Medium (HSM - William’s E fully reconstituted). The hydrogels formed as expected in both cell culture medium indicating the suitability of the ST-SC protein hydrogels for tissue engineering and liver regeneration.
2.3 What did not work

In this section I briefly acknowledged some aspects of the project that did not work and what I learnt from it. I believe it is important to show what did not work alongside what did so that future research can benefit from these findings.

Of note, the cloning detailed in the following sections was carried out in collaboration with past Regan Lab member Dr Louise Holyoake.

2.3.1 Azami Green – ST

Azami Green is a tetrameric fluorescent protein whose monomeric version is primarily used for fluorescence labelling of subcellular structures and proteins [195]. Past members of the Regan Lab worked with Azami Green and characterised it extensively [196]. In particular, Azami Green was found to express and purify very well in E. coli BL21 Gold(DE3). Given these encouraging findings, we decided to use tetrameric Azami Green as the third crosslinker alongside SasG and SasGlong to expand the purview of the ST-SC protein hydrogels.

In collaboration with Dr Holyoake, ST was cloned on Azami Green to form Azami Green - ST following the Gibson assembly protocol detailed in the Materials and Methods. After successful verification via sequencing, I expressed and purified Azami Green – ST and tested the reactivity of the construct with the SC arrays. Briefly, I mixed together 10 µL of Azami Green-ST at 50 µM with 10 µL of SC3 or SC4 at 50 µM, incubated at room temperature for an hour, and stopped the reaction by adding 5 µL of GLB. Then, I run the reaction on a SDS-PAGE to verify the presence of product bands, indicative of the successful reaction between ST and SC, Fig. 2.8.
Unfortunately, despite the successful reaction between Azami Green – ST and the SC arrays, the construct was not able to form hydrogels at any millimolar concentration investigated (1, 2, 3 mM). Preliminary microrheology and rheology experiments confirmed that the mixture of Azami Green – ST and either SC3 or SC4 did not form hydrogels (G’< G” and no gelation observed in microrheology). We hypothesised that the tetrameric nature of Azami Green with an ST on each monomer did not allow the formation of a percolated network because the termini point together in pairs rather than being far away from each other, therefore we shelved the construct.

Figure 2.8: **Purification of Azami Green – ST and reactivity test on 7.5 % SDS- page gel.** A) Purification of Azami Green - ST, 29 kDa; lysate: whole cell lysate, FT: *E. coli* proteins flowing through after incubation with Ni-NTA, IW1: 5 mM imidazole wash 1, IW2: 5 mM imidazole wash 2, ELU1: elution of protein of interest 1, 200 mM imidazole, ELU2: elution of protein of interest 2, 200 mM imidazole, dialysis: protein of interest after dialysis against distilled water. B) ST-SC reactivity test of Azami Green – ST with SC3 and SC4. At 0 minutes, the reaction is stopped immediately after mixing and the components are visible (Azami Green – ST at 29 kDa, SC3 at 41 kDa, and SC4 at 54 kDa), whilst when the reaction is carried out for 60 minutes, the components are consumed and new product bands are visible (black boxes).
2.3.2 ST-β-catenin-ST and ST-Fibcon-ST

Given the length of two ST crosslinkers available, ST-SasG- ST 17 nm and ST-SasGlong-ST 51.5 nm, we wanted to add new crosslinkers to the hydrogel library that were significantly shorter or longer to investigate how they would affect the hydrogel properties. In collaboration with Dr Chris Wells-Wood, we carried out a search over all biological units in the protein databank (PDB) with the following parameters:

- Monomeric protein (because of Azami Green-ST)
- Between 50-100 amino acids
- No cysteines (to ensure the resulting hydrogel was reliant on ST-SC)
- N-C distance > 15 Å (to ensure the protein was extended)

Amongst the results, we chose two: Fibcon, a monomeric globular protein [197] and β-catenin, an armadillo repeat protein [198]. Both proteins were very well characterised in the literature and they were significantly shorter (Fibcon, 5 nm) or longer (β-catenin, 110 nm) than the SasG crosslinkers already used in the project. In collaboration with Dr Holyoake, I designed the sequences encoding for ST-Fibcon-ST and ST-β-catenin-ST. We elected to outsource the cloning of the SpyTag peptides to either end of Fibcon and β-catenin to Twist Bioscience (https://www.twistbioscience.com) and I received the miniprepped DNA vector encoding for each protein. I successfully transformed the DNA into E. coli BL21 Gold(DE3) but unfortunately both proteins did not express. I attempted various protocols, temperatures, media, and timescales but no expression of either protein was ever seen. We concluded that there might have been an issue with the original DNA template and shelved both constructs.
2.4 Aims of this work

The overarching aim of this thesis was to develop and characterise chemically crosslinked protein hydrogels designed using the SpyTag-SpyCatcher system for tissue engineering applications and, in particular, liver regeneration. Throughout this thesis, I combined the two ST crosslinkers, ST-SasG-ST and ST-SasGlong-ST with the three SC arrays, SC3, SC4, and SC5 and investigated their physical and biological properties, Fig. 2.9.

After this brief overview on ST-SC hydrogel formation and stability, in this thesis I will show:

- Chapter 3: Microrheology - determining the speed of gelation of the ST-SC protein hydrogels.
- Chapter 4: Rheology - determining the viscoelastic properties of the ST-SC protein hydrogels.
- Chapter 5: Biocompatibility - determining the biocompatibility of the ST-SC protein hydrogels for tissue engineering and liver regeneration.
- Chapter 6: 3D printing - adapting the ST-SC hydrogel design to 3D printing.

Chapter 3

Microrheology: Investigation on the speed of gelation of the ST-SC protein hydrogels

The characterisation detailed in this chapter was carried out in collaboration with Dr Davide Michieletto who helped develop the methods used. The experiments and data analysis were carried out solely by myself but for section 3.2.3 where the data was collected by honours project student Megan Murdoch under my supervision and analysed together. Megan’s contribution is acknowledged in the specific section.

3.1 Introduction

Physical characterisation of hydrogels is crucial to ascertain their suitability to tissue engineering. In particular, measuring the gelation kinetics of a hydrogel system is critical to determine its applicability to precise therapeutic solutions. For example, injectable emulsions containing cells and growth factors have been shown to improve the rate of recovery of an injured tissue, but they require a rapid liquid-solid transition upon injection to develop into a homogenous suspension able to localise the embedded treatment into the wound and minimise inflammation and immunogenic response [144, 199]. Moreover, gelation kinetics of hydrogels also influence the possibility of adapting the hydrogel design to fabrication techniques,
such as 3D printing and electrospinning, thereby broadening the scope of their application in tissue engineering [200,201]. Therefore, determining the rate of hydrogel formation is often as important as determining the bulk rheological properties of a material. However, whilst traditional rheology is an excellent candidate for the analysis of the bulk mechanical properties of hydrogels, the rapid self-assembly kinetics of protein hydrogels cannot be resolved as easily because the transition from liquid to solid behaviour is solely dependent on small and often very quick changes in protein-protein interactions, largely undetectable by traditional rheometers.

Microrheology has emerged as a powerful complementary tool to traditional rheology to characterise the behaviour of scarce materials [202, 203]. The distinct advantage of microrheology over rheology is the amount of material needed to make the measurements, ranging from 1 µL to 10 µL as opposed to traditional rheological measurement that require samples in the order of the mL. In microrheology, tracer particles are embedded into the system and their Brownian motion is recorded over time, Fig. 3.1 [202,204]. The recording of the movement of the embedded particles is analysed via particle tracking algorithms, usually based on the weighted centroid method developed by Crocker and Grier, where the particles are located in each video, assigned a position, and their locations are linked into trajectories using a probability distribution function that accounts for the Brownian motion of a single particle [205]. This allows to calculate the mean square displacement (MSD) of the embedded particles, hence the difference over time between two positions held by the same particle. Tracking the differences in the MSD of embedded tracer particles allows to gain insight into the changing physical properties of a hydrogel system.
Microrheology can be divided into two different approaches on the basis of the driving force of the embedded particles. In active microrheology, the movement of the embedded particles is determined by the response to an external force applied to the material, typically created by optical tweezers or magnetic fields [202]. Conversely, in passive microrheology, the Brownian movement of the embedded particles is caused only by inherent thermal forces [204]. Herein, I will focus on passive microrheology, the technique used for the microrheological characterisation detailed in this chapter. It is important to note that bulk rheology can measure the gel point and the time to gelation via oscillatory time sweeps [22], but only microrheology can do so under passive conditions, i.e. when no external force is applied to the material.

3.1.1 Passive microrheology

In passive microrheology, the generalised Stoked-Einstein Relation (GSER) is typically used to transform the MSD of the embedded tracer particles to the material’s bulk rheology. However, because the driving force of passive microrheology is the inherent thermal motion of the system, the technique is limited to systems characterised by weak moduli (G’ < 4 Pa) and low viscosities [202, 206].
Nevertheless, passive microrheology is particularly useful to monitor spontaneous gelation of biomaterials over time, where at the onset of gelation the moduli of the hydrogelators are very small and the still-forming fragile structures would be easily compromised by typical stress-controlled bulk rheology [202, 204]. During gelation, very low viscosity liquids, akin to water, give a signal that is often too weak to be detected by traditional rheometers, whilst the sensitivity of passive microrheology is able to discern smaller transitions in the system with very quick gelation kinetics. Moreover, the presence of embedded microparticles in the systems is unlikely to significantly influence or break up a still-forming weak structure. For example, Larsen and Furst demonstrated the ability of microrheology to provide detailed information over the gelation kinetics of a peptide hydrogelator [207]. This pioneering work demonstrated how at the beginning of the gelation reaction, the embedded particles show free diffusion in the system, with the MSD increasing over lag time, but as the reaction proceeds, the beads’ displacement reduces until the system reaches an elastic plateau, indicating the formation of an elastic solid. The Furst group further demonstrated the uses of microrheology to investigate the gelation kinetics of β-Hairpin peptide hydrogels [208,209], and crosslinked PEG-Heparin hydrogels [210]. They also used this technique to explore hydrogel degradation [211, 212]. The group’s work has provided an efficient and accurate blueprint to investigate the gelation kinetics of hydrogels that allow to engineer tuneable networks able to closely mimic the mechanics and signalling of biological materials. Other groups successfully used particle tracking microrheology to investigate the rate of formation of a self-assembly peptide [213], the nucleation process of thermoreversible polymer gels [214], and the kinetics of gelation of physically entangled hydrogels [98] expanding and solidifying the use of this technique outside the Furst group. More recently, high throughput microrheology has been used to determine the gelation kinetics of protein hydrogels in combination with machine learning [215] and differential dynamic microscopy [216].

In this chapter I systemically explored the gelation kinetics of protein-based hydrogels designed using the SpyTag - SpyCatcher system. I carried out microrheological experiments to investigate the gelation kinetics of ST-SasG-ST and ST-SasGlong-ST in combination with SC3 and SC4. I explored the factors that influence the gelation of the hydrogels and examined how these properties can be
modulated to tailor the hydrogel to precise medical applications.

In this chapter I will show:

- When SC is present in excess of ST, the system remained a viscous liquid.
- When ST is present in excess of SC, the system showed fast gelation kinetics (≈ 10 minutes).
- Increasing the ST units increased the rate of gelation of the protein hydrogels.
- Equimolar combinations of ST:SC present slow gelation kinetics (≈ 45 minutes).
- Increasing the protein concentration of equimolar ST:SC combinations led to faster gelation kinetics.

The data will be presented in the following order: combinations of ST-SasG-ST with SC3 and SC4, followed by combinations of ST-SasGlong-ST with SC3 and SC4.

3.2 Results

The aim of this study was to investigate the gelation kinetics of the two crosslinkers ST-SasG-ST and ST-SasGlong-ST in combination with either SC3 or SC4. I systemically combined the two crosslinkers with the SC arrays and used particle tracking passive microrheology to determine the liquid - solid transition of the system as I investigated the factors that affected the gelation kinetics of the hydrogels.

The speed of gelation was investigated under different conditions to explore how gelation could be controlled, with a view to be able to optimise the ST-SC gelation rate for 3D printing applications. Controlling how fast the hydrogels gel is crucial to adapt the hydrogel design to 3D printing, for example to determine the optimal extrusion speed. The rate of gelation was measured using microrheology, in which changes in the MSD of embedded tracer particles were used to quantify changes in the liquid -solid transition of the hydrogel system. Briefly, 5 µL of SC at
the appropriate mM concentration were mixed together with 0.5 µL of tracer bead slurry and pipetted onto the masked glass slide. The glass slide was then position onto the microscope ready for recording to begin. Once both the microscope (i.e. camera focus) and recording system (i.e. duration and length of videos) were set up, 5 µL of ST at the predetermined mM concentration were pipetted onto the glass slide and mixed with the SC containing the tracer beads. The mixture was pipetted up and down to ensure mixing of the ST and SC and quickly sealed with a coverslip. 

\( t = 0 \) was determined as the moment when the recording of the first video started, which was less than 2 minutes after ST and SC were mixed and as soon as an appropriate ROI (Region of Interest) was identified (i.e. an area containing a suitable amount of beads, far from the edges and from both glass slide and coverslip). It has to be noted that the immediate and unregulated gelation of ST and SC induced limitations in the method used because the system’s gelation started when the two solutions were mixed \((t = 0_{\text{TRUE}})\) rather than when recording was able to commence \( t = 0 \). In the slow gelling systems, this limitation should not be significant but in the fast gelling systems the use of large glass microchannels, that could accommodate the viscosity of the ST solutions, or smaller tracer probes could be implemented to overcome it.

The MSD was calculated as a function of a lag time, \( \tau \), the interval between images, and the logarithmic slope of the MSD at large times \( \alpha = \frac{\log <r^2(t)>}{\log \tau} \) is a measure of the rheological state of the material. When the system behaves like a viscous liquid, \( \alpha = 1 \) as the particles exhibit free diffusion. A decrease in the slope of the MSD is indicative of the changes in the material rheology towards the formation of an elastic solid, characterised by \( \alpha = 0 \). The critical gel point, the point where the system transitions from viscous liquid to elastic solid, has been identified as \( 0.45 < \alpha < 0.55 \) for biomaterials hydrogelators [207, 209, 217]. All experiments were carried out in the passive microrheology operating regime for 1 µm tracer beads as defined by the Furst group [202] and static and dynamic errors associated with particle tracking were minimised as much as possible according to the literature [218].
For the majority of the data presented in this chapter, the time points identified were 0, 10, 20, 25, 35, and 45 minutes after mixing, where 0 minutes represents the start of the recording (t = 0\text{TRUE} was the real time of mixing of ST and SC as specified previously) and 10, 20, 25, 35, and 45 minutes represent the progressive gelation times after mixing of ST and SC. Specifically, each time represents how long after mixing the MSD of the tracer beads was measured. During the experiments, gelation time points were recorded every 2 minutes in the first 25 minutes and every 5 minutes between 25 to 45 minutes because it was observed that the majority of the changes in the system caused by progressive gelation occurred in the initial part of the recording time frame (0 - 25 minutes). The six data points plotted throughout this chapter (0, 10, 20, 25, 35, and 45 minutes) were chosen arbitrarily as good representative points of the gelation kinetics of the system and they spanned the entirety of the gelation process. When a system showed no gelation or partial gelation, like ST-SasGlong-ST combined with SC3, the recording time was progressively increased up until the samples were recorded overnight. However, when no changes in the slopes of the MSD were observed, the data was plotted from 0 to 100 minutes to reflect the increased time frame acquired.

3.2.1 Gelation kinetics of ST-SasG-ST in combinations with SC3 and SC4

I investigated the gelation kinetics of a series of ST-SC combinations keeping the molar concentration of the SC array constant at 1 mM while gradually increasing the ST component from 1 mM to 4 mM, to examine how variations in the number of ST:SC units altered the gelation kinetics of the resulting protein hydrogels.

In this section, all combinations will be referred to on the basis of their ST and SC unit content. For example, ST-SasG-ST at 2 mM has 4 units of ST (2 mM x (2 ST in each SasG chain)) and SC3 at 1mM has 3 units of SC (1 mM x 3 SC units). Therefore, ST-SasG-ST at 2 mM combined with SC3 at 1 mM will be referred to at ST:SC 1.3:1. Table 3.1 shows an overview of the ST:SC ratios of the protein hydrogels investigated in this chapter and their resulting networks for ease of understanding.
Table 3.1: Hydrogel combinations analysed for microrheology

<table>
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<tr>
<th></th>
<th>1 mM = 2 ST units</th>
<th>2 mM = 4 ST units</th>
<th>3 mM = 6 ST units</th>
<th>3.5 mM = 7 ST units</th>
<th>4 mM = 8 ST units</th>
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<td>ST</td>
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<td>SC3</td>
<td>1 mM = 3 SC units</td>
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<tr>
<td></td>
<td>0.6:1</td>
<td>1.3:1</td>
<td>2:1</td>
<td>2.3:1</td>
<td>2.6:1</td>
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<tr>
<td>SC4</td>
<td>1 mM = 4 SC units</td>
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<tr>
<td></td>
<td>0.5:1</td>
<td>1:1</td>
<td>1.5:1</td>
<td>1.75:1</td>
<td>2:1</td>
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</table>
3.2.1.1 SC3 at 1 mM and ST-SasG-ST at 1, 2, 3, 3.5, 4 mM

Combinations of ST-SasG-ST and SC3 0.6:1 showed no gelation of the system, but the beads kept diffusing freely, suggesting that the system never transitioned from a viscous liquid to an elastic solid, Fig. 3.2. Figure 3.3 shows a plot of the calculated MSD plotted against lag time (τ) for the combinations of ST-SasG-ST and SC3 1.3:1 and 2.6:1. Immediately after mixing of ST and SC, t = 0, both networks (SC3 = 1 mM and ST-SasG-ST = 2 mM or 4 mM respectively) showed liquid-like behaviour, characterised by free diffusion of the tracer particles in the system $< \Delta r^2(\tau) >= \tau$. As the time post mixing increased, the behaviour of both systems changed. The system composed of ST:SC 1.3:1 showed a progressive and continuous decrease in the magnitude of the MSD and dependence on lag time, indicative of subdiffusive behaviour. After longer time intervals (≈ 35 - 45 minutes) the microparticles’ dynamics displayed a distinct plateau and the MSD across all lag times approached a constant value, characteristic of the formation of a viscoelastic solid ($\alpha = 0$). Conversely, the system composed of ST:SC 2.6:1 showed a much faster decline of the MSD, that approached a constant value $\alpha = 0$ as early as 10 minutes. The subdiffusive dynamics observed in the slow gelling system are attributable to the development and growth of protein clusters in the pre-gel, culminating in a percolating network forming an infinite cluster throughout the entirety of the sample after the critical gelling point is reached. The subdiffusive dynamics were not clearly visible in the fast-gelling system, as this combination of ST:SC reached complete gelation too quickly, indicating that the formation of an infinite protein cluster spanning the entirety of the sample was almost immediate.
Figure 3.2: Gelation kinetics of ST-SasG-ST at 1 mM and SC3 at 1 mM, ST:SC 0.6:1. This combination shows no gelation and free diffusion of the beads, suggesting that the system never transitioned from a viscous liquid to an elastic solid.

Figure 3.3: Gelation kinetics of ST-SasG-ST and SC3 1.3:1 and 2.6:1. Immediately after mixing of ST and SC, t = 0, both networks showed liquid-like behaviour and free diffusion of the microparticles in the system (\( < \Delta r^2(\tau) > = \tau \)). As the time post mixing increased, the behaviour of both systems changed. A) The ST:SC 1.3:1 showed a progressive decrease in MSD and dependence on lag time until the system reached a plateau (\( \alpha = 0 \)), indicative of full gelation at \( \approx 45 \) minutes. B) The ST:SC 2.6:1 system showed a fast decline of MSD, that approached a constant value \( \alpha = 0 \) as early as 10 minutes.
Combinations of ST:SC at different concentrations of ST (namely ST = 3 mM and ST = 3.5 mM) showed an intermediate behaviour where complete gelation was achieved progressively faster at $\approx 25$ and $\approx 20$ minutes respectively, Fig. 3.4.

Figure 3.4: Gelation kinetics of ST-SasG-ST and SC3 2:1 and 2.3:1. Immediately after mixing of ST and SC, $t = 0$, both networks showed liquid-like behaviour and free diffusion of the microparticles in the system ($<\Delta r^2(\tau)> = \tau$). As the time post mixing increased, the behaviour of both systems changed, showing a progressive decrease in MSD and dependence on lag time until the systems reached a plateau $\alpha = 0$, indicative of full gelation at $\approx 25$ minutes and $\approx 20$ minutes respectively.
3.2.1.2 SC4 at 1 mM and ST-SasG-ST at 1, 2, 3, 3.5, 4 mM

Combinations of ST-SasG-ST and SC4 showed a similar behaviour where the speed of gelation increased with the increase in ST units into the system. As observed before, combinations of ST-SasG-ST:SC4 0.5:1 showed no gelation, indicative of the constant presence of a viscoelastic fluid, Fig. 3.5. Increasing the amount of ST led to the formation of a viscoelastic solid after \( \approx 45 \) minutes for the network consisting of ST:SC 1:1 whilst gelation was recorded within 10 minutes in the network consisting of ST:SC 2:1, Fig. 3.6. Once again, the subdiffusive dynamics of the slow gelling system were clearly visible and they can be attributed to the generation of protein clusters in the pre-gel that transition into a percolating network once the critical gelling point is reached. When gelation was complete, the MSD showed \( \alpha = 0 \), indicative of the formation of infinite elastic solid. As with SC3, in the fast-gelling system the subdiffusive dynamics were not visible, as this combination of ST:SC4 also reached complete gelation too quickly. In between combinations of ST-SasG-ST and SC4 showed progressively faster gelation, Fig. 3.7. Of note, ST-SasG-ST:SC4 1.5:1 showed very similar gelation kinetics to the ST:SC4 1:1 combination and, overall, the gelation of SC4 combined with ST-SasG-ST seemed to be slower than its SC3 counterpart, possibly due to the increased size of the SC array which would lead to slower diffusivity. For example, ST:SC4 1.5:1 gelled at \( \approx 45 \) minutes compared to ST:SC3 2:1 that gelled in \( \approx 25 \) minutes.
Figure 3.5: Gelation kinetics of ST-SasG-ST at 1 mM and SC4 at 1 mM, ST:SC 0.5:1. This combination shows no gelation and free diffusion of the beads, suggesting that the system never transitioned from a viscous liquid to an elastic solid.

Figure 3.6: Gelation kinetics of ST-SasG-ST and SC4 1:1 and 2:1. Immediately after mixing of ST and SC, t = 0, both networks showed liquid-like behaviour and free diffusion of the microparticles in the system (\( < \Delta r^2(\tau) > = \tau \)). As the time post mixing increased, the behaviour of both systems changed. A) The ST:SC 1:1 showed a decrease in MSD and dependence on lag time until the system reached a plateau \( \alpha = 0 \), indicative of full gelation at \( \approx 45 \) minutes. B) The ST:SC 2:1 system showed a fast decline of MSD, that approached a constant value \( \alpha = 0 \) as early as 10 minutes.
Immediately after mixing of ST and SC, \( t = 0 \), both networks showed liquid-like behaviour and free diffusion of the microparticles in the system \((<\Delta r^2(\tau) = \tau>)\). As the time post mixing increased, the behaviour of both systems changed, showing a progressive decrease in MSD and dependence on lag time until the systems reached a plateau \( \alpha = 0 \), indicative of full gelation at \( \approx 45 \) minutes and \( \approx 35 \) minutes respectively.

Figure 3.7: Gelation kinetics of ST-SasG-ST and SC4 1.5:1 and 1.75:1. Immediately after mixing of ST and SC, \( t = 0 \), both networks showed liquid-like behaviour and free diffusion of the microparticles in the system \((<\Delta r^2(\tau) >= \tau>)\). As the time post mixing increased, the behaviour of both systems changed, showing a progressive decrease in MSD and dependence on lag time until the systems reached a plateau \( \alpha = 0 \), indicative of full gelation at \( \approx 45 \) minutes and \( \approx 35 \) minutes respectively.
It appeared clear that the hydrogels gelled faster the more ST units were added to the system. Therefore, I set out to quantify the rate of gelation of the hydrogels to ascertain if this observed dependence was true. The constant rate of gelation, $K$, was calculated using the changes in the logarithmic slopes of the MSD with: 

$$\alpha(T)=\alpha(0)e^{-kT}$$

where $T$ is the real time at which the MSD was recorded. I determined the logarithmic slope of the MSD from the last decade of lag times, with $t > 1$ s, to prevent the known static and dynamic errors in particle tracking that limit the accuracy of the technique at short lag times [209]. Figure 3.8 shows an example of the logarithmic derivative of the MSD calculated from the data presented here as a function of real time after the gelation of the ST-SC system was initiated.

Figure 3.8: Example of the logarithmic derivative of the MSD calculated as a function of real time after spontaneous gelation was initiated in ST-SasG-ST:SC3 1.3:1 and ST-SasG-ST:SC3 2.6:1. A) Logarithmic derivative of ST-SasG-ST:SC3 1.3:1. B) Logarithmic derivative of ST-SasG-ST:SC3 2.6:1. The logarithmic slope of the MSD was determined from the last decade of lag times, with $t > 1$ s, to prevent the known static and dynamic errors in particle tracking. The values on the abscissa are adjusted to the datasets for ease of visualisation.
Figure 3.9 shows the rate of gelation $K$ for SC3 and SC4 in combination with ST-SasG-ST. $K$ linearly increases with the increase in ST units. $K$ was also slightly lower for SC4 compared to SC3, indicative of slower gelation, as intuited before.

Figure 3.9: Rate of gelation $K$ calculated using the changes in the logarithmic slopes of the MSD with: $\alpha(T) = \alpha(0)e^{-kT}$. Black: rate of gelation for ST-SasG-ST:SC3, pink: rate of gelation for ST-SasG-ST:SC4. Linear fit plotted in blue. $K$ linearly increases with the increase in ST units.
Lastly, I carried out the same microrheological experiments using combinations of ST-SasG-ST at 6 mM and SC3 or SC4 at 1 mM. However, the results showed that the speed of gelation was reduced, Fig 3.10, likely because of the very high viscosity of the ST-SasG-ST protein prep combined with the excess of ST to SC units, therefore these combinations was excluded from further analysis and 4 mM was determined to be the upper bound for the ST-SC protein hydrogels.

Figure 3.10: Gelation kinetics of ST-SasG-ST at 6 mM with SC3 or SC4 at 1mM, 4:1 and 3:1 respectively. A) ST-SasG-ST:SC3 4:1. B) ST-SasG-ST:SC4 3:1. Immediately after mixing of ST and SC, t = 0, both networks showed liquid-like behaviour and free diffusion of the microparticles in the system ($<\Delta r^2(\tau)> = \tau$). As the time post mixing increased, the behaviour of both systems changed, showing a progressive decrease in MSD and dependence on lag time. The gelation was slowed down for both SC3 and SC4, that never quite reached $\alpha = 0$. Likely, the high viscosity of ST-SasG-ST at 6 mM and the overabundance of ST units over SC are the cause of the observed slower gelation. These combinations was excluded from further analysis.
3.2.2 Gelation kinetics of ST-SasGlong-ST in combination with SC3 and SC4

3.2.2.1 SC3 at 1 mM and ST-SasGlong-ST at 1, 2, 3, 3.5, 4 mM

I moved forward the microrheological characterisation of the ST-SC protein hydrogels by investigating the gelation kinetics of ST-SasGlong-ST with SC3 and SC4. As expected, the combination of ST-SasGlong-ST:SC3 0.6:1 did not show any gelation, indicative of the permanence of a viscous liquid, Fig. 3.11. Interestingly, whilst the rate of gelation of ST-SasGlong-ST increased with the increase of ST units in the system, as expected, the logarithmic slopes of some ST-SasGlong-ST:SC3 combinations never reached $\alpha = 0$, but remained $\alpha > 0.5$. In particular, the ST:SC 1.3:1 hydrogel showed very slow progressive subdiffusive dynamics until 100 minutes and increasing the recording time did not change the slope of the curve, suggesting that the gelation of the system, as such, was complete, Fig. 3.12. The ST:SC 2:1 network also showed progressive gelation that never quite reached $\alpha = 0$, but $\alpha \approx 0.5$, indicative of the presence of a viscoelastic solid with a strong viscous component, Fig. 3.12. Further increasing the ST units into the system led to an increase in the gelation rates and to $\alpha = 0$ in the ST:SC 2.3:1 and 2.6:1 combinations that gelled within $\approx 20$ and $\approx 10$ minutes respectively, Fig. 3.13. The incomplete gelation observed in the ST-SasGlong-ST-SC3 hydrogels implied that whilst the system gelled as expected, the ST-SasGlong-ST:SC3 combinations maintained a prominent viscous component. Further research is needed to investigate this phenomenon and shed light on the specific interactions between the two proteins that leads to apparent incomplete gelation.
Figure 3.11: Gelation kinetics of ST-SasGlong-ST at 1 mM and SC3 at 1 mM, ST:SC 0.6:1. This combination shows no gelation and free diffusion of the beads, suggesting that the system never transitioned from a viscous liquid to an elastic solid.

Figure 3.12: Gelation kinetics of ST-SasGlong-ST and SC3 1.3:1 and 2:1. Immediately after mixing of ST and SC, t = 0, both networks showed liquid-like behaviour and free diffusion of the microparticles in the system \( \langle \Delta r^2(t) \rangle = \tau \). As the time post mixing increased, the behaviour of both systems changed. A) The ST:SC 1.3:1 showed a small decrease in MSD and dependence on lag time. The system never reached the plateau \( \alpha = 0 \), indicative of the presence of a viscous component in the hydrogel. B) The ST:SC 2:1 system showed a faster decline of MSD, but similarly never reached the constant value \( \alpha = 0 \).
Immediately after mixing of ST and SC, t = 0, both networks showed liquid-like behaviour and free diffusion of the microparticles in the system ($<\Delta r^2(\tau)> = \tau$). As the time post mixing increased, the behaviour of both systems changed showing a progressive decrease in MSD and dependence on lag time until the systems reached a plateau $\alpha = 0$, indicative of full gelation at $\approx 20$ minutes and $\approx 10$ minutes respectively.

Figure 3.13: Gelation kinetics of ST-SasGlong-ST and SC3 2.3:1 and 2.6:1. Immediately after mixing of ST and SC, t = 0, both networks showed liquid-like behaviour and free diffusion of the microparticles in the system ($<\Delta r^2(\tau)> = \tau$). As the time post mixing increased, the behaviour of both systems changed showing a progressive decrease in MSD and dependence on lag time until the systems reached a plateau $\alpha = 0$, indicative of full gelation at $\approx 20$ minutes and $\approx 10$ minutes respectively.
3.2.2.2 SC4 at 1 mM and ST-SasGlong-ST at 1, 2, 3, 3.5, 4 mM

I further combined the longer crosslinker ST-SasGlong-ST with SC4. As previously observed, the combinations of ST-SasGlong-ST:SC4 0.5:1 did not show any gelation and the system behaved like a viscous liquid throughout the experiment, Fig. 3.14. All other combinations of ST-SasGlong-ST and SC4 behaved as expected. The combination of ST-SasGlong-ST:SC4 1:1 was characterised by slow gelation kinetics and progressive subdiffusive dynamics until $\alpha = 0$ at $\approx 45$ minutes, Fig. 3.15. In this system, the subdiffusive dynamics were clearly visible and the transition states of the hydrogel from liquid to elastic solid were observed. Conversely, the ST-SasGlong-ST:SC4 2:1 showed very quick gelation, within $\approx 10$ minutes, and the subdiffusive dynamics were not seen due to the fast gelation rates that could not be captured with the technique used, Fig 3.15. In-between combinations of ST-SasGlong-ST and SC4 showed progressively faster gelation rates, where complete gelation was achieved after $\approx 20$ minutes for the ST:SC4 1.5:1 hydrogel and after $\approx 10$ minutes for the ST:SC4 1.75:1 hydrogel, Fig. 3.16.

Figure 3.14: Gelation kinetics of ST-SasGlong-ST at 1 mM and SC4 at 1 mM, ST:SC 0.5:1. This combination shows no gelation and free diffusion of the beads, suggesting that the system never transitioned from a viscous liquid to an elastic solid.
Figure 3.15: Gelation kinetics of ST-SasGlong-ST and SC4 1:1 and 2:1. Immediately after mixing of ST and SC, t = 0, both networks showed liquid-like behaviour and free diffusion of the microparticles in the system \( \langle \Delta r^2(\tau) \rangle = \tau \). As the time post mixing increased, the behaviour of both systems changed. A) The ST:SC 1:1 showed a decrease in MSD and dependence on lag time until the system reached a plateau \( \alpha = 0 \), indicative of full gelation at \( \approx 45 \) minutes. B) The ST:SC 2:1 system showed a fast decline of MSD, that approached a constant value \( \alpha = 0 \) as early as 10 minutes.

Figure 3.16: Gelation kinetics of ST-SasGlong-ST and SC4 1.5:1 and 1.75:1. Immediately after mixing of ST and SC, t = 0, both networks showed liquid-like behaviour and free diffusion of the microparticles in the system \( \langle \Delta r^2(\tau) \rangle = \tau \). As the time post mixing increased, the behaviour of both systems changed, showing a progressive decrease in MSD and dependence on lag time until the systems reached a plateau \( \alpha = 0 \), indicative of full gelation at \( \approx 20 \) minutes and \( \approx 10 \) minutes respectively.
As observed before, increasing the ST units in the system led to faster gelation rates. Therefore, I set out to quantify the constant rate of gelation $K$ as done before, using the changes in the logarithmic slopes of the MSD determined from the last decade of lag times, with $t > 1$ s, to prevent static and dynamic errors. Figure 3.17 shows the rate of gelation $K$ for SC3 and SC4 in combination with ST-SasGlong-ST. $K$ linearly increased with the increase in ST units. In contrast to what was observed with ST-SasG-ST, $K$ was higher for SC4 compared to SC3, given the partial gelation of the system observed in the combinations of ST-SasGlong-ST and SC3. Overall, the rate of gelation $K$ was smaller for ST-SasGlong-ST compared to ST-SasG-ST, indicating that ST-SasG-ST had faster gelation kinetics.

Figure 3.17: Rate of gelation $K$ calculated using the changes in the logarithmic slopes of the MSD with: $\alpha(T) = \alpha(0)e^{-kT}$. Black: rate of gelation for ST-SasGlong-ST:SC3, pink: rate of gelation for ST-SasGlong-ST:SC4. The linear fit is plotted in blue. $K$ linearly increases with the increase in ST units.
3.2.3 Changing the SC units

After determining the overall gelation rates of the ST-SC hydrogels by increasing only the ST content, I further characterised the gelation kinetics of the protein hydrogels by increasing the units of SC to investigate how the relationship between ST and SC affected the gelation kinetics and the limits of ST:SC in which gelation can occur. Combinations of ST-SasG-ST and SC3 were used as a model system to investigate how further changing the ratio of ST and SC influenced the gelation rates of the hydrogel.

The data presented in this section was collected by honours project student Megan Murdoch under my supervision using the methods I developed previously. All data from Megan’s honour dissertation is presented here with her consent. Of note, Fig. 3.18, 3.19, and 3.20 exhibited a lot of noise in the data. I decided to keep these datasets in my thesis to demonstrate the difficulties of accurately carrying out microrheology experiments and to acknowledge Megan’s hard work.
3.2.3.1 SC3 at 2 mM and ST-SasG-ST at 1 mM, 2mM, and 3 mM

The concentration of SC3 was increased to 2 mM (6 SC units) and systematically combined with ST-SasG-ST at 1, 2, and 3 mM. As observed previously, increasing the number of ST units led to an increase in the rate of gelation. Briefly, the combination of ST-SasG-ST and SC3 0.3:1 showed gelation within $\approx 55$ minutes, ST-SasG-ST and SC3 0.6:1 gelled within $\approx 45$ minutes, and the combination of ST-SasG-ST and SC3 1:1 exhibited rapid gelation within $\approx 10$ minutes, Fig. 3.18.

![Figure 3.18: Gelation kinetics of SC3 at 2 mM and ST-SasG-ST at 1 mM, 2mM, and 3 mM. A) ST-SasG-ST at 1mM and SC3 2 mM showed gelation within 55 minutes. B) ST-SasG-ST at 2mM and SC3 at 2 Mm gelled within $\approx 45$ minutes. C) ST-SasG-ST at 3 mM and SC3 at 2 mM exhibited rapid gelation within $\approx 10$ minutes. Data collected by honours project student Megan Murdoch.](image)

3.2.3.2 SC3 at 3 mM and ST-SasG-ST at 1 mM, 2mM, and 3 mM

Interestingly, when SC3 was further increased to 3 mM (9 SC units), the system showed a variation from the expected trend. In particular, the combination of ST-SasG-ST 1 mM and SC3 3 mM showed no gelation and the tracer beads exhibited free diffusion $\alpha \approx 1$ throughout, Fig. 3.19. Increasing the recording time did not change the gelation rates and the system remained as a viscous liquid. As expected, increasing the ST units led to gelation of the system at $\approx 35$ minutes for ST-SasG-ST 2 mM and $\approx 25$ minutes for ST-SasG-ST 3 mM, 3.19.
3.2.3.3 SC3 at 4 mM and ST-SasG-ST at 1.3 mM, 2mM, and 3 mM

The lack of gelation exhibited in the ST-SasG-ST:SC3 0.2:1 (SC3 at 3 mM combined with ST-SasG-ST at 1 mM) led to the hypothesis that a minimum ratio between ST and SC was needed in order for gelation to occur. To test this hypothesis, SC3 at 4 mM (12 SC units) was combined with ST-SasG-ST at 1.3 mM (2.6 ST units) to recreate the same ST:SC 0.2:1 ratio of SC3 at 3 mM combined ST-SasG-ST at 1 mM. As predicted, this combination failed to form a gel, confirming that ST and SC will form a hydrogel only within specific boundaries, Fig. 3.20. Decreasing the ratio of ST and SC to 0.3:1 by combining SC3 at 4 mM with ST-SasG-ST at 2 mM showed gelation within 35 minutes and combinations of SC3 at 4 mM with ST-SasG-ST at 3 mM showed gelation within $\approx 20$ minutes, Fig. 3.20.

Table 3.2 shows an overview of the gelation rates of combination of ST-SasG-ST and SC3 at different ST:SC ratio.
Figure 3.20: Gelation kinetics of SC3 at 4 mM and ST-SasG-ST at 1.3 mM, 2mM, and 3 mM. A) ST-SasG-ST 1.3 mM and SC3 4 mM showed no gelation. Increasing the ST units led to gelation of the system. B) ST-SasG-ST 2 mM and SC3 at 4 mM showed gelation in ≈ 35 minutes. C) ST-SasG-ST 3 mM and SC3 at 4 mM showed gelation in ≈ 20 minutes. Data collected by honours project student Megan Murdoch.

Table 3.2: Gelation rates of ST-SasG-ST and SC3 at different ST:SC ratio

<table>
<thead>
<tr>
<th>ST 1 mM = 2 ST units</th>
<th>SC3 1 mM = 3 SC units</th>
<th>SC3 2 mM = 6 SC units</th>
<th>SC3 3 mM = 9 SC units</th>
<th>SC3 4 mM = 12 SC units</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6:1 no gel</td>
<td>0.3:1, gel in ≈ 55 mins</td>
<td>0.2:1 no gel</td>
<td>ST 1.3 mM = 2.6 units 0.2:1 no gel</td>
<td></td>
</tr>
<tr>
<td>ST 2 mM = 4 ST units</td>
<td>1.3:1 gel in ≈ 45 mins</td>
<td>0.6:1 gel in ≈ 45 mins</td>
<td>0.4:1 gel in ≈ 35 mins</td>
<td>0.3:1 gel in ≈ 35 mins</td>
</tr>
<tr>
<td>2:1 gel in ≈ 25 mins</td>
<td>1:1 gel in ≈ 10 mins</td>
<td>0.6:1 gel in ≈ 25 mins</td>
<td>0.5:1 gel in ≈ 20 mins</td>
<td></td>
</tr>
</tbody>
</table>
The analysis of ST-SasG-ST combined with SC3 showed that when ST is progressively increased compared to SC, the rate of gelation is increased, as observed before, table 3.2, blue. This small case study also showed that an increase in the rate of gelation was also recorded as the ratio between SC3 and ST-SasG-ST remained the same (ST:SC 0.6:1), but the overall protein concentration was increased table 3.2, orange. Interestingly, approximately equimolar combinations of ST-SasG-ST and SC3 also showed faster gelation kinetics at increased protein concentrations 3.2, teal. Expanding the ratio of ST and SC beyond 0.2:1 resulted in failure to form a hydrogel, likely because all the available ST is unproductively crosslinked with the overabundant SC, leaving unbound SC and resulting in a viscous liquid. Further characterisation of the gelation kinetics of ST-SasG-ST and SC3 is needed to expand the blueprint offered by the experiments carried out in this project.

3.3 Discussion

Engineering hydrogels for precise medical applications requires a thorough understanding of the gelation kinetics that lead to gel formation. In this chapter, the gelation kinetics of the ST-SC hydrogels were investigated using particle tracking microrheology that allowed to capture the changes in the material’s rheology over time. I determined the rate of gelation of different combinations of the crosslinkers ST-SasG-ST and ST-SasGlong-ST by systematically combining them with either SC3 or SC4.

The microrheological characterisation clearly identified that the gelation speed of the ST-SC hydrogels increased with the increase in ST units. This was expected as the increase in peptide concentration leading to faster gelation kinetics was observed before [98, 109, 208]. Overall, the gelation kinetics appeared to be similar for most of the combinations analysed. Briefly, the ST:SC combinations failed to form a hydrogel when the SC units were present in excess compared to ST. This is likely due to mostly unproductive crosslinking between ST and the overabundant SC, that hinders the formation of a percolating network [109, 219]. Conversely, when the ST units were present in excess compared to SC, the combinations exhibited fast gelling kinetics with complete gelation reached in less than
10 minutes. Intuitively, the increase in ST units led to higher chances of chain encounter and, consequently, faster crosslinking [109]. In the fast-gelling systems, the subdiffusive dynamics were not clearly visible due to the rapid formation of an infinite protein cluster spanning the entire sample. Finally, when ST and SC were present at roughly equimolar concentrations the combinations exhibited slow gelation kinetics and complete gelation was reached in about \( \approx 45 \) minutes. The slow gelation kinetics exhibited by equimolar combinations of ST:SC allowed to observe the subdiffusive dynamics of the system. The formation and propagation of protein clusters in the pre-gel are responsible for the observed dynamics that culminated in a percolating network creating an infinite cluster spanning the entire sample when the critical gel point was achieved. Progressively increasing the units of ST compared to SC led to a progressive increase in the speed of the gelation kinetics from \( \approx 45 \) minutes to less than 10 minutes. The smaller and thorough case study of the gelation kinetics of ST-SasG-ST and SC3 also highlighted that increasing the total protein concentration of equimolar combinations of ST and SC led to faster gelation kinetics.

The combination of ST-SasGlong-ST and SC3 deviated from the identified trend and showed partial gelation at equimolar concentrations and, even when the ST units were increased compared to SC, the gelation of ST-SasGlong-ST remained partially incomplete. This deviation from the observed trend indicated that there are complexities in the gelation reaction of the ST-SC protein hydrogels that microrheology does not capture. For example, the potential formation of intermolecular loops between ST and SC and steric hindrances of crosslinking due to the large ST-SasGlong-ST molecules could be responsible for the observed changes. The presence of potential steric hindrance due to large molecules has already been identified as the probable cause of the deviation from Flory-Stockmayer theory of covalently crosslinked PEG-Heparin hydrogels [210] and the physical hydrogel system MITCH [98]. Since the combination of ST-SasGlong-ST and SC4 did not exhibit deviations from the observed trend, I hypothesised that the increased length of SC4 compared to SC3 could neutralize the potential steric hindrances and allow the binding of multiple ST-SasGlong-ST onto the same SC4 array, resulting in the typical gelation kinetics observed. Interestingly, the systematic rheological characterisation detailed in Chapter 4 also hinted at the presence of an underlying phenomenon
influencing the crosslinking of ST-SasGlong-ST and SC3 that deviated from the rheological trend observed in all other combinations. In the conclusions of this thesis, I tried to rationalise both the microrheological and rheological behaviour of ST-SasGlong-ST and SC3. Further characterisation of the specific interactions between ST-SasGlong-ST and SC3 was beyond the scope of this project, but molecular dynamics simulations and cluster aggregation analysis could help shed light on this particular phenomenon.

Moreover, the microrheological characterisation also showed that ST-SasG-ST is the most efficient crosslinker, allowing gel formation at the lowest protein concentration in the shortest amount of time. Depending on the chosen therapeutic application, this could be advantageous for engineering tuneable protein hydrogels. Combinations of ST-SasG-ST and SC3 showed the fastest gelation kinetics (K), closely followed by combinations of ST-SasG-ST and SC4. This is likely due to the smaller size of ST-SasG-ST compared to ST-SasGlong-ST that would lead to faster diffusivity. The same reasoning would also explain why combinations ST-SasG-ST and SC3 (smaller) gelled marginally faster that ST-SasG-ST combined with SC4 (bigger).

As previously mentioned, another interesting aspect discovered in the microrheological characterisation was that the subdiffusive dynamics of the fast-gelling combinations, when ST was present in excess compared to SC, were not observed. At higher ST content, the self-assembly process is faster and the movement of the tracer beads are restrained by the hydrogel network at shorter times compared to combinations with lower ST content. In the fast-gelling systems the kinetics transitioned from viscous liquid at 0 minutes (\(\alpha \approx 1\)), hence when ST and SC were first combined, to the presence of an elastic solid within 10 minutes (\(\alpha \approx 0\)). Data collected in between 0 and 10 minutes did not show any significant differences as the system formed a percolated network essentially immediately, making it impossible to register the fast gelation kinetics with the methods used. In addition, the fast gelation kinetics recorded were within the operational regime of passive microrheology for tracer beads of 1 µm in diameter \([202]\), but they were at their bottom limit. Taken together, these observations would suggest that the use of smaller tracer particles could be useful to investigate a system that gels quickly.
The Furst group evaluated the effects of probe particle size on the gelation of fast gelling systems [208]. Furst et al. identified that the structural length scales at the gel point of fast gelling systems are small enough that 1 µm diameter particles are unable to discern such small and fast changes and demonstrated identical behaviours ($\alpha = 0$). However, the structures of the developing gel still provided enough free volumes for particles with 0.75 µm in diameter to exhibit diffusive behaviour for longer time before their motion became subdiffusive due to the forming entanglements [208]. Therefore, it would be interesting to investigate the gelation kinetics of the fast-gelling ST-SC hydrogels within the first 10 minutes using smaller tracer probes and ascertain when precisely these combinations transition from viscous liquid to elastic solid. However, one would have to be careful to choose a probe radius that would be larger than the average size of the hydrogel mesh throughout the entire gelation process. This is because if, during gelation, the mesh size of the forming hydrogel is larger than the average probe radius, the probes would be unable to register the viscoelastic response of the hydrogel but they would reflect free diffusion in a heterogenous network [208, 220]. Given that the overall gelation kinetics of the ST-SC hydrogels were observable using 1 µm diameter tracer particles, I hypothesise that the overall mesh size of the protein hydrogels, hence their porosity, is smaller than 1 µm.

Another interesting factor to consider when analysing the different reaction speeds of the ST-SC protein hydrogels is that the gelation kinetics investigated in this chapter could also lead to the formation of hydrogels with different microstructural properties. In fact, according to fractal hydrogel theory, fast gelling systems, hence diffusion limited systems, typically generate structures with lower fractal dimension compared to reaction limited systems, hence hydrogels with slow gelation kinetics [221]. Characterisation of the fractal dimension of the ST-SC protein hydrogels was beyond the scope of this project, but it would be interesting to determine the fractal dimension of the ST - SC hydrogels and investigate if and how it correlates to the observed changes in gelation kinetics. For example, SAXS or SANS have been used previously to successfully investigate the microstructural properties of protein hydrogels [148].
The thorough microrheological characterisation detailed in this chapter was used to determine the appropriate speed of gelation needed to 3D print a ST-SC protein hydrogel as described in Chapter 6. Moreover, the thorough analysis of the gelation kinetics expands the purview of the potential applications of the ST-SC system for tissue engineering. In fact, depending on the therapeutic application, the time of gelation could vary from seconds to up to several minutes. For example, slow gelation kinetics such as the ones exhibited by equimolar combination of ST and SC at low protein concentration could be suitable for surgical delivery into an area of the body with little void space and high tortuosity, such as the spinal cord. Fast gelation in these applications could lead to clogging up of the delivery needle and failure of the material to diffuse in the affected area. Conversely, the fast gelation kinetics caused by the excess in ST would be better suited to targeted injectable delivery of therapeutics.

Finally, it would be remiss of me if I did not stress the experimental limitations of the microrheological characterisation carried out in this chapter. Namely, given the spontaneous and unregulated gelation of the ST-SC system, it was very difficult to capture the 0 minutes gelation point, as the ST and SC had to be mixed together onto the glass slide, the coverslip applied, the chamber put onto the microscope and a Region of Interest (ROI) had to be found before the recording of the movement of the tracer probes could begin. The process was streamlined and I trained myself to be as quick as possible. Any combination where it took me than 3 minutes to begin recording was discarded and I would start anew. However, it should be noted that perhaps 2 minutes should be added to the gelation kinetics of the ST-SC protein hydrogels detailed in this chapter.
3.4 Conclusions

The aim of this chapter was to investigate the gelation kinetics of the ST-SC protein hydrogels using particle tracking passive microrheology. I showed that by subtly changing the ratio of ST:SC in the system one can control the speed of gelation of protein-based hydrogels from slow gelation kinetics (≈ 45 minutes) to fast gelation (≈ 10 minutes). Moreover, by changing the ST:SC ratios more dramatically, I was able to determine the ratios beyond which no gelation occurred (due to one of the components being present in large excess). Within these established boundaries, I observed that the rate of gelation was directly proportional to the number of ST units and that, at equimolar combinations of ST and SC, increasing the overall protein concentration led to faster gelation kinetics.

The work carried out in this chapter offers a blueprint of the gelation kinetics of the ST-SC protein hydrogels that allowed me to adapt the hydrogel design to 3D printing. In addition, the characterisation carried out here could also expand the uses of the ST-SC hydrogel system to specific therapeutic applications depending on the desired gelation speed. Overall, understanding the gelation kinetics of the ST-SC protein hydrogels increased the capabilities of synthetic biology to target different therapeutic applications and allowed synthetic biology to interface with tissue engineering and nanotechnology.
Chapter 4

Rheology: Investigation on the viscoelastic properties of the ST-SC protein hydrogels

The text and figures in this chapter are adapted from the following article which was published in 'Gels' following peer review:

“Modulating the viscoelastic properties of covalently crosslinked protein hydrogels”

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In order to provide a structure better suited to this thesis, certain sections have been merged and rearranged or renamed. The SpyCatcher array SC5 was cloned, expressed, and purified by Regan lab PhD student Fokhrul Islam. All other experimental work was carried out solely by myself.

4.1 Introduction

In essence, organs are complex materials with specific properties and structures that drive a unique set of biological events. Tissue engineering is tasked with recreating this complexity and building a biomaterial able to fulfil the biomechanical functions of an injured or diseased tissue and restoring its functionality.
The concept of organ stiffness goes hand in hand with tissue engineering [1] but, since its inception, the concept has evolved to reflect the newfound complexities of the native physiological environment. Initially, the proposition was simple: to successfully replace native tissue a biomaterial should have similar mechanical properties. Hence, tissue engineering started focusing on hard scaffolds to regenerate bone structures, or on softer hydrogels for most other organs. In both cases, the biomaterials were essentially used as support structures for embedded cells. However, this simplistic model rapidly changed when it was discovered that cells feel and respond to the stiffness of their substrate [7]. Therefore, the structure of the biomaterial affects how the cells respond to it and to the surrounding environment.

Nowadays, when engineering a new hydrogel, it is crucial to not only consider how the material will biomechanically integrate with the host tissue, but also how the correct biomechanical signalling will be transmitted to the embedded cells, primarily via the material’s stiffness, to ensure the correct regulation of cell proliferation, attachment, and differentiation [8].

The field of rheology explores the behaviour of matter under deformation, i.e. how forces imposed onto a material change its stiffness [139, 222]. Investigating the bulk mechanical properties of hydrogels is fundamental to ascertain the suitability of the material to a specific tissue engineering application. Given the complexity of the human physiological environment, the stiffness of living tissues spans a large scale from the soft neural tissue (1-3 kPa) to hard bone (1.28 – 1.97 GPa) [8]. Because of this, the final stiffness of hydrogels is a crucial characteristic that directly impacts the ability of the material to be used successfully in tissue engineering. In particular, this project targeted liver regeneration and the stiffness of the hydrogels was investigated against the organ’s reported stiffness, $G' = 10,500$ Pa, and decellularised liver tissue, i.e. liver ECM without cells, $G' = 1,180$ Pa [223].
In addition, rheological properties are also invaluable in determining the processability and the suitability of hydrogels to specific delivery systems. For example, shear thinning hydrogels able to self-heal, hence fully or partially regain their properties once the stress is removed, are excellent candidates for injectable applications, whilst shear thickening hydrogels are more suited to surgical implantation where a degree of shearing prior to positioning is to be expected [139].

Therefore, it appears clear that studying and understanding material stiffness with the aim of mimicking the stiffness of the native tissue is crucial to engineering lifelike biomaterials.

### 4.1.1 Small deformation rheology

The main method to quantitatively assess the mechanical properties of a material is small deformation rheology. Small deformation rheology is carried out on macroscopic samples of materials and within the linear viscoelastic region (LVR). The LVR is where the response of the material to deformation is independent from the magnitude of the imposed stress or strain [139]. Typically, small deformation tests are defined as small amplitude oscillatory shear (SAOS) measurements, where the sample is continuously oscillated around its equilibrium position.

SAOS measurements can be carried out using strain or stress-controlled rheometers, where strain is defined as the deformation of the material caused by an external force, the stress. In both cases, using a parallel plate geometry, the sample is positioned in the rheometer between two plates at a known and fixed gap (h) and the upper plate oscillates periodically at a predetermined stress or strain, Fig. 4.1. It is important to notice that the gap between the plates has to be selected to allow the material to spread evenly between the two plates without imposing deformation, to avoid risks linked to the uneven distribution of the material that could induce artefacts in the rheology experiment. However, once the gap has been set, it does not change for the duration of the experiment and the material is only subjected to the oscillation of the plates. In a strain-controlled rheometer, the sample is subjected to deformation, hence a strain, and the torque (i.e. the stress) needed
to impose such strain is calculated. Conversely, in a stress-controlled rheometer, a known torque is imposed onto the upper plate and the resulting strain imposed onto the material is calculated [224]. Herein, I will focus on stress-controlled rheometers like the TA DHR-2 used for the rheological measurements carried out in this chapter.

Figure 4.1: Schematics of a rheometer equipped with a parallel plate geometry The sample is positioned between the two plates at a known and fixed gap (h) and the top plate oscillates periodically at a predetermined stress or strain. Once set, the gap is not altered for the duration of the experiment. Created with BioRender.com
The ratio of the applied stress to the calculated strain gives the complex modulus (G*), a quantitative measure of the stiffness of the material and its ability to resist deformation. Using complex notation, the G* of a material can be further subdivided into its two constituents: \( G* = G' + iG'' \), with \( G' \) being the real component of the material and \( G'' \) its counterpart, the imaginary component. \( G' \) represents the storage modulus, or elastic component of the material and it is a measurement of the amount of energy stored by the material during deformation. This stored energy will be the driving force that will allow the material to revert back to its original shape (in the ideal case) once the stress has been removed. A purely elastic materials can be modelled as a spring obeying Hooke’s law where the applied stress is proportional to the resulting strain, Fig. 4.2 [224].

Conversely, \( G'' \) represents the loss modulus, or viscous component, and it is a measurement of how much energy is lost by the material during deformation due to internal frictions. This energy is mostly dissipated via heat, and the deformation imposed onto the material is permanent. A purely viscous material, or Newtonian fluid, is modelled as a dashpot, Fig. 4.2, [224].

In a parallel plate SAOS measurement, the motion of the system is represented by a sinusoidal wave where the shear stress is applied at a determined angular frequency (\( \omega \)) and delta (\( \delta \)) is the loss factor \( \delta = G''/G' \). In an ideally elastic material, stress and strain are in phase and \( \delta = 0^\circ \). Conversely, for a purely viscous material, stress and strain are considered out of phase and \( \delta = 90^\circ \). Most real materials exhibit viscoelastic behaviour, that is a behaviour between that of an ideal elastic solid and an ideal viscous liquid. Specifics depend on the material’s composition and microstructure. Therefore, for real materials the phase difference will fall somewhere between 0° and 90° according to how much the viscous and elastic components of the material contribute to the material’s total stiffness (G*). As an example, the Kelvin-Voigt model, a spring and a dashpot connected in parallel, is the simplest representation of a viscoelastic solid, Fig. 4.2, [139].
Figure 4.2: **Schematic representation of a SAOS measurement.** The ratio of the applied stress to the calculated strain gives the complex modulus $G^*$, divided into its constitutive components $G'$ and $iG''$. $G'$ is the elastic component, the energy stored by the material during deformation under stress which allows the material to restore its shape. A purely elastic material is modelled as a spring obeying Hooke’s law where the applied stress is proportional to the resulting strain. In an ideally elastic material, stress and strain are in phase and $\delta = 0^\circ$. $G''$ represents the viscous component, the energy lost by the material during deformation and dissipated via heat. An ideally viscous material is modelled as a dashpot. In a purely viscous material, stress and strain are out of phase and $\delta = 90^\circ$. Real materials show viscoelastic behaviour, depending on the material’s composition and microstructure, with $0^\circ < \delta < 90^\circ$. Viscoelastic solids are represented by the Kelvin-Voigt model, a spring and a dashpot connected in parallel. Created with BioRender.com.
4.1.2 Strain and frequency sweep

In hydrogel rheology, $G'$ and $G''$ are primarily measured as a function of strain or frequency. Briefly, if $G' > G''$ ($\tan(\delta) < 1$), the material behaves like an elastic solid, whilst if $G' < G''$ ($\tan(\delta) > 1$), the material behaves like a viscoelastic liquid [225]. Of note, $G'$ and $G'$ can also be measured as a function of time, a time sweep, to determine how the material’s structure changes over time. Time sweeps are particularly useful to determine the gelation time of a hydrogel, visualised as the crossover point between $G'$ and $G''$ [22]. In this thesis, it was decided not to carry out time sweeps as the speed of gelation of the ST-SC hydrogels was already determined via microrheology in a passive system (i.e. with no external force imposed). The information gathered during the microrheological characterisation was deemed sufficient enough to carry out other experiments, such as 3D printing, therefore the material was conserved and subsequently used. $G'$ and $G''$ were analysed as a function of strain and frequency to gather crucial information regarding the structure and stability of the ST-SC protein hydrogels as detailed below.

Determining the behaviour of $G'$ and $G''$ against strain establishes the linear viscoelastic region (LVR) of the material, where the properties of the hydrogel are independent from the shear stress applied. In the LVR, the applied stresses are not enough to yield the material, i.e. cause structural degradation, therefore the material’s properties at rest can be characterised. Outside of this region, the material’s properties are changing with stress and the microstructural properties cannot be observed as easily. Strain sweep experiments determine the LVR by periodically applying an increasing oscillation strain at a constant angular frequency $\omega$, Fig. 4.3A. The limit of the LVR is indicated by the point where the structure begins to yield, known as the yield stress [224]. If the structure presents $G' > G''$ in the LVR, the material is a viscoelastic solid and the yield stress is the value at which $G'$ becomes dependant on stress until $G' = G''$, where the material starts to flow, the flow point, Fig. 4.3B. Below the yield stress and inside the LVR, the material will deform linearly and its properties can be observed. The yield stress of a material depends on its structural composition. Typically, for a gel, yielding will result in viscoelastic liquid behaviour showing shear thinning [225].
Figure 4.3: **The behaviour of $G'$ and $G''$ against strain.** A) Strain sweeps determine the LVR by periodically applying an increasing oscillation strain at a constant angular frequency $\omega$; B) the limit of the LVR is point where the structure begins to yield, the yield stress. The material starts to flow at the crossover point $G' = G''$, the flow point. Created with BioRender.com
Investigating $G'$ and $G''$ as a function of frequency measures the time dependency of a material by decreasing (or increasing) the frequency ($\omega$) of a constant applied stress, Fig. 4.4A. Because frequency is inversely proportional to time ($t$), $\omega = 1/t$, high frequencies correspond to short time scales and low frequencies correspond to long timescales. Frequency sweeps are particularly useful to investigate the differences between the microstructure of physically entangled gels and chemically crosslinked hydrogels [22]. In physically entangled gels, the $G'$ and $G''$ values are highly frequency dependant and the entangled gels will only appear solid over an appropriate time scale [22]. Specifically, physically entangled gels present storage modulus significantly higher than the loss modulus at high frequencies whilst they will relax and diffuse over long time scales ($G'' > G'$). Conversely, for irreversibly chemically crosslinked hydrogels, both $G'$ and $G''$ are independent from frequency, and $G'$ exceeds $G''$ towards infinitely low frequencies and long timescales [139, 225]. The absence of a crossover frequency, i.e. the frequency where $G' = G''$, indicates the presence of permanently crosslinked hydrogels, Fig. 4.4B, and the magnitude of the $G'$ and $G''$ plateaus are proportional to the stiffness of the hydrogels [22].

Figure 4.4: **The behaviour of $G'$ and $G''$ against frequency.** A) Frequency sweeps determine the time dependency of a material by increasing (or decreasing) the frequency ($\omega$) of a constant applied stress. B) Irreversibly chemically crosslinked hydrogels show $G'$ and $G''$ independent from frequency, and $G'$ exceeds $G''$ throughout the entire range of frequency analysed. The absence of a crossover frequency indicates the presence of permanently crosslinked hydrogels. Created with BioRender.com.
In this chapter I systemically explore the rheological properties of protein-based hydrogels designed using the SpyTag - SpyCatcher system. I carried out both strain and frequency sweeps to investigate the changes in elastic and loss moduli and determine what conditions alter the rheological nature of the hydrogels. Specifically, I investigated how the identity of the protein pair, the ST:SC ratio, and the protein concentration affect the final viscoelastic properties of the hydrogels. I will demonstrate how a clear trend emerged from the rheological investigation which allowed me to develop a blueprint of the viscoelastic behaviour of ST - SC protein hydrogels for tissue engineering applications. Finally, I will discuss the potential reasons behind this trend and the factors that could influence the microstructure of the ST-SC hydrogels.

In this chapter I will show numerous graphs which will depict the full spectrum of the systematic characterisation that was carried out. However, I have noted below the core graphs for each section to facilitate the reading and the understanding of the chapter. The core graphs highlight the behaviour detailed in each section whilst the other graphs are supporting evidence of the same rheological behaviour, or variations thereof, using other combinations of proteins. Figure 4.6 reports the entire systematic characterisation carried out.

In this chapter I will show:

- Viscoelastic changes in response to the identity of the protein pair, core graph: Figure 4.6.
- Viscoelastic changes in response to ST:SC unit ratio, core graph: Figure 4.8.
- Viscoelastic changes in response to total protein concentration, core graph: Figure 4.20.
- Viscoelastic changes in response to the unfolding of the protein building blocks, core graph: Figure 4.27.
4.2 Results

The aim of this chapter was to characterise the rheological properties of the ST-SC protein hydrogels to ascertain their suitability for tissue engineering and, specifically, with the aim of using them for liver tissue regeneration. I present a thorough investigation of the viscoelastic properties of the protein hydrogels by systematically combining the two crosslinkers ST-SasG-ST and ST-SasGlong-ST with the SC arrays SC3, SC4, and SC5. Figure 4.5 shows an example of a self-standing ST-SC protein hydrogel taken out of an Eppendorf tube and positioned onto the sandblasted bottom plate used for the rheological characterisation detailed here.

![Figure 4.5: Self-standing ST-SC protein hydrogel positioned on the sandblasted bottom plate used for rheology. ST and SC were mixed at the required mM concentrations inside an Eppendorf tube. After gelation was complete, the hydrogel was delicately taken out of the tube and positioned onto the rheometer.](image)

It is important to notice that the 8 mm parallel plate geometry used in all experiments detailed in this chapter lacked a solvent trap due to its small size, therefore the temperature could not be thoroughly controlled. However, the temperature was controlled as much as possible by maintaining the bottom plate of the rheometer at 25°C and by keeping the temperature of the room where the rheometer was located at 23 °C. No evaporation or drying of the sample was observed for the duration of the experiments. Another limitation of the rheological experiments
detailed in this chapter is linked to the transfer of the samples from the Eppendorf tubes to the bottom plate of the rheometer. All hydrogels were made in Eppendorf tubes by mixing together ST and SC at the appropriate mM concentrations and, as such, the hydrogels assumed the concave geometry of the bottom of the tube, Fig. 4.5. All hydrogels were made inside Eppendorf tubes of the same form and size, however pipetting errors and small changes in the tube’s geometry could have induced changes in the sample’s thickness and shape. The Eppendorf tubes were cut in half to facilitate the extraction of the hydrogels, that were taken out as delicately as possible, positioned on the sandblasted bottom plate of the rheometer and left to equilibrate. The top plate was lowered at slow speed to reduce any orientation or stress effects and the samples were allowed to rest for 5 minutes after lowering of the top plate to maintain consistency with the conditions of operation before measurements were taken. All efforts were made to ensure that the hydrogels’ temperature, thickness, and geometry were stable and consistent and damages to the structures due to sample transfer were minimised as much as possible. Each experiment was repeated three times to ensure errors or artifacts introduced were minimised. For example, if the hydrogels were visibly fractured or damaged during transfer, the sample was discarded and a new one prepared. Clearly, a damaged hydrogel would have altered the rheological results as it is unlikely that the sample would have sustained sufficient elastic deformation or stress. The data presented in this chapter is of high quality as the error bars plotted are well within the margin of errors of published standards, but the limitations of the methods linked to sample transfer and suboptimal control over the hydrogel’s temperature, thickness, and geometry have to be acknowledged.

4.2.1 Viscoelastic changes in response to the identity of the protein pairs

During the rheological characterisation, it appeared clear that the identity of the protein pair involved in hydrogel formation was a crucial factor affecting the resulting hydrogel properties. Specifically, altering either side of the protein building blocks, ST crosslinkers or SC arrays, changed the viscoelastic response of the final hydrogels.
I collected all my findings in Fig. 4.6 to showcase the differences in the rheological behaviour of the ST-SC hydrogels formed by ST-SasG-ST or ST-SasGl-ong-ST combined with SC3, SC4 or SC5 at the same molar ratios.

When possible in this chapter, all hydrogels are referred to on the basis of their ST and SC unit content. For example, ST-SasG-ST at 2 mM has 4 units of ST (2 mM x (2 ST in each SasG chain)) and SC3 at 1 mM has 3 units of SC (1 mM x 3 SC units). Therefore, ST-SasG-ST at 2 mM combined with SC3 at 1 mM will be referred to at ST:SC 1.3:1. Table 4.2 shows an overview of the ST:SC molar ratios investigated in this chapter and their resulting networks for ease of understanding.

Table 4.1: Hydrogel combinations analysed for rheology

<table>
<thead>
<tr>
<th>ST</th>
<th>1 mM = 2 ST units</th>
<th>2 mM = 4 ST units</th>
<th>3 mM = 6 ST units</th>
<th>3.5 mM = 7 ST units</th>
<th>4 mM = 8 ST units</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC3</td>
<td>1 mM = 3 SC units</td>
<td>0.6:1</td>
<td>1.3:1</td>
<td>2:1</td>
<td>2.3:1</td>
</tr>
<tr>
<td>SC4</td>
<td>1 mM = 4 SC units</td>
<td>0.5:1</td>
<td>1:1</td>
<td>1.5:1</td>
<td>1.75:1</td>
</tr>
<tr>
<td>SC5</td>
<td>1 mM = 5 SC units</td>
<td>0.4:1</td>
<td>0.8:1</td>
<td>1.2:1</td>
<td>1.4:1</td>
</tr>
</tbody>
</table>

Combining my findings also showed a clear trend covering all ST-SC combinations, indicating the presence of underlying rules responsible for the overall viscoelastic changes in the protein hydrogels, Fig. 4.6. Specifically, regardless of the protein pair used, when SC is present in great excess compared to ST, the structure results in a viscoelastic liquid rather than a hydrogel (G’ < G”). Conversely, when the ST crosslinker is present in great excess compared to SC, the resulting hydrogels will exhibit G’ = 1,000 Pa no yielding up until 100% strain. Approximately equimolar combinations of ST and SC will create a hydrogel characterised by G’ = 10,000 Pa and critical yield stress at ≈ 10% strain. By increasing the total protein concentration, equimolar combinations of ST-SC will generate a hydrogel with G’
$= 1,000$ Pa and no yielding up until $100\%$ strain. There are two notable exceptions to this trend. Specifically, all combinations of ST-SasGlong-ST and SC3 exhibited the same physical characteristics, i.e. $G' = 10,000$ Pa and critical yield stress at $\approx 10\%$ strain, regardless of ST:SC ratio and protein concentration. Combinations of ST-SasGlong-ST and SC3 also deviated from the observed trend during the micro-rheological characterisation detailed in Chapter 3, suggesting the presence of an underlying phenomenon linked to potential steric hindrances that limits additional crosslinking in this combination. This phenomenon will be discussed thoroughly later on. Combinations of ST-SasG-ST and SC5 also deviated from the observed trend insofar as approximately equimolar combinations of ST-SasG-ST and SC5 exhibited $G' = 5,000$. Ongoing investigation in the Regan Lab on the nature of SC5 are showing the potential presence of truncated proteins, indicating the presence of other SpyCatchers in the SC5 protein preps. This could explain the physical properties observed as SC5 might have parts of SC3 and SC4 and behave in the middle of the two. Further characterisation to ascertain this phenomenon is ongoing.

Having presented all my results, I will now proceed to illustrate the variables investigated, i.e. the changes in response to the ST:SC unit ratio and the total protein concentration, in detail. In each section, data will be presented as follows: combinations of SC3 with ST-SasG-ST and ST-SasGlong-ST, followed by combinations of SC4 with ST-SasG-ST and ST-SasGlong-ST, and finally combinations of SC5 with ST-SasG-ST and ST-SasGlong-ST.
Figure 4.6: Core Graph. Systematic rheological characterisation of the ST-SC hydrogels. Top row: combinations of ST-SasG-ST with A) SC3 (black), B) SC4 (pink), and C) SC5 (green). Bottom row: combinations of ST-SasGlong-ST with D) SC3 (black), E) SC4 (pink), and F) SC5 (green). O: viscous liquid (G’<G’’), X: G’ = 10,000 Pa, *: G’ = 5,000 Pa, + : G’ = 1,000 Pa. A trend emerged from the characterisation: when SC is present in great excess compared to ST, the structure results in a viscoelastic liquid rather than a hydrogel (G’<G’’); when ST is present in great excess compared to SC, the hydrogels exhibit G’ = 1,000 Pa and no yielding up until 100% strain; approximately equimolar combinations of ST and SC result in a hydrogel with G’ = 10,000 Pa and critical yield stress at 10% strain. By increasing the total protein concentration, equimolar ST-SC combinations generate a structure with G’ = 1,000 Pa and no yielding until 100% strain. Combinations of ST-SasGlong-ST and SC3 showed consistent physical properties with G’ = 10,000 Pa and critical yield stress at 10%. Combinations of ST-SasG-ST and SC5 exhibited G’ = 5,000 Pa potentially due to the presence of truncated proteins.
4.2.2 Viscoelastic changes in response to ST:SC unit ratio

I made a series of hydrogels, keeping the molar concentration of the SC array constant at 1 mM while gradually increasing the ST component from 1 mM to 4 mM, to examine how variations in the number of ST:SC units altered the rheological characteristics of the resulting protein hydrogels. I carried out strain and frequency sweeps to test the changes in the hydrogels’ shearing behaviour. Strain sweeps were carried out with increasing strain from 0.1 to 100% with constant frequency 100 rad/s and frequency sweeps were carried out with decreasing frequency from 100 to 0.1 rad/s at constant strain 1%.

4.2.2.1 Combinations of SC3 with ST-SasG-ST and ST-SasGlong-ST

Combinations of ST-SasG-ST:SC3 0.6:1 resulted in a viscoelastic liquid rather than a hydrogel (G’ < G’’), Fig. 4.7. Increasing the ST component to 1.3:1 led to the formation of a hydrogel and the network exhibited gel like behaviour, with G’ = 10,000 Pa and G’ > G’’ throughout the entire range of frequency analysed, as expected from a permanently crosslinked hydrogel, Fig. 4.8B (black). This network exhibited critical yield stress at 10% strain, where the material yielded and a rapid decrease in the elastic modulus occurred until G’ fell below G’’, indicative of viscous liquid behaviour, Fig. 4.8A (black). Further increase of the ST units led to a 10-fold reduction in G’ to 1,000 Pa and no apparent yield stress up to 100% strain as shown by the ST:SC 2:1, 2.3:1 and 2.6:1 combinations, Fig. 4.8.
Figure 4.7: **Combinations of ST-SasG-ST and SC3 at 0.6:1** Strain sweeps were carried out with strain increasing from 0.1 to 100% and fixed frequency $\omega = 100$ rad/s. B) Frequency sweep of ST-SasG-ST and SC3 0.6:1. Frequency sweeps were carried out inside the established LVR by decreasing frequency from 100 to 0.1 rad/s at constant strain 1%. $G'$ = full squares, $G''$ = empty squares. Combinations of ST-SasG-ST:SC3 0.6:1 resulted in a viscoelastic liquid rather than a hydrogel ($G' < G''$). Error bars represent the standard deviation with number of repeats per conditions $N = 3$.

Figure 4.8: **Core graph. Example of behaviour at varying ST:SC unit ratio.** A) strain sweep of ST-SasG-ST and SC3. Strain sweeps were carried out with increasing strain from 0.1 to 100% with constant frequency 100 rad/s; B) frequency sweep of ST-SasG-ST and SC3. Frequency sweeps were carried out with decreasing frequency from 100 to 0.1 rad/s at constant strain 1%. $G'$ = full squares, $G''$ = empty squares. Black: ST-SasG-ST:SC3 1.3:1 exhibited gel like behaviour, with $G' = 10,000$ and $G' > G''$, as expected from a permanently crosslinked hydrogel, and critical yield stress at $\approx 10\%$ strain. Combinations of ST-SasG-ST and SC3 at 2:1 (pink), 2.3:1 (green), and 2.6:1 (purple) showed $G' = 1,000$ Pa and no yielding up until 100% strain. Error bars represent the standard deviation with number of repeats per conditions $N = 3$. 
Similarly, combinations of ST-SasGlong-ST and SC3 0.6:1 showed the permanence of a viscoelastic liquid, Fig. 4.9, and increasing the ST unit led to the formation of permanently crosslinked hydrogels. However, combinations of ST-SasGlong-ST and SC3 consistently exhibited the same physical properties characterised by $G' = 10,000$ Pa, $G' > G''$, and critical yield stress at $\approx 10\%$ strain at all the ST:SC ratio analysed, Fig. 4.10.

Figure 4.9: **Combinations of ST-SasGlong-ST and SC3 at 0.6:1** A) strain sweep of ST-SasGlong-ST and SC3 at 0.6:1; B) Frequency sweep of ST-SasGlong-ST and SC3 0.6:1. $G'$ = full squares, $G'' = \text{empty squares}$. Combinations of ST-SasGlong-ST:SC3 0.6:1 resulted in a viscoelastic liquid rather than a hydrogel ($G' < G''$). Error bars represent the standard deviation with number of repeats per conditions $N = 3$. 
Figure 4.10: **Combinations of ST-SasGlong-ST and SC3**

A) strain sweep of ST-SasGlong-ST and SC3. B) Frequency sweep of ST-SasGlong-ST and SC3. $G'$ = full squares, $G''$ = empty squares. Combinations of ST-SasGlong-ST and SC3 at 1.3:1 (grey), 2:1 (pink), 2.3:1 (green), and 2.6:1 (purple) showed $G' = 10,000$, $G' > G''$ over all frequencies as expected from a permanently crosslinked hydrogel, and critical yield stress at $\approx 10\%$ strain. Error bars represent the standard deviation with number of repeats per conditions $N = 3$. 
4.2.2.2 Combinations of SC4 with ST-SasG-ST and ST-SasGlong-ST

I further investigated the behaviour of SC4 paired with ST-SasG-ST or ST-SasGlong-ST. The ST-SasG-ST behaviour previously observed in combination with SC3 was replicated when ST-SasG-ST was combined with SC4. The ST-SasG-ST:SC4 0.5:1 combination resulted in a viscoelastic liquid (G’<G”), Fig. 4.11. Once the ST units were increased, the equimolar ST-SasG-ST:SC4 combination 1:1 exhibited G’ = 10,000 Pa, G’ > G”, and critical yield stress at 10% strain. Further increase of the ST units led to the formation of permanently crosslinked hydrogels characterised by G’ = 1,000 Pa and no yielding until 100% strain, Fig. 4.12.

Figure 4.11: Combinations of ST-SasG-ST and SC4 at 0.5:1 A) strain sweep of ST-SasG-ST and SC4 at 0.5:1; B) Frequency sweep of ST-SasG-ST and SC4 0.5:1. G’ = full squares, G” = empty squares. Combinations of ST-SasG-ST:SC4 0.5:1 resulted in a viscoelastic liquid rather than a hydrogel (G’ < G”). Error bars represent the standard deviation with number of repeats per conditions N = 3.
Figure 4.12: **Combinations of ST-SasG-ST and SC4.** A) strain sweep of ST-SasG-ST and SC4; B) frequency sweep of ST-SasG-ST and SC4. \( G' \) = full squares, \( G'' \) = empty squares. Black: ST-SasG-ST:SC4 1:1 exhibited gel-like behaviour, with \( G' = 10,000 \) and \( G' > G'' \), as expected from a permanently crosslinked hydrogel, and critical yield stress at \( \approx 10\% \) strain. Combinations of ST-SasG-ST and SC3 at 1.5:1 (pink), 1.75:1 (green), and 2:1 (purple) showed \( G' = 1,000 \) Pa and no yielding up until 100\% strain. Error bars represent the standard deviation with number of repeats per conditions \( N = 3 \).
As expected, combinations of ST-SasGlong-ST and SC4 0.5:1 resulted in a viscoelastic liquid (G’ < G’’), Fig. 4.13, and increasing the ST units led to the formation of permanently crosslinked hydrogels. The ST-SasGlong-ST:SC4 1:1 combination was characterised by G’ = 10,000 Pa, G’ > G’’ and critical yield stress at ≈ 10%, whilst combinations containing an excess of ST units compared to SC resulted in hydrogels characterised by G’ = 1,000 Pa, G’ > G’’, and no yielding up until 100% strain, Fig. 4.14. The behaviour of ST-SasGlong-ST combined with SC4 is in contrast with the behaviour of ST-SasGlong-ST combined with SC3 where the hydrogels presented constant physical characteristics (G’ = 10,000 Pa).

Figure 4.13: **Combinations of ST-SasGlong-ST and SC4 at 0.5:1** A) strain sweep of ST-SasGlong-ST and SC4 at 0.5:1; B) Frequency sweep of ST-SasGlong-ST and SC4 0.5:1. G’ = full squares, G’’ = empty squares. Combinations of ST-SasGlong-ST:SC4 0.5:1 resulted in a viscoelastic liquid rather than a hydrogel (G’ < G’’). Error bars represent the standard deviation with number of repeats per conditions N = 3.
Figure 4.14: **Combinations of ST-SasGlong-ST and SC4.** A) strain sweep of ST-SasGlong-ST and SC4; B) frequency sweep of ST-SasGlong-ST and SC4. $G'$ = full squares, $G''$ = empty squares. Grey: ST-SasGlong-ST:SC4 1:1 exhibited gel-like behaviour, with $G' = 10,000$ and $G' > G''$, as expected from a permanently crosslinked hydrogel, and critical yield stress at $\approx 10\%$ strain. Combinations of ST-SasGlong-ST and SC3 at 1.5:1 (pink), 1.75:1 (green), and 2:1 (purple) showed $G' = 1,000$ Pa and no yielding up until 100% strain. Error bars represent the standard deviation with number of repeats per conditions $N = 3$. 
4.2.2.3 Combinations of SC5 with ST-SasG-ST and ST-SasGlong-ST

Combinations of ST-SasGlong-ST and SC5 exhibited a similar behaviour to the one observed so far. When SC was present in great excess compared to ST, i.e. in the ST-SasGlong-ST:SC5 0.4:1 combination, the mixture resulted in a viscoelastic liquid ($G' < G''$), Fig. 4.15. Progressive increase of the ST unites led to the formation of permanently crosslinked hydrogels. At approximately equimolar combinations of ST and SC, the resulting hydrogel was characterised by $G' = 10,000$ Pa, $G'> G''$, and critical yield stress at $\approx 10\%$, Fig. 4.16. When ST was present in great excess compared to SC, the resulting hydrogels exhibited $G' = 1,000$ Pa, $G'> G''$, and no yielding up until 100% strain, Fig. 4.16.

Figure 4.15: Combinations of ST-SasGlong-ST and SC5 at 0.4:1 A) strain sweep of ST-SasGlong-ST and SC5 at 0.4:1; B) Frequency sweep of ST-SasGlong-ST and SC5 0.4:1. $G'$ = full squares, $G''$ = empty squares. Combinations of ST-SasGlong-ST:SC5 0.4:1 resulted in a viscoelastic liquid rather than a hydrogel ($G' < G''$). Error bars represent the standard deviation with number of repeats per conditions $N = 3$. 
Figure 4.16: Combinations of ST-SasGlong-ST and SC5. A) strain sweep of ST-SasGlong-ST and SC5; B) frequency sweep of ST-SasGlong-ST and SC5. $G'$ = full squares, $G''$ = empty squares. Grey: ST-SasGlong-ST:SC5 0.8:1 exhibited gel like behaviour, with $G' = 10,000$ and $G' > G''$, as expected from a permanently crosslinked hydrogel, and critical yield stress at $\approx 10\%$ strain. Combinations of ST-SasGlong-ST and SC5 at 1.2:1 (pink), 1.4:1 (green), and 1.6:1 (purple) showed $G' = 1,000$ Pa and no yielding up until 100\% strain. Error bars represent the standard deviation with number of repeats per conditions $N = 3$. 

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Combinations of SC5 and ST-SasG-ST led to a partial variation in the observed behaviour. As expected, combinations of SC5 with ST-SasG-ST at a 0.4:1 ratio resulted in a viscoelastic liquid rather than a hydrogel (G' < G") at lower protein concentrations compared to SC3 and SC4. The partial deviation from the observed behaviour could be linked to the increased flexibility of the SC5 arrays due to its longer glycine rich linker (GGS)₄GSG compared to that of SC3 and SC4 (GGS)₂RS that could allow the formation of crosslinks with ST-SasG-ST with different spatial orientation. However, as mentioned before, current investigation ongoing in the Regan lab has shown the potential presence of truncated proteins in the SC5 array that could be responsible for the recorded G' = 5,000 as the SC5 could behave ‘in-between’ SC3 and SC4. Of note, combinations involving the longer SC5 array supported the formation of hydrogels (G' > G") at lower protein concentrations compared to SC3 and SC4. For example, the 0.8:1 ST:SC5 network resulted in a hydrogel rather than a viscous liquid for both SasG long, Fig. 4.16 and SasG, Fig. 4.18. It is possible that the quantity of SC units in 1 mM of SC5 compared to SC3 and SC4 lowered the threshold needed to form a hydrogel due to the presence of enough SC able to form crosslinks with ST. Extrapolating from this finding, lengthening the SC array could allow the formation of a hydrogel at even lower protein concentrations, thereby creating a hydrogel with the desired physical characteristics using less protein.
Figure 4.17: **Combinations of ST-SasG-ST and SC5 at 0.4:1**

A) strain sweep of ST-SasG-ST and SC5 at 0.4:1; B) Frequency sweep of ST-SasG-ST and SC5 0.4:1. $G'$ = full squares, $G''$ = empty squares. Combinations of ST-SasG-ST:SC5 0.4:1 resulted in a viscoelastic liquid rather than a hydrogel ($G' < G''$). Error bars represent the standard deviation with number of repeats per conditions $N = 3$.

Figure 4.18: **Combinations of ST-SasG-ST and SC5**

A) strain sweep of ST-SasG-ST and SC5; B) frequency sweep of ST-SasG-ST and SC5. $G'$ = full squares, $G''$ = empty squares. Grey: ST-SasG-ST:SC5 0.8:1 exhibited gel like behaviour, with $G' = 10,000$ and $G' > G''$, as expected from a permanently crosslinked hydrogel, and critical yield stress at $\approx 10\%$ strain. Pink: ST-SasG-ST and SC5 exhibited $G' = 5,000$ Pa and yielding at $\approx 50\%$ strain. Combinations of ST-SasG-ST and SC5 at 1.4:1 (green), and 1.6:1 (purple) showed $G' = 1,000$ Pa and no yielding up until 100% strain. Error bars represent the standard deviation with number of repeats per conditions $N = 3$. 

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Figure 4.19: Combinations ST-SasG-ST and SC5 1.2:1 and 1.4:1. A) strain sweep of ST-SasG-ST and SC5; B) frequency sweep of ST-SasG-ST and SC5. \( G' \) = full squares, \( G'' \) = empty squares. Pink: ST-SasG-ST and SC5 exhibited \( G' = 5,000 \) Pa and yielding at \( \approx 50\% \) strain. Green: ST-SasG-ST and SC5 at 1.4:1 showed \( G' = 1,000 \) Pa and no yielding up until 100\% strain. Error bars represent the standard deviation with number of repeats per conditions \( N = 3 \).
4.2.3 Viscoelastic changes in response to total protein concentration

I expanded the rheological analysis on the ST-SC hydrogels to determine if and how the previously observed viscoelastic properties change in response to alterations in the total protein concentration of the building blocks. I created a new series of hydrogels where the ST:SC unit ratio was kept constant at 1:1 but the total protein concentration was progressively increased and carried out the same strain and frequency sweeps as in 4.2.2. Tables 4.2 and 4.3 detail the millimolar concentrations and the corresponding number of SC or ST units analysed in this section, concentration 1 being the lowest and concentration 4 being the highest. The number of ST and SC units were chosen \textit{a priori} and the corresponding mM concentrations were subsequently calculated. For example, 1.25 units of SC in SC3 corresponds to 0.41 mM because: \textit{mM concentration} $= \frac{1.25 \text{ units}}{3 \text{ SC in SC3}} = 0.41 \text{ mM}$. Similarly, 1.25 ST units in ST-SasG-ST correspond to 0.625 mM because: \textit{mM concentration} $= \frac{1.25}{2 \text{ ST in ST-SasG-ST}} = 0.625 \text{ mM}$. In this section, hydrogels will be referred to based on their concentration because their ST:SC units’ ratio was always 1:1.
Table 4.2: Molar concentration and number of SC and ST units of SC3 (A), SC4 (B), SC5 (C) in combination with ST-SasG-ST.

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<th>SC5 [mM]</th>
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Table 4.3: Molar concentration and number of SC and ST units of SC3 (A), SC4 (B), SC5 (C) in combination with ST-SasGlong-ST (L).

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4.2.3.1 Combinations of SC3 with ST-SasG-ST and ST-SasGlong-ST

At low protein concentrations, A1 and A2, SC3 combined with ST-SasG-ST resulted in a viscoelastic liquid rather than a hydrogel (G’<G”), Fig. 4.20. Once the protein concentration was increased, A3 and A4, combinations of ST-SasG-ST and SC3 resulted in permanently crosslinked hydrogels. In particular, A3 exhibited G’ = 10,000 Pa and yielding at 10% strain, and by increasing the total protein concentration in A4, the hydrogel exhibited to G’ = 1,000 Pa and no yielding up until 100% strain, Fig. 4.20. Combinations of ST-SasGlong-ST and SC3 also resulted in viscoelastic liquids (G’ < G”) rather than hydrogels at low protein concentrations, AL1 and AL2, Fig. 4.21. Increasing the total protein concentration led to the formation of permanently crosslinked hydrogels characterised by G’ = 10,000 Pa and critical yield stress at ≈ 10% strain at all other protein concentration analysed, AL3 and AL4, Fig. 4.21. As observed before, the rheological behaviour of the ST-SasGlong-ST – SC3 hydrogels remained unchanged regardless of the ST:SC unit ratio or the total protein concentration.

Figure 4.20: Core Graph. Example of transitionary behaviour with increasing total protein concentration. Combinations of ST-SasG-ST and SC3 with increasing (from A1 to A4) total protein concentration and ST:SC = 1. A) strain sweep of ST-SasG-ST and SC3; B) frequency sweep of ST-SasG-ST and SC3. G’ = full squares, G” = empty squares. Black: A1 ST-SasG-ST 0.625 mM + SC3 0.4 mM resulted in a viscoelastic liquid, G’ < G”. Pink A2: ST-SasG-ST 1.25 mM + SC3 0.83 resulted in a viscoelastic liquid, G’ < G”. Green A3: ST-SasG-ST 2.5 mM + SC3 1.6 mM exhibited gel like behaviour, with G’ = 10,000 and G’ > G”, as expected from a permanently crosslinked hydrogel, and critical yield stress at ≈ 10% strain. Purple A4: ST-SasG-ST 3.75 mM + SC3 2.5 mM exhibited G’ = 1,000 Pa and no yielding up until 100% strain. Error bars represent the standard deviation with number of repeats per conditions N = 3.

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Figure 4.21: Combinations of ST-SasGlong-ST and SC3 with increasing (from AL1 to AL4) total protein concentration and ST:SC = 1. A) strain sweep of ST-SasGlong-ST and SC3; B) frequency sweep of ST-SasGlong-ST and SC3. G' = full squares, G'' = empty squares. Grey: AL1 ST-SasGlong-ST 0.625 mM + SC3 0.4 mM resulted in a viscoelastic liquid, G' < G''. Pink AL2: ST-SasGlong-ST 1.25 mM + SC3 0.83 resulted in a viscoelastic liquid, G' < G''. Green AL3: ST-SasGlong-ST 2.5 mM + SC3 1.6 mM exhibited gel behaviour, with G' = 10,000, G' > G'', as expected from a permanently crosslinked hydrogel, and critical yield stress at ≈10% strain. Purple AL4: ST-SasGlong-ST 3.75 mM + SC3 2.5 mM exhibited gel behaviour with G' = 10,000 Pa, G' > G'', as expected from a permanently crosslinked hydrogel, and critical yield stress at ≈10% strain. Error bars represent the standard deviation with number of repeats per conditions N = 3.
4.2.3.2 Combinations of SC4 with ST-SasG-ST and ST-SasGlong-ST

I moved forward the rheological characterisation and systematically analysed combinations of SC4 with ST-SasG-ST and ST-SasGlong-ST. At low protein concentrations, B1 and B2, combinations of SC4 with ST-SasG-ST resulted in viscoelastic liquids, $G' < G''$, Fig. 4.22. Once the total protein concentration was increased, B3 and B4, the ST-SasG-ST – SC4 hydrogels exhibited $G' > G''$, $G' = 1,000$ Pa and no yielding up until 100% strain, Fig. 4.22. Of note, a hydrogel characterised by $G' = 10,000$ Pa and critical yield stress at 10% strain was achieved at lower total protein concentration in the 1:1 network composed of ST-SasG-ST 2 mM and SC4 1 mM, Fig. 4.14. Combinations of ST-SasGlong- ST and SC4 resulted in viscoelastic liquids at low protein concentration, BL1 and BL2, as expected, Fig. 4.23. Increasing the protein concentration led to the formation of permanently crosslinked hydrogels characterised by $G' = 10,000$ Pa and critical yield stress at 10% (BL3) and $G' = 1,000$ Pa and no yielding up until 100% strain (BL4), Fig. 4.23.

Figure 4.22: Combinations of ST-SasG-ST and SC4 with increasing (from B1 to B4) total protein concentration and ST:SC = 1. A) strain sweep of ST-SasG-ST and SC4; B) frequency sweep of ST-SasG-ST and SC4. $G'$ = full squares, $G''$ = empty squares. Black: B1 ST-SasG-ST 0.625 mM + SC4 0.312 mM resulted in a viscoelastic liquid, $G' < G''$. Pink B2: ST-SasG-ST 1.25 mM + SC4 0.625 mM resulted in a viscoelastic liquid, $G' < G''$. Green B3: ST-SasG-ST 2.5 mM + SC4 1.25 mM exhibited gel like behaviour, with $G' = 1,000$ and $G' > G''$, as expected from a permanently crosslinked hydrogel, and no yielding until 100% strain. Purple B4: ST-SasG-ST 3.75 mM + SC4 1.875 mM exhibited $G' = 1,000$ Pa and no yielding up until 100% strain. Error bars represent the standard deviation with number of repeats per conditions N = 3.
Figure 4.23: Combinations of ST-SasGlong-ST and SC4 with increasing (from BL1 to BL4) total protein concentration and ST:SC = 1. A) strain sweep of ST-SasGlong-ST and SC4; B) frequency sweep of ST-SasGlong-ST and SC4. $G'$ = full squares, $G''$ = empty squares. Grey: BL1 ST-SasGlong-ST 0.625 mM + SC4 0.312 mM resulted in a viscoelastic liquid, $G' < G''$. Pink BL2: ST-SasGlong-ST 1.25 mM + SC4 0.625 resulted in a viscoelastic liquid, $G' < G''$. Green BL3: ST-SasGlong-ST 2.5 mM + SC4 1.25 mM exhibited gel like behaviour, with $G' = 10,000$ and $G' > G''$, as expected from a permanently crosslinked hydrogel, and critical yield stress at 10% strain. Purple BL4: ST-SasGlong-ST 3.75 mM + SC4 1.875 mM exhibited $G' = 1,000$ Pa and no yielding up until 100% strain. Error bars represent the standard deviation with number of repeats per conditions N = 3.
4.2.3.3 Combinations of SC5 with ST-SasG-ST and ST-SasGlong-ST

As expected, combining ST-SasG-ST and SC5 at very low protein concentration, C1, resulted in a viscoelastic liquid, $G' < G''$, Fig. 4.24. Increasing the total protein concentration led to the formation of permanently crosslinked hydrogels. Specifically, C2 resulted in $G' = 10,000$ Pa and yielding at 10% strain, C3 exhibited $G' = 5,000$ Pa and critical yield stress at $\approx 10\%$ strain, and, when the total protein concentration was increased in C4, the hydrogels exhibited $G' = 1,000$ Pa and no yielding up until 100% strain, Fig. 4.24. It also worth noticing that the difference between concentration C3 (ST-SasG-ST 2.5 mM and SC5 1 mM, hence 5 units each of ST and SC) and the ST:SC5 network 1.2:1 (ST-SasG-ST 3 mM and SC5 1 mM, hence 6 units of ST and 5 of SC) in Fig. 4.19 was only 0.5 mM of ST-SasG-ST in favour of the ST:SC 1.2:1 network. However, the strain sweep showed that the critical strain for the 1.2:1 network was $\approx 50\%$ whilst C3 yielded at $\approx 10\%$, demonstrating how a small increase in only one component led to an appreciable difference in the viscoelastic behaviour of the resulting system, Fig. 4.25. This led to hypothesise that there is a strong level of cooperativity between ST and SC in the ST-SC protein hydrogels. However, as mentioned before, research is currently ongoing in the Regan Lab to ascertain the purity of the SC5 construct. Combinations of ST-SasGlong-ST and SC5 behaved as expected. Briefly, the combination of ST-SasGlong-ST and SC5 resulted in a viscoelastic liquid at low protein concentration, CL1, Fig. 4.26. Increasing the total protein concentration led to the formation of permanently crosslinked hydrogels. Specifically, CL2 resulted in $G' = 10,000$ Pa and yielding at 10% strain whilst CL3 and CL4 exhibited $G' = 1,000$ Pa and no yielding up until 100% strain, Fig. 4.26. Notably, SC5 allowed the formation of a permanently crosslinked hydrogels at lower protein concentrations compared to SC3 and SC4, as observed before, likely due to the increased length of the array.
Figure 4.24: Combinations of ST-SasG-ST and SC5 with increasing (from C1 to C4) total protein concentration and ST:SC = 1. A) strain sweep of ST-SasG-ST and SC5; B) frequency sweep of ST-SasG-ST and SC5. $G'$ = full squares, $G''$ = empty squares. Black: C1 ST-SasG-ST 0.625 mM + SC5 0.25 mM resulted in a viscoelastic liquid, $G' < G''$. Pink C2: ST-SasG-ST 1.25 mM + SC5 0.5 mM exhibited $G' = 10,000$ Pa and yielding at 10% strain. Green C3: ST-SasG-ST 2.5 mM + SC5 1 mM exhibited gel like behaviour, with $G' = 10,000$ and $G' > G''$, and yielding at $\approx 10\%$ strain. Purple C4: ST-SasG-ST 3.75 mM + SC5 1.5 mM exhibited $G' = 1,000$ Pa and no yielding up until 100% strain. Error bars represent the standard deviation with number of repeats per conditions N = 3.

Figure 4.25: Strain sweep of ST:SC5 1.2:1 (pink - as in Fig. 4.19) compared to ST-SasG-ST – SC5 concentration C3 (green). $G'$ = full squares, $G''$ = empty squares. The difference between C3 (ST-SasG-ST 2.5 mM and SC5 1 mM) and the ST:SC5 1.2:1 network (ST-SasG-ST 3 mM and SC5 1 mM) was 0.5 mM of ST-SasG-ST. The strain sweep showed critical strain for the 1.2:1 network at $\approx 50\%$ and $\approx 10\%$ for C3. Error bars represent the standard deviation with number of repeats per conditions N = 3.
Figure 4.26: Combinations of ST-SasGlong-ST and SC5 with increasing (from CL1 to CL4) total protein concentration and ST:SC = 1. A) strain sweep of ST-SasGlong-ST and SC5; B) frequency sweep of ST-SasGlong-ST and SC5. $G' = \text{full squares}, G'' = \text{empty squares}$. Grey: CL1 ST-SasG-ST 0.625 mM + SC5 0.25 mM resulted in a viscoelastic liquid, $G' < G''$. Pink CL2: ST-SasG-ST 1.25 mM + SC5 0.5 mM exhibited $G' = 10,000$ Pa and yielding at $\approx 10\%$ strain. Green CL3: ST-SasGlong-ST 2.5 mM + SC5 1 mM exhibited gel like behaviour, with $G' = 1,000$ and $G' > G''$, and no yielding up until 100% strain. Purple CL4: ST-SasGlong-ST 3.75 mM + SC5 1.5 mM exhibited $G' = 1,000$ Pa and no yielding up until 100% strain. Error bars represent the standard deviation with number of repeats per conditions $N = 3$. 

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4.2.4 Viscoelastic changes in response to the unfolding of the protein building blocks

I further expanded the rheological analysis on the ST-SC hydrogels to test the influence of protein folding on the hydrogel’s properties. Tissue engineering relies on the ability of hydrogels to mimic the properties of the native tissue, therefore the mechanical properties of hydrogel networks have been modulated using a wealth of different methods. Recently, changing protein thermodynamic and mechanical stability has been shown to alter the hydrogel macroscopic properties and allow control over the network’s architecture and mechanics [148, 149]. For example, control over protein unfolding was linked to the ability to program shape memory into biomaterials, that ‘remembered’ bulk shape after large deformations [148]. Therefore, I investigated the viscoelastic changes in response to the unfolding of the building blocks via high concentrations of urea to gain insight into how the protein thermodynamic and kinetic stability affect the bulk mechanics of mature hydrogels.

In particular, I investigated how the viscoelastic response of the hydrogels would change if the protein building blocks were unfolded but the covalent crosslinks between ST and SC remained in place. Wang et al. showed that a concentration of 3.2 M urea was enough to unfold stable telechelic protein catenates built via the SpyTag-SpyCatcher complex [226]. Therefore, to ensure protein unfolding in the hydrogels, after gelation, I soaked the hydrogel ST-SasG-ST:SC3 2.6:1 (4mM ST-SasG-ST and 1 mM of SC3) in 8 M urea overnight and I carried out the same strain and frequency sweep as before. Whilst I could not quantify the extent of protein unfolding within the hydrogel, it is reasonable to assume that significant protein unfolding occurred after treatment in 8 M urea given the findings of Wang et al. I expected the urea to unfold the protein building blocks but leave the covalent bonds between ST and SC intact. Prior to urea treatment, the hydrogel exhibited permanently crosslinked gel behaviour, G’ > G” throughout the entire range of frequency, G’ = 1,000 Pa and no yielding up until 100% strain, as shown in Fig. 4.8 (purple). After urea treatment, Fig. 4.27, the hydrogel still exhibited permanently crosslinked gel behaviour indicative of the stability of the predominantly unaffected covalent bonds between ST and SC, but both G’ and G” were increased. The increase in G” was particularly significant as the loss modulus went from ≈ 100 Pa before urea
treatment to $\approx 5,000$ Pa after, whilst the increase in $G'$ was less considerable, from $\approx 1,000$ Pa to $\approx 8,000$ Pa. Therefore, the considerable increase in $G''$ combined with the smaller increase in $G'$ led to an increase in the loss ratio $\delta (G''/G')$, a marker of the level of protein unfolding in folded protein hydrogels [149], confirming substantial unfolding of the protein building blocks. Specifically, $\delta$ without urea was calculated to be: $\approx 0.1$ and $\delta$ after urea was: $\approx 0.625$. Further characterisation on the influence of protein unfolding on the hydrogel properties is needed. In particular, this experiment aimed at the complete denaturation of the protein building blocks to test the network’s rigidity in response to the unfolding of the proteins, but further work could focus on the partial unfolding of the system using lower concentrations of urea to investigate how the ratio between folded and unfolded building blocks influences the stability of the entire network, with a view of optimising the ST-SC hydrogels for stiffer or softer organs compared to liver.

Figure 4.27: Viscoelastic response of the hydrogel ST-SasG-ST:SC3 2.6:1 with (black) and without (purple – as in Fig. 4.8) overnight soaking in 8 M urea. A) strain sweep of ST-SasG-ST and SC3; B) frequency sweep of ST-SasG-ST and SC3. $G'$ = full squares, $G''$ = empty squares. Black: ST-SasG-ST:SC3 2.6:1 after urea treatment; purple: ST-SasG-ST:SC3 2.6:1 without urea treatment. After soaking in 8 M urea, the hydrogel exhibited permanently crosslinked gel behaviour ($G' > G''$) with an increase in $G'$ and $G''$. Error bars represent the standard deviation with number of repeats per conditions $N = 3$. 

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4.3 Discussion

Due to the precise control that protein engineering offers over the resulting hydrogel properties, protein-based hydrogels are becoming increasingly significant in tissue engineering, biophysics, and soft matter. Understanding how to further adapt this innovative class of material into precise and accurate applications requires a fundamental knowledge of protein hydrogel mechanics as well as insights into the underlying formation and deformation mechanisms of the protein building blocks.

In this chapter, I rheologically characterised protein hydrogels developed using the SpyTag-SpyCatcher system. By systemically combining one of the two crosslinkers, ST-SasG-ST or ST-SasGlong-ST, with the SC arrays, SC3, SC4, and SC5, I demonstrated that changes in the protein building blocks affect the viscoelastic response of the resulting hydrogels. Specifically, I showed that variations in the ST:SC unit ratio and the total protein concentration influence the rheological properties of the ST-SC hydrogels. Moreover, all hydrogels presented in this Chapter exhibited $G' > G''$ towards infinitely low frequencies, confirming that the ST-SC system generates permanently crosslinked hydrogels. A distinct pattern emerged from the systematic rheological characterisation: the resulting structure will be a viscoelastic liquid rather than a hydrogel ($G' < G''$) when SC is present in great excess compared to ST. Conversely, when ST is present in great excess of SC, the hydrogels exhibit $G' = 1,000$ Pa and no yielding up until 100% strain. When ST and SC are equimolar, or nearly equimolar, the resulting hydrogels exhibit $G' = 10,000$ and critical yield stress at 10% strain. Raising the total protein concentration in equimolar combinations of ST and SC generates a hydrogel characterised by $G' = 1,000$ Pa and no yielding up until 100% strain. The results presented here are consistent with the work of Mulyasasmita et al. on the physically entangled system MITCH, Mixing-Induced Two-Component Hydrogel [98]. Briefly, Mulyasasmita et al. showed that by increasing the total concentration, equimolar combinations of MITCH transitioned from a viscous liquid to a progressively stiffer hydrogel. The authors also showed that by changing the ratio of the two MITCH components at the same concentration, the MITCH system transitioned from viscoelastic liquid to stiff hydrogel. However, great excess of one component compared to the
other led to a decrease in hydrogel stiffness. Other authors have reported similar findings where the ratio of the building blocks led to changes in the viscoelastic behaviour of their hydrogels, resulting in either no gel formation or weakening of the hydrogel [109, 162, 165]. Since all viscoelastic responses of a material are due to its internal structure, I proceeded to hypothesise the reasons behind the rheological behaviour of the ST-SC protein hydrogels.

It is possible to consider the ST crosslinkers akin to telechelic chains given the similarities between the two structures [191, 226]. Telechelic polymers are linear chains containing associating ‘sticky’ groups only at the chain ends and, similarly, the ST crosslinkers were engineered to have two sticky ST at each end of the stiff, rod-like protein SasG or SasGlong [225].

The association of telechelic chains was modelled by Winnik and Yekta as a function of concentration. Winnik and Yekta demonstrated how telechelic chains associate in two primary conformations, named ‘loops’ and ‘bridges’, Fig. 4.28 [227]. Briefly, at low concentrations, if the telechelic chains are separated from each other by a distance greater that the chain’s radium of gyration, a telechelic chain is likely to form an isolated loop onto itself because it cannot stretch enough to associate with another chain. The isolated loops are deemed ‘inactive’ and they do not contribute to the modulus of the polymer. Conversely, at high concentrations, active bridges connect multiple telechelic chains spanning the entire network. These bridges are deemed ‘active’ and they contribute to the stability of the modulus by connecting two different chains [227].

I hypothesised that the combination of ST-SasG-ST and ST-SasGlong-ST with the SC arrays could result in loop and bridge conformations that are comparable to those of telechelic chains. Specifically, at low concentrations of ST, i.e. when SC is present in great excess compared to ST, the number ST crosslinkers is insufficient to form enough active bridges and the excess SCs could remain unbound, or bound to only one ST, and act as inactive loops throughout the system. The presence of excess binding domains prevents the development of an interconnected network, such that the system behaves as a viscoelastic liquid (G’<G”).
At equimolar concentrations the number of active bridges is maximised as the higher number of ST crosslinkers is able to join enough SC belonging to different arrays, creating intermolecular crosslinks and inducing the development of active bridges throughout the entire hydrogel network that, in turn, translate into a stiff matrix (G' = 10,000 Pa). Intuitively, equimolar combinations of ST and SC yield the stiffest hydrogels, as any deviation from this ratio would lead to excess binding domains that do not contribute to the network’s structure. However, the lack, or low presence, of excess binding domains could mean that the hydrogel is more brittle (critical yield stress at 10%) because when one ST crosslinker frees itself from a SC array, it will take longer for a new ST-SC connection to form given that most other crosslinkers will be already occupied, therefore the network relaxes rapidly.

Conversely, when ST is present in great excess of SC, the excess ST crosslinkers cause the formation of dangling ends, where the crosslinkers have only one ST bound to one SC array and the other ST remains unbound due to the lack of available SC. These dangling ends, akin to inactive loops, do not contribute to the hydrogel’s network, thereby weakening it and generating G' = 1,000 Pa. However, the presence of dangling ends and the excess of ST could induce the fast formation of new ST-SC crosslinks once one ST-SC breaks, allowing the hydrogel to deform up until 100% strain. Similarly, if the protein concentration in solution is increased above a certain threshold, the crosslinking of the chains could be limited by steric hindrances, leading to a reduction in the hydrogel’s stiffness but an increased resistance to deformation due to the presence of more ST able to reform a broken ST-SC connection. Figure 4.28 is a schematic representation of the loops and bridges conformations in telechelic chains as modelled by Winnik and Yekta with the corresponding conformations hypothesised in the ST – SC hydrogels.
Figure 4.28: Schematic representation of the loops and bridges conformations in telechelic chains as modelled by Winnik and Yekta and the corresponding conformations hypothesised in the ST – SC hydrogels. A) At low concentrations, a telechelic polymer chain cannot stretch enough to associate and form an isolated loop. In the ST-SC system, the number ST crosslinkers is insufficient to form enough active bridges and the excess SCs could remain unbound, or bound to only one ST, and act as inactive loops throughout the system. B) At equimolar concentrations the telechelic chains are connected together by active bridges forming an extend network. In the ST-SC system, the ST crosslinker joins two SC belonging to two different arrays, creating intermolecular crosslinks and inducing the development of active bridges throughout the entire hydrogel network. C) At higher concentrations the presence of dangling ends does not contribute to the polymer’s modulus, thereby weakening it. In the ST-SC system the dangling ends caused by excess ST weaken the hydrogel’s matrix and induce the faster formation of new ST-SC crosslinks once one ST-SC breaks, allowing the hydrogel to deform up until 100% strain. Dangling ends are highlighted by black arrows.
This model can be further supported by calculating the molecular weight between entanglements/crosslinks using Equation 1 [225].

Equation 1. \( G_N^0 = \frac{\rho RT}{M_e} \)

Where \( \rho \) is the protein density (calculated to be 1.35 g/cm\(^3\) and particularly independent from molecular weight [228]), \( M_e \) is defined as the molecular weight between entanglements, and \( G_N^0 \) is the entanglement plateau modulus of G’ in the appropriate frequency range [225]. The curves presented here are all in the relevant frequency region where G’ is nearly constant, therefore G’ corresponds to \( G_N^0 \). \( M_e \) can be visualised as the distance between two corners in a square grid, where the network is represented by the edges of the boxes and the crosslinks are at the corners of each box, Fig. 4.29. Briefly, more crosslinks between different binding domains lead to shorter molecular weight between them, i.e a strong network, whilst fewer crosslinks translate into larger distances between them that lead to larger molecular weights and lower \( G_N^0 \). Thus, when the number of crosslinks is maximised in equimolar ST-SC combinations, the molecular weight between crosslinks \( M_e \) is low and \( G_N^0 \) is high (G’ = 10,000 Pa). Conversely, when ST is present in great excess of SC, the hydrogel weakens (G’ = 1,000 Pa), indicating that there are fewer linkers joining up ST and SC due to the presence of dangling ends, i.e. ST attached to one end of the SC array but not linked to anything else, therefore \( M_e \) increases and \( G_N^0 \) decreases.
Figure 4.29: **Schematic representation of the relationship between $G_N^0$ and $M_e$.** A): At equimolar combinations of ST and SC, the number of crosslinks is maximised: $M_e$ is low and $G_N^0$ is high ($G' = 10,000$ Pa). B) When ST is present in great excess of SC, fewer linkers join ST and SC due to the presence of dangling ends, $M_e$ increases and $G_N^0$ decreases, resulting in a weaker hydrogel ($G' = 1,000$ Pa).
Notably, combinations of ST-SasGlong-ST and SC3 exhibited $G' = 10,000$ Pa and critical yield stress at 10% strain at all protein concentration and all ratios analysed. I hypothesised that unique and specific topological interactions between the longer SasGlong protein and the shortest SC3 array are responsible for this deviation from the observed behaviour. For example, chain connectivity and spacer rigidity of larger molecules have been shown to limit the degree of freedom of large binding domains that could be sterically inaccessible [98,210]. Therefore, it is possible that the increased bulk and rigidity of ST-SasGlong-ST combined with the short length of the SC3 array maximises the formation of crosslinks in the system whilst hindering the formation of dangling ends due to steric limitations, i.e. excess ST-SasGlong-ST remains unbound because there is not enough space for it to bind to an available array regardless of the increase in ST or protein concentration. Further experiments and molecular modelling are needed to ascertain the details of the behaviour underlying the viscoelastic response of ST-SasGlong-ST and SC3.

Another interesting explanation for the structural differences between the protein hydrogels might be their differing reaction speeds [229]. In brief, at high reaction rates, the rate of transport is the limiting factor to gelation, and the reaction is called diffusion limited, but at slow reaction rates, the reaction rate itself is the limiting element, and the system is called reaction limited. Evidenced from the microrheological investigation detailed in Chapter 3, the protein hydrogels here described present considerably different gelation speeds. The hydrogels that have fast gelation kinetics are characterised by $G' = 1,000$ Pa whilst the hydrogels that gel slowly present $G' = 10,000$ Pa. According to fractal hydrogel theory, diffusion limited systems, hence gels with fast gelation kinetics, typically generate structures with lower fractal dimension, $Df$ [221]. Fractal dimension is also tightly correlated with $G'$ insofar as $G'$ is predicted to increase exponentially with $Df$ [230, 231]. This would suggest that the fast-forming crosslinks between ST and SC create a structure with lower fractal dimension and, consequently, lower $G'$ ($G' = 1,000$ Pa) when compared to the same intermolecular clusters forming at slower rates ($G' = 10,000$ Pa). Further molecular dynamic characterisation of this phenomenon was beyond the scope of this project, but it would be interesting to determine the fractal dimension of the ST - SC hydrogels and investigate if and how $Df$ correlates to the changes in gelation kinetics and viscoelastic behaviour [232].
Of note, the diffusion coefficient of the ST-SC hydrogels could be measured by dynamic light scattering (DLS), where light is scattered as a function of the size and shape of the macromolecules and used to characterise the diffusion behaviour of particles in solution [233]. Briefly, the Brownian motion of macromolecules in solution causes the intensity of scattered light to fluctuate and the intensity fluctuations can be related back to the diffusion coefficient of the solution, as large particles diffuse slowly, resulting in similar positions at different time points, compared to small particles that move faster and do not stay in a specific position [233].

The folded nature of the protein hydrogels was also investigated, and it was shown how the bulk mechanical properties of the hydrogel were dependant on the folded structure of ST-SasG-ST and SC. Before and after urea denaturation the hydrogels exhibited elastically dominated behaviour, $G' > G''$, but the presence of urea led to an increased level of viscosity in the hydrogel, demonstrated by the significant increase in $G''$, likely due to the increased presence of unfolded proteins behaving as wormlike chains losing energy upon deformation due to chain rearrangements [149]. Hence, the increase in $G''$ shown here can be directly correlated with the increase in the amount of unfolded protein in the hydrogel, as expected due to the very high concentration of the denaturant urea. Surprisingly, the unfolded hydrogel also showed a smaller increase in $G'$ compared to the folded network. Increases in the storage modulus of unfolded hydrogels have been reported before and they have been linked to the presence of additional chemical [148] or physical [149] crosslinks in the network forming over time. I hypothesised that, in this case, the increase in $G'$ could be due to the formation of additional physical crosslinks in the network rather than chemical because the ST – SC complex needs to be folded for the covalent bond to be created and the high molarity of urea used (8 M) was expected to denaturate all proteins. Moreover, the $G'$ visibly reduced towards low frequencies, potentially indicating the presence of physical entanglements forming and relaxing over long timescales. However, ascertaining the specific direct contribution of either physical or chemical entanglements on the modulus of a hydrogel is extremely complicated [234] and beyond the scope of this project. Of note, the denaturation of the hydrogels carried out during this project occurred post gelation and it would be interesting to see if denaturation of the protein building blocks during
gelation changed the hydrogel architecture and how modulating the concentration of urea could create partially unfolded states in the SC arrays. Of course, due to the unregulated reactivity of ST-SC, it would be quite complicated to determine when exactly gelation starts and finishes, but the microrheological characterisation detailed in Chapter 3 could offer a blueprint for this future work.

In conclusion, the rheological results reported here are instructive and contribute not only to the understanding of the ST-SC system, but also provide a clear roadmap of rheological behaviour for precise tissue engineering applications. Based on my findings, the molar ratio and size of the proteins required to generate a hydrogel with the necessary physical properties may be calculated. For example, a mixture with excess ST relative to SC will exhibit strong resistance to deformation due to the fast creation of new ST-SC connections, but the opposite combination (ST « SC) is unlikely to form a self-sustaining network. Furthermore, increasing the length of the SC array reduces the amount of protein required to generate a hydrogel in terms of both ST:SC unit ratio and protein concentration. As a result, by genetically engineering a longer version of the SC array, one could be able to push the formation of a hydrogel rather than a viscoelastic liquid at even lower protein concentrations. This pattern can already be seen in the transitional behaviours between SC3, SC4, and SC5, where the ST-SasG-ST:SC5 0.8:1 ratio already allowed the creation of a self-sustaining network (G’ = 10,000 Pa and critical yield stress at 10%).

Finally, the G’ of the hydrogels are comparable to that of medium viscoelastic organs such as the liver and kidneys, highlighting their viability for tissue engineering applications [8]. More specifically, the hydrogels characterised by G’ = 1,000 Pa are in good accordance with the G’ of decellularised liver tissue, reported at 1,180 Pa, and the hydrogels characterised by G’ = 10,000 Pa present similar stiffness to that of liver tissue, reported at 10,500 Pa, highlighting their suitability for liver tissue regeneration [223].
4.4 Conclusions

In this chapter, I moved forward the understanding of protein hydrogel rheology, increasing the capabilities of synthetic biology to target tissues with different viscoelasticity and allowing synthetic biology to interface with tissue engineering. I identified a trend encompassing the rules underlying the ST – SC rheological behaviour and I critically hypothesised why this behaviour might be present by identifying parallelisms between the structures of the ST crosslinkers and telechelic polymer chains. I presented three potential explanations for the behaviour exhibited by the ST - SC hydrogels: the presence of inactive loops or active bridges, the inverse proportionality between $G_N^0$ and the molecular weight between entanglements $M_e$, and the differences in reaction speeds. Taken together, these theories are overlapping and all point to differences in the formation of the hydrogel’s microstructure due to differences in crosslinking between ST and SC. The crosslinking between ST and SC lead to the formation of either a fully formed and homogenous network at equimolar combinations or weakened gels due to the potential presence of dangling ends within the system.

Although my results are indeed informative, further characterisation of the protein hydrogels is needed to understand the mechanisms underlying ST – SC hydrogel rheology. However, this characterisation is complicated by the nature of the ST - SC system. For example, due to unregulated spontaneous and immediate gelation, it is impossible to analyse pregel suspensions via SAXS as successfully done previously [232]. Nevertheless, SAXS or SANS could be used on fully formed hydrogels to determined their microstructure and investigate the differences in ST-SC crosslinking. Moreover, modelling of cluster aggregation kinetics and molecular dynamics tools could be used to shed light onto the aggregation kinetics of the ST - SC system and reveal new information about the precise mechanisms in which the system gels and how these affect the resulting viscoelastic properties. The rational understanding of the mechanics behind the formation of ST - SC hydrogels would be an advantageous predictive tool to move towards the identification of proteins with the desired physical properties, such as mechanical stability and elasticity, able to expand the scope of the SpyTag-SpyCatcher system beyond this study and increase the sophistication of protein-based biomaterials.
Chapter 5

Biocompatibility: ST-SC protein hydrogels as 2D and 3D encapsulation matrices

The SC3 RGD array was cloned, expressed, and purified by Regan lab PhD student Fokhrul Islam. The HEK293 cell experiment was carried out in collaboration with Dr Dirk-Jan Kleinjan, Rosser lab. All other experiments and data analysis were carried out solely by myself.

5.1 Introduction

Bridging synthetic biology and tissue engineering via novel biomaterials able to regenerate tissues that have lost functionalities due to injury or disease, is an exciting new field that remains very challenging [235]. After determining the physical properties of any new material, the next step in the process to investigate its suitability for tissue engineering is to demonstrate its biocompatibility.

Biocompatibility goes hand in hand with tissue engineering and it is broadly defined as the ability of a material to coexist and interact with human tissue or physiological systems without inflicting damage [1]. Given that the ultimate goal for a new biomaterial is to restore loss of function due to injury or disease, it is important to establish the relationship between the material itself and the physiological
environment that will surround it. The concept of biocompatibility has evolved alongside the field of tissue engineering from a simple ‘do no harm’ paradigm to a more active role [236]. Nowadays, it is no longer sufficient for a biomaterial to not induce an immunogenic reaction in the host, but it should also actively promote cell growth, adhesion, and proliferation. Moreover, the dynamic setting between the cells and their microenvironment and the physical forces involved in it are fundamental players in a large number of physiological processes, including organ formation and tissue repair, and a successful biomaterial should be able to mimic their pathways [236, 237].

Mechanotransduction, the conversion of physical and mechanical forces from the microenvironment into biochemical information, influences cellular behaviours, such as proliferation, spreading, migration, and differentiation [237]. The architectural control of mechanotransduction is unique and specific to each organ. For example, fluid shear stress is particularly important for cardiac myocytes, whilst mechanical loads are critical for bone cells [237]. Naturally, the complex physiology of the human body makes these environments even more dynamic and further complications can arise due to local pathologies.

Mechanotransduction in vivo is regulated by the extracellular matrix (ECM), a 3D network of proteins, primarily collagen, that contribute to cell signalling and cell behaviour [238]. Another important component is integrins, heterodimeric transmembrane receptors that play an essential role in cell adhesion and motility [238]. Given the complexity of physiological environments, biomaterials aimed at tissue regeneration have been developed to present defined cues mimicking the natural ECM of the target organ, including mechanical stiffness and cell adhesion [7, 239].

Furthermore, seminal work by Bissel and co-workers demonstrated disparities in cell function between 2D and 3D environments, by showing that breast epithelial cells developed tumour like features when cultured in 2D but they reverted back to normal growth when cultured in 3D systems mimicking their natural environment [240]. Subsequent findings have identified further issues with 2D cell culture, where cells are constrained in a planar environment that limits the complex
morphologies observed \textit{in vivo}. For example, in 2D cell culture only a segment of the cell membrane can interact with the ECM of the neighbouring cells, leading to unnatural mechanotransduction that affects intracellular signalling and cell phenotype \cite{241,242}. 2D cell culture also exposes cells to a homogeneous concentration of nutrients and cell growth factors via the bulk cell culture media and lacks the dynamic spatial gradients that influence cell-cell communication \textit{in vivo} \cite{243}. Taken together these findings suggest that investigating cell behaviour in 2D is not sufficient to thoroughly understand cell physiology, mechanotransduction, and tissue morphogenesis: therefore cells should be cultured in 3D culture platforms that closely mimic the native ECM.

Hydrogels are uniquely suitable to 3D cell culture due to their high water content and their ability to simulate the nature of most soft to medium viscoelastic tissues \cite{239,244,245}. Disentangling the chemical, physiological, and physical mechanisms triggered by the contact of the biomaterial with the cells and understanding the consequences of these interactions are key to determine the biocompatibility of the hydrogels and their suitability for 3D cell culture and, ultimately, for tissue regeneration.

Having established that the viscoelastic properties of the ST-SC protein hydrogels designed in this project were suitable for tissue engineering applications, I set out to investigate their biocompatibility \textit{in vitro}. This was not only complicated by the traditional challenges of tissue engineering, such as potential immunogenicity and cytotoxicity of the materials, but also by the bacterial origin of the protein used for the hydrogels, that were produced in \textit{E. coli}.

The ST-SC hydrogels were designed with a view of targeting organs with medium viscoelasticity and, more specifically, the focus was on creating a substrate for liver regeneration. Therefore, following initial studies in the widely used HEK293 cell line, the biocompatibility studies detailed in this chapter were carried out in a modified hepatocyte cell line: the HepG2 derived cell line HepG2-CYC1-GFP, in which a GFP reporter gene was knocked-in into the cytochrome c1 (CYC1) gene to allow visualisation of the cells by fluorescence \cite{246}. CYC1 encodes for a heme protein in the inner mitochondrial membrane of mammalian cells. The
HepG2-CYC1-GFP reporter cell line was initially developed to monitor the stress response to known stress-inducing compounds of the mitochondria in hepatotoxicity studies [246]. This thesis was not focussed on the mitochondria of cells, but the GFP reporter gene was useful to improve cell visualisation and to assess cell health, as no fluorescence would have implied a stressor on the mitochondria. The cell line was donated by Prof. Bob van der Water of Leiden University via the intercession of Prof. David Hay. From here on this cell line will be referred to as HepG2-GFP.

5.2 Results

In this chapter I investigated the biocompatibility of the protein hydrogels created via the SpyTag-SpyCatcher system using the HEK293 and the HepG2 – GFP cell lines. Combinations of ST-SasG-ST with SC3 or SC4 were investigated to ascertain their suitability to tissue engineering. Preliminary experiments aimed at verifying that the ST-SC hydrogels were stable in aqueous and cell culture medium and that they had good swelling properties were detailed in Chapter 2.

In this chapter I will show:

- Protein hydrogels sustain HEK 293 and HepG2 - GFP cell cultures
- Protein hydrogels can encapsulate HepG2 - GFP in 3D
- HepG2 – GFP attach preferentially to protein hydrogels
- Addition of the integrin binding site RGD to the SC3 array increases protein hydrogel biocompatibility
- Cell viability depends on the viscoelastic properties of the protein hydrogels
5.2.1 Protein hydrogels sustain HEK293 and HepG2 – GFP cell cultures

Endotoxins, lipopolysaccharides associated with the outer membrane of gram-negative bacteria, are an unwanted by-product of the purification of recombinant proteins from *E. coli* [247]. Endotoxins are inherently toxic to mammalian cells as they affect cell biochemical signalling, influencing *in vitro* experiments [247]. In addition, the presence of endotoxins could cause immunogenic reactions and inflammation *in vivo*. Therefore, it was crucial to ascertain if endotoxins were present in the purified ST-SC hydrogels to verify their biocompatibility. First, I set out to investigate the biocompatibility of the ST-SC protein hydrogels by simply exploring their suitability as a 2D substrate for cell culture.

Initially, the 2D biocompatibility of the ST-SC protein hydrogels was investigated using the cell line HEK 293, human embryonic kidney 293 cells, because the cell line was readily available, straightforward to grow in tissue culture, and generally used as standard by the Rosser Lab [219]. Briefly, ST-SasG-ST at 4 mM was mixed together with SC3 or SC4 at 1mM in the wells of a 96 well plate at room temperature to initiate spontaneous gelation. Once gelation was complete, HEK293 were seeded on top. The cells were incubated with the hydrogels for 3 days at 37 °C and imaged on the third day. After 3 days, the morphology of the HEK293 cells incubated with the hydrogels was similar to the morphology of the cells incubated only with media, indicating that the hydrogels were not cytotoxic and they did not induce apoptosis in the cells, Fig. 5.1. To confirm this observation, the cells were stained using Trypan Blue 0.4%. Trypan Blue is a stain widely used to quantify live mammalian cells by labelling exclusively dead cells [248]. Trypan Blue can penetrate the membrane of dead cells and enter the cytoplasm causing dead cells to become darker whilst, because live cells have intact membranes, Trypan Blue is unable to enter, leaving the cells clear. The presence of a very large proportion of unstained cells in the wells containing the hydrogels indicated good biocompatibility of the ST-SC protein hydrogels, Fig. 5.1.
Figure 5.1: **ST-SC protein hydrogels supports HEK293 cell culture** HEK293 after 3 days of incubation with the ST-SC protein hydrogels. First column: ST-SasG-ST at 4 mM mixed with SC3 at 1mM, second column: ST-SasG-ST at 4 mM mixed with SC4 at 1mM, third column: control, cells incubated with media only. Top row: unstained cells. Bottom row: cells stained with Trypan Blue 0.4%. The morphology of the HEK293 incubated with the hydrogels was similar to the morphology of the control cells, suggesting good levels of cell viability and biocompatibility of the hydrogels. Staining with Trypan Blue confirmed good cell viability by showing predominantly clear cells. Darker cells are highlighted by red arrows. Magnification 10x, scale bar = 100 µm. Created with Biorender.com
The experiment was repeated using the cell line of interest for liver regeneration, HepG2 - GFP. ST-SasG-ST at 4 mM was mixed together with SC3 or SC4 at 1 mM to initiate spontaneous gelation. Once gelation was complete, HepG2 – GFP cells were seeded on top. The cells were incubated with the hydrogels for 3 days at 37 °C and imaged on the third day. After 3 days, the morphology of the HepG2 – GFP incubated with the hydrogels was compared to the morphology of the cells incubated only with media. Morphological similarities and a similar level of GFP expression suggested good levels of cell viability, Fig. 5.2. Of note, at 488 nm excitation, the hydrogels exhibited some unexpected fluorescence background.

Figure 5.2: **HepG2 – GFP after 3 days of incubation with the ST-SC protein hydrogels.** First column: ST-SasG-ST at 4 mM mixed with SC3 at 1 mM, second column: ST-SasG-ST at 4 mM mixed with SC4 at 1 mM, third column: control, cells incubated with media only. The morphology of the HepG2 – GFP incubated with the hydrogels was similar to the morphology of the control cells and the cells showed a similar level of GFP expression, suggesting good levels of cell viability and biocompatibility of the hydrogels. Magnification 40x, scale bar = 88.1 µm. Created with Biorender.com
I had modified the batch protein purification protocol used throughout the project by adding an extra purification step using cold Triton X-114. Cold Triton has been shown to remove endotoxins and lipopolysaccharides from \textit{E. coli} purified proteins \cite{247}. The improved protocol was tested on the SC arrays and the purification was successful. However, given the good results obtained by the initial biocompatibility experiments, the extra cold Triton step was not needed and the proteins used in the biocompatibility experiments were purified using the batch method described in Materials and Methods.

\subsection*{5.2.2 Protein hydrogels encapsulate HepG2 – GFP in 3D}

Having established that the ST-SC proteins hydrogels are biocompatible in 2D, I set out to investigate their ability to encapsulate the HepG2 - GFP in 3D. Encapsulation of the cells was achieved by mixing ST-SasG-ST at 4 mM and SC3 at 1mM or SC4 at 1 mM together with the cells as done previously \cite{186}. The components were mixed together manually to initiate gelation. I anticipated that the cells would prosper in the hydrogel environment due to its biological origin and suitable viscoelastic properties. After 24 hours incubation in medium in the tissue culture incubator, the cells were visualised by scanning confocal microscopy.

Figure 5.3 shows z-stacks through the hydrogels. I observed cells throughout the hydrogels, confirming good encapsulation. Cells presented the characteristic clusters or islands and expressed GFP, good indicators of cell viability. Other indicators of cell viability could be the quantification of albumin or $\alpha$-fetoprotein via ELISA assays, as done in our publication \cite{192}, as well as glucose secretion and lactate dehydrogenase activity via enzymatic assays. The straightforward encapsulation conditions contributed to make the ST-SC protein hydrogels a compelling choice as a platform for tissue engineering and regenerative medicine.
Figure 5.3: Scanning confocal imaging of the HepG2 - GFP cells encapsulated in the ST-SC protein hydrogels after 24 hours. Top row: ST-SasG-ST at 4 mM combined with SC3 at 1 mM. Bottom row: ST-SasG-ST at 4 mM combined with SC4 at 1 mM. Z-stacks show cells throughout the hydrogels. Cells presented the characteristic clusters or islands and expressed GFP, good indicators of cell viability. Magnification 10x, scale bar = 110.2 µm. Created with Biorender.com
Given the good results obtained after 24 hours, I repeated the experiment encapsulating the HepG2 – GFP in the hydrogels for 3 days (72 hours). The HepG2 – GFP were encapsulated as described before, and imaged every day. Figures 5.4 and 5.5 show z-stacks at 24, 48, and 72 hours for ST-SasG-ST + SC3 and ST-SasG-ST + SC4 respectively. As observed after 24 hours, after 3 days cells continued to present the characteristic clusters or islands and expressed GFP, good indicators of cell viability.

Figure 5.4: Scanning confocal imaging of the HepG2 - GFP encapsulated in the ST-SC protein hydrogels after 24, 48, and 72 hours. ST-SasG-ST at 4 mM combined with SC3 at 1 mM. Z-stacks show cells throughout the hydrogels. Cells presented the characteristic clusters or islands and expressed GFP, good indicators of cell viability. Magnification 10x, scale bar = 110.2 µm. Created with Biorender.com
Figure 5.5: Scanning confocal imaging of the HepG2 - GFP encapsulated in the ST-SC protein hydrogels after 24, 48, and 72 hours. ST-SasG-ST at 4 mM combined with SC4 at 1 mM. Z-stacks show cells throughout the hydrogels. Cells presented the characteristic clusters or islands and expressed GFP, good indicators of cell viability. Magnification 10x, scale bar = 110.2 µm. Created with Biorender.com
5.2.3 HepG2 – GFP attach preferentially to protein hydrogels

I moved forward with the investigation on the biocompatibility of the ST-SC protein hydrogels by exploring the ability of the HepG2 – GFP to attach to the hydrogels. I manually mixed together the ST and SC components in situ in a 96 well plate and, once gelation was complete and the hydrogels covered the entire bottom of the well, I seeded HepG2 – GFP on top. After the cells were incubated for 24 hours at 37 °C, the wells were washed to remove unattached or floating cells, fresh media was gently added and the cells were incubated for a further 24 hours. The wells were washed again and the remaining cells were then detached via gentle and repetitive pipetting from representative wells and counted. Three samples were taken per well with N wells = 3 per condition to ensure accurate quantification. Because I had washed the wells to ensure the removal of unattached cells, the cell count could be translated to cell attachment. Quantification of the attached cells showed preferential cell attachment of the HepG2 - GFP to the protein hydrogels when compared to tissue culture plastic-only control, Fig. 5.6. In addition, the ST-SasG-ST + SC3 and ST-SasG-ST + SC4 hydrogels allowed the attachment of comparable levels of HepG2 – GFP (p >0.05). Imaging of the cells also confirmed HepG2 - GFP cell attachment and a good level of GFP expression, suggesting good cell viability, Fig. 5.7. Fluorescence intensity analysis carried out on the cell attachment images confirmed the quantification of the cell attachment by showing that both hydrogels allowed the attachment of comparable levels of HepG2 - GFP, Fig. 5.7.
Figure 5.6: Quantification of HepG2 - GFP cell attachment on ST-SC protein hydrogels. Quantification of the attached cells showed preferential cell attachment of the HepG2 - GFP to the protein hydrogels when compared to tissue culture plastic-only control (p < 0.01). ST-SasG-ST + SC3 or ST-SasG-ST + SC4 hydrogels allowed the attachment of comparable levels of HepG2 – GFP (p > 0.05). Data is presented as ± SD with N = 3.
Figure 5.7: Fluorescence imaging of HepG2 – GFP on ST-SC protein hydrogels. Fluorescence imaging showed cell attachment and a good level of GFP expression, suggesting good cell viability. Petri dish: schematics of HepG2 - GFP attached onto spotted hydrogels. First column: ST-SasG-ST at 4 mM mixed with SC3 at 1mM, second column: ST-SasG-ST at 4 mM mixed with SC4 at 1mM. Magnification 4x, scale bar = 550.9 µm. Fluorescence intensity analysis confirmed that ST-SasG-ST + SC3 and ST-SasG-ST + SC4 hydrogels allowed the attachment of comparable levels of HepG2 – GFP (p > 0.05). Data is presented as ± SD with N = 5. Created with Biorender.com
5.2.4 Addition of RGD to the SC3 array increases hydrogel biocompatibility

I further investigated if I could increase the biocompatibility of the ST-SC protein hydrogels by adding cell attachment features in the sequences of the protein building blocks. The ability to genetically encode new features, such as cell attachment sequences and binding or degradation sites, is one of the main advantages of using a recombinant protein hydrogel approach to tissue engineering, given the impossibility of modifying the backbone of polymers in such a way. It was decided to add arginylglycylaspartic acid (RGD), the most common peptide motif responsible for cell attachment to the ECM, at the end of the SpyCatcher sequence. RGD was chosen because of its well documented ability to increase cell attachment in biomaterials for tissue engineering applications [25] and the location at the end of the SC array was deemed the most suitable to ensure good availability of the RGD binding site to the HepG2 – GFP cells. Cloning of the RGD to the SC3 array, expression in E. coli, and protein purification were carried out by Regan lab PhD student Fokhrul Islam. All biocompatibility experiments and imaging were carried out by myself.

Cell attachment and cell encapsulation experiments were carried out as described before. Quantification of the attached cells showed an increase in HepG2 - GFP cell attachment in the ST-SasG-ST + SC3 RGD when compared to the hydrogels without RGD, Fig. 5.8. Imaging of the cells also confirmed increased HepG2 - GFP cell attachment and a good level of GFP expression on the ST-SasG-ST + SC3 RGD hydrogels. Fluorescence intensity analysis carried out on the cell attachment images confirmed the quantification of the cell attachment by showing increased levels of HepG2 – GFP cell attachment on the ST-SasG-ST + SC3 RGD hydrogels, Fig. 5.9. Likely, the increased levels of fluorescence were due to the higher number of cells attached, but it could also be possible that GFP expressed better on the RGD hydrogels compared to their non-RGD counterpart. Of note, I observed that the addition of RGD seemed to increase HepG2 - GFP clustering. In general, HepG2 favour excessive clustering over spreading [249], but it could be possible that the cells are migrating towards the integrin binding sites forming bigger clusters.
Figure 5.8: Quantification of HepG2 - GFP cell attachment on ST-SC protein hydrogels with and without RGD. Quantification of the attached cells showed increased HepG2 – GFP cell attachment on the ST-SasG-ST + SC3 RGD compared to the hydrogels without RGD (p < 0.01). Data is presented as ± SD with N = 3.
Figure 5.9: **Fluorescence imaging of HepG2 – GFP on ST-SC protein hydrogels with and without RGD.** Fluorescence imaging showed increased HepG2 – GFP cell attachment on the ST-SasG-ST + SC3 RGD compared to the hydrogels without RGD and good GFP expression. Petri dish: schematics of HepG2 - GFP attached onto spotted hydrogels. First column: ST-SasG-ST at 4 mM mixed with SC3 at 1mM, second column: ST-SasG-ST at 4 mM mixed with SC4 at 1mM, third column ST-SasG-ST at 4 mM mixed with SC3 RGD at 1 mM. Magnification 4x, scale bar = 550.9 µm. Fluorescence intensity analysis confirmed increased HepG2 – GFP cell attachment on the ST-SasG-ST + SC3 RGD compared to the hydrogels without RGD (p < 0.01). Data is presented as ± SD with N = 5. Created with Biorender.com
Having established that the addition of RGD in the SC sequence increased HepG2 – GFP cell attachment, I set out to investigate if the presence of RGD affected the 3D encapsulation conditions of the cells. Encapsulation of the cells was achieved as described before by manually mixing together the ST and SC components with the HepG2 – GFP. Given the good results obtained so far in the 3D encapsulation experiments, the HepG2 – GFP cells were encapsulated for 4 days (96 hours) and imaged every day. Figures 5.10 and 5.11 show z-stacks at 24, 48, 72, and 96 hours for ST-SasG-ST + SC3 and ST-SasG-ST + SC3 RGD respectively. As observed before, cells presented a healthy epithelial - like morphology and expressed GFP after 4 days. In addition, at 96 hours I noticed cracks in the protein matrix that were only present in the ST-SasG-ST + SC3 RGD hydrogels, Fig. 5.11. This led me to hypothesise that the addition of the RGD to the hydrogels did indeed increase cell attachment and cell proliferation, allowing the cells to manipulate the matrix by pulling and pushing at it. This could indicate that the cells are degrading the hydrogels by expanding and proliferating. Further longer-term experiments are needed to ascertain if this is the case and what precisely is the rate of degradation of the ST-SC protein hydrogels by the HepG2 – GFP, but it is nevertheless a promising result that should encourage the use of the ST-SC protein-based approach in tissue engineering.
Figure 5.10: Scanning confocal imaging of the HepG2 - GFP encapsulated in the ST-SasG-ST + SC3 protein hydrogels after 24, 48, 72, and 96 hours. ST-SasG-ST at 4 mM combined with SC3 at 1 mM. Z-stacks show cells throughout the hydrogels. Cells presented a healthy epithelial-like morphology and expressed GFP. Magnification 10x, scale bar = 110.2 µm. Created with Biorender.com
Figure 5.11: Scanning confocal imaging of the HepG2 - GFP encapsulated in the ST-SasG-ST + SC3 RGD protein hydrogels after 24, 48, 72, and 96 hours. ST-SasG-ST at 4 mM combined with SC3 RGD at 1 mM. Z-stacks show cells throughout the hydrogels. Cells presented a healthy epithelial-like morphology and expressed GFP. At 96 hours, cracks in the protein hydrogels are visible. The cracks are highlighted by red arrows. Magnification 10x, scale bar = 110.2 µm. Created with Biorender.com
5.2.5 Cell viability depends on the formulation of the protein hydrogels

So far, I have shown good cell viability of the HepG2 – GFP cells with the hydrogels by morphological analysis and equal levels of GFP expression. Overall, these are good indicators of cell viability, but I have further ascertained the biocompatibility of the hydrogels by quantifying the levels of HepG2 – GFP cell viability on the ST-SC protein hydrogels via an MTT assay. The MTT assay is a widely used colorimetric assay used to determine cell metabolic activity via the reduction of MTT, a yellow tetrazole, to purple formazan in living cells [250, 251]. The amount of purple formazan produced by live cells, measured via absorbance at 570 nm, is directly proportional to the number of living cells in the culture and it can be readily translated into a percentage of cell viability [251].

So far, I have used hydrogels that gelled quickly and had G’ = 1,000 Pa, according to the microrheology and rheology characterisation carried out in Chapters 3 and 4 respectively. This was a requirement imposed by the encapsulation experiments that required a hydrogel to gel quickly in order to encapsulate the cells. Attempts to use slower gelling combinations led to the HepG2 - GFP cells sinking to the bottom of the well before the hydrogels formed and had time to encapsulate them. However, given that the MTT assay does not require cell encapsulation, I was able to quantify the viability of the HepG2 - GFP cells on ST-SasG-ST hydrogels characterised by G’ = 10,000 Pa, alongside the hydrogels with G’ = 1,000 Pa used so far. The stiff hydrogels (G’ = 10,000 Pa) were composed of ST-SasG-ST at 2 mM + SCx at 1 mM, whilst the soft hydrogels (G’ = 1,000 Pa) were composed of ST-SasG-ST 4 mM + SCx at 1 mM. I expected the quantification of cell viability to lead to the same results, given the common origin of the protein hydrogels, but the cell viability was remarkably different. Figure 5.12 shows the raw absorbance levels at 570 nm (A) and the translation to percentage of cell viability (B). Herein, I will focus on the percentage of cell viability.
ST-SasG-ST at 4 mM combined with SC3 at 1mM or SC4 at 1mM and having G’ = 1,000 Pa showed good cell viability, comparable to that of control (p > 0.05) HepG2 – GFP incubated with only cell culture medium on plastic, with 91.25 ± 7.90 % cell viability for ST-SasG-ST + SC3 and 88.28 ± 5.19 % cell viability for ST-SasG-ST + SC4. Conversely, ST-SasG-ST at 2 mM combined with the same SC3 at 1 mM or SC4 at 1mM and having G’ = 10,000 Pa showed reduced cell viability with 62.79 ± 8.14% viable cells for ST-SasG-ST + SC3 (p < 0.05) and 59.91 ± 5.09 % viable cells for ST-SasG-ST + SC4 (p < 0.01). This suggested that the hydrogel’s viscoelastic properties have an effect on cell viability and that the stiffer hydrogels are less suitable to supporting the viability of liver cells.

Even more remarkably, the addition of RGD to the SC array seemed to increase the cell viability in the G’ = 10,000 Pa hydrogels. The ST-SasG-ST + SC3 RGD hydrogels showed comparable levels (p > 0.05) of cell viability regardless of G’ = 10,000 Pa (80.22 ± 3.72% viable cells) or G’ = 1,000 Pa (90.67 ± 9.07% viable cells). In addition, statistical analysis of the differences between all the stiffer formulations (G’ = 10,000 Pa) showed increased levels of cell viability on ST-SasG-ST + SC3 RGD when compared to the hydrogels without RGD, p < 0.05 for ST-SasG-ST + SC3 and p < 0.01 for ST-SasG-ST + SC4. This finding suggested that the addition of RGD does indeed increase the biocompatibility of the ST-SC protein hydrogels.
Figure 5.12: **MTT assay quantifying cell viability on the ST-SC protein hydrogels.** A) Raw absorbance levels at 570 nm. B) Translation of the absorbance levels to percentage of cell viability. Black: ST-SasG-ST + SC3, pink: ST-SasG-ST + SC4, green: control HepG2-GFP cells on plastic, purple: ST-SasG-ST + SC3 RGD. Block colour: G’ = 1,000 Pa, square pattern: G’ = 10,000 Pa. ST-SasG-ST at 4 mM combined with SC3 at 1mM or SC4 at 1mM and having G’ = 1,000 Pa showed good cell viability (p > 0.05) comparable to control. ST-SasG-ST at 2 mM combined with SC3 at 1 mM or SC4 at 1mM and having G’ = 10,000 Pa showed reduced cell viability, p < 0.05 for ST-SasG-ST + SC3 and p < 0.01 for ST-SasG-ST + SC4. The addition of RGD to the SC array increased cell viability in the G’ = 10,000 Pa hydrogels. The ST-SasG-ST + SC3 RGD hydrogels showed comparable levels (p > 0.05) of cell viability regardless of G’. Analysis of the stiffer formulations (G’ = 10,000 Pa) showed increased levels of cell viability in ST-SasG-ST + SC3 RGD when compared to the ST-SasG-ST + SC3 and ST-SasG-ST + SC4 hydrogels without RGD (p < 0.05 for SC3 and p < 0.01 for SC4). Data is presented as ± SD with N = 3.
5.3 Discussion

In this chapter I have investigated the biocompatibility of the ST-SC protein hydrogels for liver regeneration. I have shown that the ST-SC protein hydrogels can be used as 2D structural support for liver cells, can encapsulate the cells for up to 4 days without loss of cell viability, can drive increased cellular attachment, and that the addition of the integrin binding site RGD to the SC array increases the biocompatibility of the hydrogels. I also demonstrated good cell viability on the hydrogels via a colorimetric MTT assay. Overall, these results demonstrate the good biocompatibility of the ST-SC protein hydrogels. In addition, whilst I was not able to precisely quantify the pore size and the overall porosity of the protein hydrogels, the successful 3D encapsulation suggested that the hydrogel porosity is enough to allow gaseous exchanges and exchanges of nutrients with the surrounding medium preventing necrosis of cells.

The main challenge of using *E. coli* derived protein-based hydrogels is the potential presence of endotoxins or lipopolysaccharides in the protein preps. These components would make the hydrogels cytotoxic and unsuitable for tissue engineering. The first two experiments detailed in this chapter demonstrated that the protein purification protocol carried out to obtain the protein stocks was enough to get rid of potential *E. coli* induced toxicity. In addition, the good cell viability showed by the 3D encapsulation experiments suggested that the endotoxins were not simply trapped inside the hydrogels, but that they were not present at all, or that they were present to undetectable levels, in the protein preps. This is in line with previous findings that showed no cytotoxicity on *E. coli* derived ST-SC protein hydrogels [186, 187].

The next step in assessing the biocompatibility of the ST-SC protein hydrogels should be to test their potential immunogenicity. Immunogenicity studies were not carried out because they can only be done in animal models in vivo, which was beyond the scope of this project. However, the SpyTag - SpyCatcher system has been used successfully to produce virus-like particles and nanoparticles to investigate the cross-reactive immune response to mice and bovine viruses [176,177,252]. These findings suggest that the SpyTag – SpyCatcher system is not immunogenic.
in itself and they pave the way for successful use of the ST-SC hydrogels in tissue engineering. In addition, recent studies have also shown that it is possible to map the amino acids responsible for an immunogenic reaction and mutate them to less immunogenic residues [253–255]. Therefore, if future immunogenic studies carried out on the ST-SC protein hydrogels would reveal an immunogenic response, it should be possible to map the amino acids responsible and genetically mutate the sequences of ST and SC to avoid this issue.

An unexpected challenge in this project was the presence of high background fluorescence caused by the protein hydrogels at 488 nm excitation (GFP excitation). I first noticed it when imaging the hydrogels and the HepG2 – GFP cells in 2D and realised that the morphology of the cells was partially concealed by the hydrogels. This was unfortunate as the primary reason for using the hepatocyte cell line HepG2 – GFP was to ease cell visualisation and quantification of cell attachment via GFP fluorescence. Nevertheless, I first tried to ascertain cell attachment onto the hydrogels by quantifying the levels of GFP fluorescence in the wells after 48 hours incubation. I repeated the experiment several times, tweaking the number of cells seeded, the number of washes, the GFP gain settings, and the volume of hydrogel used. Unfortunately, regardless of the changes to the protocol, the results remained inconclusive due to the high background fluorescence caused by the hydrogels. Therefore, in consultation with the Regan, Hay, and Rosser labs, we devised the new protocol based on cell counting using an automated hemacytometer reported here. The background fluorescence of the hydrogels was drastically reduced at 570 nm, the absorbance level of purple formazan used to quantify cell viability onto the ST-SC protein hydrogels via the MTT assay, thus I was able to subtract the small hydrogel-induced background fluorescence and accurately quantify cell viability.

One of the main results presented in this chapter was the successful addition of the integrin binding site RGD to the SC array that led to increased cell attachment and biocompatibility of the ST-SC protein hydrogels. RGD was added seamlessly to the SC3 array sequence by PhD student Fokhrul Islam, demonstrating the advantage of using a genetically encoded protein approach to tissue engineering. In fact, given the well documented benefits of adding RGD to biomaterials [25], several in-
ert polymer-based biomaterials have added this integrin binding site. For example, RGD coating on a titanium implant increased the cellular osteogenic behaviours of mesenchymal stem cells [256] and RGD-modulated surface functionalisation increased cell proliferation and cell adhesion on polylactic acid scaffolds [257]. However, these hybrid networks are limited by the structural identities of the synthetic materials that cannot be genetically modified and are usually constrained to coating, conjugation or immobilisation of RGD peptides on the surface of the scaffolds [258–260]. By contrast, the use of the ST - SC system allows to not only genetically encode one integrin binding site, as done in this project, but also other features enabling protein immobilisation in the hydrogels [182] or the formation of complex topologies [180], functionalising and customising the hydrogels to specific tissue engineering applications.

Moreover, the addition of RGD to the SC3 array allowed the HepG2 – GFP cells to start degrading the hydrogels. I anticipated that the HepG2 – GFP would be able to degrade, at least to an extent, the protein-based hydrogels because cells secrete matrix metalloproteinases (MMPs), zinc dependant proteolytic enzymes that degrade various proteins in the ECM [261]. However, as shown by the presence of cracks only in the RGD containing protein matrix, the addition of RGD increased cell attachment, which in turn, sped up the degradation process. It is likely that the HepG2 – GFP would be able to degrade the protein hydrogels even without the RGD binding site present, but the process would take longer than four days. Furthermore, because of the genetically encodable nature of the ST-SC system, MMP cleavage sites could be added to the sequence of the protein building blocks, as done for the RGD binding site, encouraging cell migration and allowing the cells to degrade the hydrogels in a controllable manner [262]. By precisely quantifying and modulating the number and the position of RGD binding sites and MMP cleavage sites onto the hydrogels, one could design bespoke ST-SC protein hydrogels that would allow increased cell spreading and cell migration into the hydrogels, whilst inducing degradation matching the rate of regeneration of the target organ [263, 264]. For example, HepG2 – GFps express high levels of MMP3 protease [265], therefore genetically adding the sequence encoding for a MMP3 cleavage site into the protein building blocks would allow the cells to degrade the hydrogels in a controllable manner, matching the speed of regeneration of liver.
The results from the quantification of cell viability via the MTT assay were quite unexpected and remarkable. The results showed in the previous experiments anticipated good HepG2 - GFP cell viability on the ST – SC protein hydrogels. Indeed, this was the case as the soft hydrogels exhibited ≈ 90% cell viability. However, there was a clear difference between the formulations where the stiffer hydrogels (G’ = 10,000 Pa) showed a significant reduction in cell viability (≈60%) compared to their softer counterparts (G’ = 1,000 Pa). Given that the only difference between the hydrogel formulations was the concentration of the ST component (either ST-SasG-ST at 2 mM for the stiffer hydrogels or ST-SasG-ST at 4 mM for the softer ones), I concluded that the observed decrease in HepG2 - GFP cell viability was due to the viscoelastic properties of the hydrogels.

The reported elastic modulus of decellularised liver tissue is 1,180 Pa [223], therefore it is possible that the mechanotransduction signalling of the stiffer hydrogels (G’ = 10,000 Pa) is incompatible with HepG2 - GFP, whilst the biosignalling of the softer hydrogels (G’ = 1,000 Pa) more closely mimics the viscoelasticity of liver. Hence, the softer hydrogels present a more suitable environment to the HepG2 – GFP that more closely resembles the characteristics of native liver, allowing the cells to proliferate. Interestingly, the reported stiffness of liver is between 9,000 - 10,500 Pa [8, 223], which suggest that the stiffer matrix should be more suitable to liver regeneration. However, it has been shown that cells confer a certain degree of rigidity to the organs [223]. Thus, when designing a new biomaterial, one should aim for a hydrogel that matches the stiffness of the decellularised target organ as the increased stiffness will be induced by the seeded cells. The differences between the two mechanical environments compared to that of native liver could explain the differences in cell viability of the HepG2 – GFP reported in this chapter. Even more interestingly, the addition of RGD increased HepG2 - GFP cell viability in the stiff protein hydrogels to comparable levels to those of the softer ones. Perhaps, if the viscoelastic properties of the hydrogels are not too unsuitable, the hypothesised suboptimal mechanotransduction caused by the stiffer hydrogels could be counteracted by the increased cell binding induced by RGD.
It is important to acknowledge the limitations of the results presented in this chapter. First, throughout this chapter, I made use of an immortalised cell line, HepG2 - GFP. Immortalised cell lines have several advantages, including their genetic identity, stability, and ease of maintenance, however, they are normally sturdier and less fragile compared to cells derived from primary cultures, therefore it is possible that the ST-SC hydrogel could be cytotoxic, at least partially, to a more delicate cell line such as a primary human hepatocyte culture. Moreover, the lack of long term cultures, beyond the one week reported here, limits the potential cytotoxic effects that could be observed, as it is possible that long term incubation of the hydrogels with the cells, i.e. one month, could unveil previously unseen toxic effects. Finally, the addition of RGD to the hydrogels could have affected their rheological properties, that, in turn, could have influenced the cell viability results. Whilst initial rheological tests were carried out on a few samples to ascertain that the physical properties of the RGD hydrogels were similar to the non-RGD hydrogels, a full rheological panel was not carried out, therefore I cannot be certain that the rheological properties were completely unaffected.

To strengthen the results presented here, further biocompatibility experiments should focus on the use of primary cell cultures from various tissues, including but not limited to liver, rather than immortalised cell lines combined with long term culture studies (> 30 days) to determine the potential toxic effects of the ST-SC protein hydrogels. Moreover, biodegradation studies focussing on the investigation of hydrogel debris after cellular degradation could also be carried out to determine the presence of potential side effects or immune reactions. In addition, specific assays aimed at determining cell viability beyond the MTT assay used in this project could be used to analyse cell health, such as apoptosis or oxidative stress assays.

An interesting experiment to investigate the differences in the mechanotransduction of both stiff and soft hydrogels would be to test the differentiation of pluripotent stem cells (PSCs). The differentiation of PSCs into hepatocytes is modulated by numerous factors, including growth factors and proteins such as Oncostatin M, but one of the most important ones is the microenvironment surrounding them [266, 267]. Therefore, it would be interesting to investigate if there are any
differences in the differentiation profile of the PSCs seeded on stiff or soft ST-SC hydrogels. Based on the results detailed in this chapter, one would anticipate that the PSCs will differentiate into functional hepatocytes better on the soft matrices rather than the stiff ones, but other factors could modulate and affect the differentiation.

5.4 Conclusions

In this chapter, using the hepatocyte cell line HepG2 – GFP, I have demonstrated that the ST-SC protein hydrogels designed in this project are biocompatible with mammalian cells, despite their bacterial origin. Specifically, I have shown that the ST-SC protein hydrogels can sustain cell culture in 2D, can encapsulate mammalian cells without loss of cell viability for up to 4 days, and can drive increased cell attachment. I also showed that the addition of RGD to the SC3 array not only increased cell attachment and proliferation, but also potentially induced faster biodegradation of the hydrogels by the HepG2 – GFP cells. Quantification of cell viability via an MTT assay confirmed good cell viability but showed a remarkable difference in between stiff and soft hydrogel formulations, with the former significantly decreasing cell viability and the latter showing cell viability comparable to untreated cells.

The experiments carried out in this chapter demonstrated the overall suitability of the ST-SC protein hydrogels for tissue engineering, and specifically for liver regeneration. Further longer-term biocompatibility and biodegradation studies are needed to confirm if and how the HepG2 - GFP are degrading the protein hydrogels and for how long 3D cell culture can be successfully maintained. Genetically modifying the protein hydrogels by adding more RGD binding sites or MMP cleavage sites in the sequences of the protein building blocks could be an interesting avenue to develop bespoke and tuneable hydrogels that are not only biocompatible, but also biodegradable at a speed matching that of the regeneration of the target organ.
Chapter 6

3D printing: adapting the ST-SC hydrogel design to biofabrication

6.1 Introduction

Bridging synthetic biology and tissue engineering remains challenging. One of the main advantages of traditional tissue engineering is the ability to fabricate stable structures by casting, electrospinning, melt-electrowriting, or 3D printing suitable scaffolds and seed them with living cells [235]. The idea is that the cells will migrate and populate the scaffolds and form functional tissue, with the ultimate goal of surgically implanting it into the patient in need. The customisable scaffold is built specifically to suit a particular defect, cell line, and material via appropriate biofabrication techniques. This approach has been quite effective in traditional tissue engineering, especially due to the ease of processing of polymer materials via a variety of different techniques. For example, electrospun PVA/PEG/silk nanofibers were engineered for neural tissue regeneration after ischemic stroke [70], meltelectrowritten PCL scaffolds were successfully tested in vivo for wound healing [268], and ceramic-based casted scaffolds were successfully built as bone replacements [269].

In particular, amongst the biofabrication techniques available, 3D printing holds great promise for tissue engineering as it can provide a low-cost, rapid, and functional approach to assemble tissue in vitro [270]. 3D printing creates a 3D ob-
ject layer-by-layer using a computer aided design (CAD) or computed tomography (CT) scan, that can be based on a patient’s imaging data [271]. The main advantage of 3D printing is the possibility of defining a specific set of microarchitectures in the CAD design that not only provide structural and mechanical support, but also allow gaseous exchanges and nutrient supply to the seeded cells, thus creating a 3D dimensional space well suited to cell culture able to mimic the native environment and adapt to specific defects [270]. 3D printing of polymers and ceramics has been especially efficacious for bone tissue engineering and chondrogenesis as evidenced by the plethora of reviews available on the subject detailing numerous successful applications [272, 273]. 3D printing has also shown great promise for soft and medium tissues, demonstrating the versatility of this technique and its widespread impact in tissue engineering and regenerative medicine [274, 275].

It is evident that the ability of traditional tissue engineering to mould and shape a material into a suitable structure is fundamental to expanding the purview of materials and increasing the capabilities of the material itself to repair tissues. Synthetic biology still lacks this ability as the biofabrication of protein-based materials is complicated by the difficulties in processing proteins by traditional biofabrication techniques due to their, often suboptimal, physical properties. However, automated platforms aimed at bridging synthetic biology and tissue engineering have shown good potential to increase the capabilities of this technology. For example, Hay and co-workers developed a fully automated stem cell-based tissue engineering platform that allowed self-assembly of human liver tissue in vitro [266]. This system produced phenotypically stable liver tissue that was used to model human liver disease more accurately compared to other non-automated systems. Similarly, Kizawa and colleagues demonstrated that bioprinted liver tissue could maintain the active metabolism of drugs, glucose, and lipids for several weeks [276]. Moreover, advances in the use of microfluidics in synthetic biology has shown great promise for drug analysis, investigation of diseases, and the development of organ like architectures [277]. For example, the liver-on-chip developed by Freag and co-workers successfully mimicked fatty liver disease [278]. Therefore, developing methodologies to successfully combine biofabrication techniques with synthetic biology could improve the capabilities of synthetic biology to interface with tissue engineering.
In this chapter, I adapted the design of the protein-based hydrogels developed using the SpyTag-SpyCatcher system to 3D printing. I developed, tested, and optimised the methods to 3D print a self-standing structure using a dual nozzle 3D printer, TissueStart bioprinter, from TissueLabs (https://www.tissuelabs.com/). Combinations of ST-SasG-ST and SC3 were used to test and improve the methods.

In this chapter I will show:

- Methods development for 3D printing using a T junction
- 3D printing of a two-tier structure using a T junction
- Methods development for 3D printing using independent syringes

6.2 Results

First, 3D printing relies on the creation of a CAD based model that is converted into a stereo-lithography file (STL) [271]. Essentially, the STL file breaks down the model into its cross-sectional layers that allow the model to be 3D printed [271]. For the 3D printing trials carried out in this project, I designed a simple 4x4 mm square using SolidWorks (https://www.solidworks.com/) and imported the STL file in the TissueLabs proprietary software, Fig. 6.1. I decided to print a very simple 4x4 mm square as it was a clear structure with 90 degrees angles easily supported by the 3D printer used in this project and it required small amounts of materials. The amount of protein needed to print a self-standing structure was substantial and, because of the recombinant nature of the protein used in this project, material conservation was fundamental. Unless otherwise specified, all 4x4 mm squares were 3D printed using at least \( z = 4 \) layers as preliminary trials determined this to be the minimum amount needed to 3D print a visible and self-standing hydrogel whilst conserving material.
Figure 6.1: Screenshot of the TissueLabs proprietary software used for 3D printing. The screenshot shows the main interface of the software with the CAD design of the small 4x4 mm square and the controls over the parameters of the 3D printer.
Second, 3D printing relies on the deposition of the material via a nozzle to build a structure layer-by-layer [271]. The microrheological characterisation carried out in Chapter 3 was instrumental in developing the methodologies needed to adapt the ST-SC hydrogel design to 3D printing. In fact, the methods developed were based on the balance between needing combinations of ST-SasG-ST and SC3 that would gel quickly enough to be self-standing and sustain the weight of another layer being printed on top the first one, but not too quickly to block the nozzle and hinder extrusion. Given these parameters, ST-SasG-ST at low millimolar concentration, ST-SasG-ST = 2 mM, was excluded *a priori* because of the longer gelling times identified during the microrheological characterisation (≈45 minutes). Combinations of ST-SasG-ST at 3, 3.5, or 4 mM with SC3 at 1mM were tested for 3D printing.

Due to the unregulated and spontaneous gelation of ST and SC, the use of a dual nozzle 3D printer was crucial because it allowed to keep the two components separate, one in each syringe, until extrusion could commence. In all experiments, 3 mL syringes with 8.66 mm diameter were used. Considering the size of the trailing ring connected to the plunger, the minimal viable volume required to 3D print a 4x4 mm square was calculated at ≈ 1.3 mL each of ST and SC. Therefore, I needed at least 1.3 mL of each component at the specific millimolar concentration to begin 3D printing. Figure 6.2 shows a schematic representation of the two syringes used to 3D print in this project.
Figure 6.2: Schematic representation of the two syringes used to adapt the ST-SC hydrogel design to 3D printing. 3 mL syringes with 8.66 mm diameter were used. ST and SC were kept separate in each syringe until extrusion could commence. Created with BioRender.com.
6.2.1 Methods development for 3D printing using T junction

Initially, I trialled adapting the hydrogel design to 3D printing using a T junction, Fig. 6.3. The ST and SC components were kept separate, one in each syringe, and the two syringes were connected together by a T junction allowing simultaneous extrusion of the material via a 21-gauge needle. In the T junction configuration, the pistons acted on the two syringes simultaneously with the same set of parameters, as it was impossible to control the two syringes independently with the 3D printer used. Therefore, the parameters had to be optimised for both proteins, ST-SasG-ST and SC3, at the same time despite differences in their viscosities, namely SC3 was always used at 1 mM, whilst ST-SasG-ST was used at higher mM concentrations, 3, 3.5, and 4 mM.

Figure 6.3: Dual nozzle 3D printer with T junction. A) Schematic representation, B) the 3D printer in real life. The ST and SC components were kept in separate syringes and the two syringes were connected together by a T junction allowing simultaneous extrusion of the material via a 21-gauge needle. Schematic representation created with BioRender.com.
First, I tried 3D printing using ST-SasG-ST at 3 mM and SC3 at 1 mM because the viscosities of the two liquids were the most similar. According to the microrheological characterisation detailed in Chapter 3, this combination achieved full gelation in ≈ 25 minutes. I was hoping that the system would gel enough to allow another layer to be printed on top of the first even if complete gelation was not yet achieved. Unfortunately, when I trialled 3D printing with this combination, the resulting structure was too liquid and weak, therefore even if the mixture could be extruded by the nozzle, it could not be 3D printed, Fig. 6.4.

Figure 6.4: **3D printing using ST-SasG-ST at 3 mM and SC3 at 1 mM.** The mixture could be extruded by the nozzle, but the resulting structure was too liquid and weak.
Given the unsuccessful trial using ST-SasG-ST at 3 mM and SC3 at 1 mM, I decided to increase the viscosity of the ST component by using ST-SasG-ST at 4 mM in combination with SC3 at 1 mM. According to the microrheological characterisation carried out in chapter 3, this combination gelled very quickly in less than 10 minutes. However, when I tried using ST-SasG-ST at 4 mM combined with SC3 at 1 mM, I encountered the opposite problem to the one I had before. This combination gelled too quickly, essentially upon contact, and gelled inside the T junction, clogging up the nozzle and blocking extrusion. Increasing the pressure on the pistons led to the quick expulsion of a fully gelled hydrogel, Fig. 6.5. The hydrogel was not 3D printed, but rather ejected by the nozzle in an uncontrollable manner. This trial was also unsuccessful.

Figure 6.5: **3D printing using ST-SasG-ST at 4 mM and SC3 at 1 mM.** The combination gelled upon contact and gelled inside the T junction, clogging up the nozzle and blocking extrusion. The hydrogel was ejected by the nozzle uncontrollably.
Lastly, I tried 3D printing with the combination in between the two I tried until now, and 3D printed using ST-SasG-ST at 3.5 mM with SC3 at 1mM. According to microrheology, this combination gelled in \( \approx 20 \) minutes. 3D printing with this combination was successful and I was able to establish a continuous and consistent extrusion flow. The structure was very delicate at the end of the 3D printing process (after printing 4+ layers) and it was left to gel for 20 minutes. After 20 minutes, the resulting square was gelled, maintained its shape, and was self-standing, Fig 6.6. I also optimised the 3D printing parameters, specifically the extrusion volume and the printing speed for ST-SasG-ST at 3.5 mM with SC3 at 1 mM, and determined the optimal printing speed to be 2 mm/s and the extrusion volume at 50 µL. Table 6.1 details the results of the optimisation trials.

![Image of 3D printing process](image)

**Figure 6.6:** 3D printing using ST-SasG-ST at 3.5 mM and SC3 at 1 mM at 2 mm/s and 50 µL extrusion volume. The structure was very delicate at the end of the 3D printing process (layer 4+) and it was left to gel for 20 minutes. After 20 minutes the square was gelled and the hydrogel was self-standing.
Table 6.1: Optimisation parameters for 3D printing of ST-SasG-ST at 3.5 mM and SC3 at 1 mM using the T junction.

<table>
<thead>
<tr>
<th>Extrusion [µL]</th>
<th>Printing speed [mm/s]</th>
<th>Print?</th>
<th>Notes/comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.5</td>
<td>No</td>
<td>Nothing comes out</td>
</tr>
<tr>
<td>100</td>
<td>1</td>
<td>Yes</td>
<td>Very, very intermittent flow</td>
</tr>
<tr>
<td>100</td>
<td>1.5</td>
<td>Yes</td>
<td>Very, very intermittent flow</td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>Yes</td>
<td>Speed too fast, can extrude but nothing sticks to platform</td>
</tr>
<tr>
<td>20</td>
<td>1</td>
<td>No</td>
<td>Nothing comes out</td>
</tr>
<tr>
<td>20</td>
<td>1.5</td>
<td>No</td>
<td>Nothing comes out</td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td>No</td>
<td>Nothing comes out</td>
</tr>
<tr>
<td>50</td>
<td>1</td>
<td>No</td>
<td>Nothing comes out</td>
</tr>
<tr>
<td>50</td>
<td>1.5</td>
<td>Yes</td>
<td>Intermittent flow</td>
</tr>
<tr>
<td>50</td>
<td>2</td>
<td>Yes</td>
<td>Consistent flow without blockages</td>
</tr>
</tbody>
</table>
6.2.1.1 3D printing of a two-tier structure using the T junction

Once I determined the correct ST-SasG-ST and SC3 combination and the parameters needed to 3D print, I pushed further the biofabrication of the protein hydrogels by trying to 3D print a two-tier structure, i.e. two separate squares one on top of the other. This approach was trialled to increase the complexity of the 3D printed design with a view of creating a more concave structure able to increase gaseous and nutrient exchanges between the seeded cells and the surrounding environment.

Given that I determined the right ST-SasG-ST - SC3 combination and the optimal parameters needed to print, 3D printing of the two squares was not an issue anymore. However, the main problem encountered was that the two squares would melt into one another and not remain separate as two distinct structures. First, I 3D printed a square with the parameters determined in section 6.2.1, I let the first square gel for 20 minutes and 3D printed the second on top. Unfortunately, the second structure immediately started melting into the first during printing, resulting in one homogenous square, Fig. 6.7.

Figure 6.7: Attempt to 3D print two distinct structures using ST-SasG-ST at 3.5 mM and SC3 at 1 mM at 2 mm/s and 50 µL extrusion volume. A) first structure; B) second structure melted into the first during printing.
To solve this problem, I decided to try printing a denser first square with $z = 9$ layers rather than $z = 4$ layers as done before. The idea was that a denser square would be stronger and be able to sustain the weight of the second structure being 3D printed on top, whilst maintaining its mechanical integrity. I 3D printed the first structure, let it gel for 40 minutes, accounting for the increased quantity of material needing to gel, and 3D printed the second structure on top. Unfortunately, as seen before, the two structures started melting into one another soon after printing of the second tier started, Fig. 6.8.

![First structure](image1.png) ![Second structure](image2.png)

Figure 6.8: Attempt to 3D print two distinct structures using ST-SasG-ST at 3.5 mM and SC3 at 1 mM at 2 mm/s and 50 µL extrusion volume. A) first structure $z = 9$ layers; B) second structure melted into the first during printing.

Given that a denser structure did not solve the problem, I decided to revert back to the original structure with $z = 4$ layers to conserve material, but I increased the gelation time of the first structure to ensure complete stabilisation. I 3D printed the first structure, let it gel for 35 minutes, and 3D printed the second structure on top. This trial was quite successful. I successfully 3D printed the two structures and they remained separate with two distinctive borders, rather than melting into one another, Fig 6.9. However, optimisation is needed as the second layer was not completely homogeneous.
6.2.2 Methods development for 3D printing using independent syringes

The main disadvantage of using the T junction configuration is that it is impossible to consider the two syringes as independent, meaning that the pistons will act on both syringes at the same time with the same parameters. Given the differences in viscosities between ST-SasG-ST 3.5 mM and SC3 1 mM, SC3 is extruded faster than ST-SasG-ST, likely resulting in a partially unbalanced mixture of material forming the 3D printed hydrogels. To overcome this issue, I tried 3D printing using an independent syringe configuration, Fig. 6.10. Briefly, I loaded SC3 in one syringe and ST-SasG-ST in the other as done before, but I removed the T junction and 3D printed one layer of SC3 using one syringe, immediately followed by a layer of ST-SasG-ST with the other syringe. This way I could optimise the parameters for each protein and ensure a 50:50 mixture of the resulting hydrogel.
Figure 6.10: **Dual nozzle 3D printer without T junction.** A) Schematic representation, B) the 3D printer in real life. The ST and SC components were kept in separate syringes and 3D printing was carried out layer-by-layer with independently defined parameters. Schematic representation created with BioRender.com.
I was able to 3D print using the independent syringe configuration and I optimised the 3D printing parameters for both SC3 at 1 mM and ST-SasG-ST at 3.5 mM, determined to be 2 mm/s printing speed and 50 µL extrusion volume for ST-SasG-ST at 3.5 mM and 1 mm/s printing speed and 50 µL extrusion volume for SC3 at 1 mM. Table 6.2 details the results of the optimisation trials. Intuitively, the optimal parameters for 3D printing of ST-SasG-ST 3.5 mM by itself were the same as the ones determined for 3D printing of both SC3 and ST-SasG-ST, 6.2.1, because this protein was the more viscous of the two.

Table 6.2: Independently optimised parameters for 3D printing of ST-SasG-ST at 3.5 mM and SC3 at 1 mM

<table>
<thead>
<tr>
<th>Extrusion [µL]</th>
<th>Printing speed [mm/s]</th>
<th>Print?</th>
<th>Notes/comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.5</td>
<td>No</td>
<td>Nothing comes out</td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>No</td>
<td>Almost nothing comes out</td>
</tr>
<tr>
<td>20</td>
<td>0.5</td>
<td>No</td>
<td>Nothing comes out</td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td>Yes</td>
<td>Very intermittent flow</td>
</tr>
<tr>
<td>50</td>
<td>0.5</td>
<td>Yes</td>
<td>Intermittent flow</td>
</tr>
<tr>
<td>50</td>
<td>1</td>
<td>Yes</td>
<td><strong>SC3 good, SasG needs optimisation</strong></td>
</tr>
<tr>
<td>50</td>
<td>1.5</td>
<td>Yes</td>
<td><strong>SC3 fast, SasG needs optimisation</strong></td>
</tr>
<tr>
<td>50</td>
<td>2</td>
<td>Yes</td>
<td><strong>SC3 too fast, SasG good</strong></td>
</tr>
</tbody>
</table>
However, the resulting hydrogel never completely gelled and it remained a fairly weak structure compared to the stronger self-standing hydrogel observed with the T junction configuration, Fig 6.11. I rationalised that, despite the two materials being printed with their respective optimised parameters, it is likely that the delays in the 3D printing process due to the continuous starting and stopping needed to change the parameters, led to issues in the mixing of the proteins. Moreover, because the two materials were 3D printed separately, it is possible that they mixed only at their interface rather than in the entire structure, resulting in a weaker and more unstable hydrogel. The poor properties of the resulting hydrogel combined with the lengthy and complicated 3D printing process, harder to automate for potential future applications, led us to shelf the independent syringe configuration and establish the T junction as the best way to 3D print the ST-SC protein hydrogels.

![Figure 6.11: 3D printing using ST-SasG-ST at 3.5 mM and SC3 at 1 mM using the independent syringe configuration.](image)

Figure 6.11: 3D printing using ST-SasG-ST at 3.5 mM and SC3 at 1 mM using the independent syringe configuration. ST-SasG-ST printed at 2 mm/s and 50 µL extrusion volume, SC3 printed at 1 mm/s and 50 µL extrusion volume. 3D printing was successful but the resulting hydrogel never completely gelled and it remained a fairly weak structure.
6.3 Discussion

In this chapter I adapted the ST-SC hydrogel protein design to 3D printing using a dual nozzle 3D printer. I developed, tested, and optimised the methods needed to 3D print the hydrogels using combinations of ST-SasG-ST at 3.5 mM and SC3 at 1 mM, determined the optimal printing parameters, and concluded that 3D printing with a T junction led to better structural results compared to 3D printing layer-by-layer with independent parameters. Finally, I successfully 3D printed a two-tier structure composed of two squares one on top of the other, albeit optimisation is still needed. Whilst 3D printing is not new, to the best of my knowledge, this is the first time that successful 3D printing has been reported using only recombinant protein hydrogels.

Adapting the ST-SC protein hydrogel design to 3D printing was an exercise in balance between choosing the right combination of ST-SasG-ST and SC3 and the 3D printing parameters. The ST-SasG-ST and SC3 combination needed to gel fast enough to sustain its own weight whilst being extruded but not too fast to clog up the nozzle. The trial-and-error investigation showed that ST-SasG-ST at 3.5 mM combined with SC3 at 1 mM presented the right gelling characteristics. However, 3D printing this combination using the T junction configuration forced me to optimise the 3D printing parameters for the more viscous solution, the ST-SasG-ST in this case, despite SC3 being extruded a bit too quickly due to its lower viscosity. This disparity is evidenced also in the optimisation trials carried out for the independent syringe configuration where the optimal 3D printing parameters identified for SC3 are different to the ones determined for ST-SasG-ST. I rationalised that this discrepancy is the reason why the first few attempts to 3D print a two-tier structure did not work. Specifically, the microrheological characterisation carried out in Chapter 3 demonstrated that the combination of ST-SasG-ST at 3.5 mM and SC3 gelled in 20 minutes. This appeared to be consistent with the hydrogel 3D printed using the T junction that gelled after 20 min. However, when I tried to 3D print a second structure on top of the first using the same gelation time of 20 minutes, the two structures melted into one another. This result suggested that the gelation of the first structure was not as completed as previously thought. This observation was further validated when I increased the gelation time of the first structure to 35
minutes that allowed me to 3D print another distinct structure on top, indicative of complete gelation. It is possible that the increased amount of SC3 extruded into the hydrogel due to the lower viscosity of this protein changed the ST:SC ratio in the resulting 3D printed hydrogel. The ST:SC imbalance led to slower gelation compared to the precise 2.3:1 ST:SC ratio used in the microrheology experiments.

The spontaneous gelation of ST-SC could be considered a disadvantage for 3D printing when compared to more common photocrosslinked systems, where gelation can be controlled readily by modulating the illumination intensity [271]. However, once accurate control over the ST-SC gelation is achieved, the 3D printed ST-SC hydrogels will be highly biocompatible thanks to the lack of crosslinking agents, as increased exposure to UV light can be cytotoxic [22].

Moreover, another avenue exploiting the spontaneous gelation of ST and SC that could be investigated is the use of a single syringe containing a mixture of ST and SC with slow gelation kinetics. For example, from the microrheological characterisation detailed in Chapter 3 of this thesis, it is clear that combinations of ST-SasG-ST at 2 mM and SC3 at 1 mM achieve complete gelation in 45 minutes. In fact, this combination was excluded from the 3D printing trials detailed in this chapter as it was deemed too slow. However, the slow gelation kinetics of this system could be advantageous because they allow to premix ST and SC in one syringe and extrude the ST-SC mixture simultaneously before the gelled system clogs up the nozzle. In addition, this route could overcome the limitations linked to the unbalanced extrusion of either ST or SC loaded in two different syringes because the two components could be accurately pre-mixed together at the correct mM concentrations and extruded simultaneously using the same set of parameters. However, due to the slow gelation kinetics, one could not start 3D printing immediately after mixing as the mixture would be too weak to self-sustain but a predetermined setting time would be required to ensure that once 3D printing commences, the system is able to sustain its own shape and the extrusion process.

The natural next step to further bridge synthetic biology and tissue engineering is to 3D print cells directly inside the materials. Successful 3D printing of mammalian cells inside hydrogels has already been shown multiple times [271, 279],
and given that I demonstrated that mammalian cells can be encapsulated inside the hydrogels in 3D and the lack of potentially cytotoxic crosslinking agents, I am confident that direct 3D printing of cells inside the ST-SC protein hydrogels will be possible. In particular, the large cylindrical nozzle and the overall low pressure needed to 3D print with the 3D printer used in this project are good predictors of good cell viability in the 3D printed hydrogels [280].

3D printing of more complex shapes should also be investigated, as the proof-of-concept showed here only 3D printed a small square. However, research should first focus on increasing the yield of the protein building blocks in order to have more purified protein available to 3D print bigger and more sophisticated objects. When protein production will be scaled-up, the methods developed in this chapter offer a suitable blueprint for the 3D printing of complex shapes using a dual nozzle 3D printer.

Further expansion of this work is to adapt the hydrogel design to other biofabrication techniques, such as the Rotational Internal Flow Layer Engineering (RIFLE) technology developed by the Davies Lab [281]. Briefly, in RIFLE technology hydrogels are suspended as liquid layers on the internal surface of a rotating tube. The addition of gelling agents gels the initial liquid layer before the addition of further layers, creating a macroscale tubular construct [281]. This system has been evaluated with alginate and CaCl$_2$ and it is currently being tested with ST and SC. A step further in expanding this system could be to spray the materials inside the rotational tube under the guidance of an electric current, akin to electrospinning, increasing control over the deposition and the mechanical properties of the fibres [282]. Moreover, the ST-SC hydrogels could also be adapted to the fully automated stem cell-based tissue engineering platform built by Hay and co-workers [266], allowing self-assembly of human liver tissue directly inside the protein-based hydrogels that could be implemented as drug testing devices.
6.4 Conclusions

In this chapter I developed and optimised the methods needed to 3D print the ST-SC hydrogels via trial-and-error using a dual nozzle 3D printer. The results detailed here could improve the capabilities of synthetic biology to interface with tissue engineering by opening the field of biofabrication to protein-based hydrogels. Future research should focus on 3D printing mammalian cells directly inside the hydrogels and ascertain cell viability, as well as increasing the sophistication of the dual nozzle 3D printing methods developed here. Broadening the types of biofabrication techniques that could be used with the SpyTag – SpyCatcher system should also be investigated to increase the potential of protein hydrogels in tissue engineering.
Chapter 7

Conclusions and Future Directions

In this thesis I developed and characterised protein-based hydrogel designed using the SpyTag-SpyCatcher system. I determined the speed of gelation, the stiffness, and the biocompatibility of the hydrogels, and adapted the design to 3D printing. Herein, I will give a brief final overview of my results in the context of the literature and I will discuss the potential future directions of this project.

The physical characterisations detailed in Chapters 3 and 4 showed an underlying trend in the formation of the protein hydrogels. Specifically, both the microrheological and the rheological characterisations showed that when SC is present in great excess compared to ST the systems remain a viscous liquid and the hydrogel does not form. Moreover, roughly equimolar combinations of ST and SC led to slow gelation kinetics (gel formation in \( \approx 35-45 \) minutes) and strong but brittle hydrogels (\( G' = 10,000 \) Pa and critical stress at \( \approx 10\% \) strain), whilst excess of ST over SC induced fast gelation kinetics (gel formation progressively faster until a gel formed in less than \( \approx 10 \) minutes) and weaker hydrogels strongly resistant to deformation (\( G' = 1,000 \) Pa and no critical strain up to 100\% strain).

Given the highly specific crosslinking properties of ST-SC, it is reasonable to assume that chemical crosslinks are the only crosslinking mechanism involved in the formation of the hydrogels, therefore, changes in the crosslinking between ST and SC led to changes in the network morphology that, in turn, affected their viscoelastic properties. Moreover, because of the highly cooperative nature of ST and SC, I hypothesise that the crosslinking between ST and SC does change, but not...
the structure of one crosslink. That is to say that all crosslinks formed in the protein hydrogels are likely to be the same, i.e. the covalent bond between ST and SC, but the number of bound/unbound ST and SC in the network influences the resulting viscoelastic properties. Similar findings are present in the literature and the authors largely ascribed the variation in the physical properties of their protein hydrogels to differences in the crosslinking density.

For example, the Regan group already investigated this phenomenon in another type of protein hydrogel, an α-helix based hydrogel formed using tetra tri copeptide (TPR) domains concatenated into arrays and crosslinked with their corresponding peptide attached to a 4-arm-PEG crosslinker [109]. Similar to the findings described here, the authors showed that the rate of gelation is strongly dependant on the concentration of the components, namely when TPR and PEG are equimolar the hydrogel formed slowly and increasing PEG led to faster hydrogel formation. This is fairly intuitive, as more components in the same volume lead to a higher probability of chain encounter and, consequently, faster crosslinking, compared to smaller quantities of components in the same volume where the chains have to stretch in order to crosslink. However, when TPR is present in excess compared to PEG, a hydrogel did not form. The authors hypothesised that an excess in available peptide binding sites did not lead to productive crosslinking and the formation of a percolating network. Christoffersson et al. reported very similar results where an excess in one component compared to the other hindered the formation of a hydrogel [165]. This phenomenon is clearly similar to the one described in this thesis, where an increase in ST led to faster gelation kinetics, but an excess of SC hindered the formation of a hydrogel. Therefore, an excess of binding sites offered by the large quantity of SC caused most ST to bind unproductively, hindering the formation of a matrix and a percolating network.

Other authors observed changes in the physical properties of their protein-based hydrogel due to changes in the ratio of the components. For instance, Heise et al. developed chemically crosslinked polypeptide interpenetrating networks (IPNs) by orthogonally crosslinking bis-PEG-N3 with varying molar ratios of pentaerythritol tetraacrylate [162]. The authors reported that when the thiol:acrylate ratio was increased to 1:2 the hydrogel’s elastic modulus was reduced from 3677 kPa to
604 kPa and ascribed this phenomenon to the excess acrylate moieties yielding a heterogenous structure. Once again, the results reported by Heise and co-workers are very similar to the results presented here, where an excess in ST over SC caused a reduction in the hydrogel storage modulus. In Chapter 4, I hypothesised the presence of ‘dangling ends’, conceptually similar to the ‘heterogenous structure’ proposed by Heise. Briefly, in combinations where ST >> SC, the ST binds to an SC array but it is possible that only one end of the ST-SasG-ST construct is bound and the other one is not crosslinked resulting in a ‘dangling end’, due to the lack of available SC arrays. The dangling ends do not participate to the modulus of the hydrogel and, consequently, weaken the hydrogel.

Another interesting article focused on physically entangled Nap-based hydrogels showed that the kinetics of assembly of the hydrogels had a clear effect on the ability of the networks to withstand strain, where the critical strain changed from 1% to 5% with increased glucono-δ-lactone (GdL) concentrations [94]. Contrary to the results presented here, the authors showed that despite the different kinetics pathways in which the networks are formed, the final plateau values of G’ and G” were very similar, suggesting that the final hydrogels all have similar mechanical properties. This could be ascribed to the authors using GdL to regulate the final pH of the hydrogel and, consequently, the kinetic assembly, rather than an increase or decrease in the components’ concentration. Nevertheless, the authors reported changes in critical stress due to different gelation kinetics, indicating that gelation kinetics do play a crucial role in the crosslinking of protein hydrogels that, in my case, affect the resulting viscoelastic properties.

Notably, throughout this thesis, combinations of ST-SasGlong-ST and SC3 failed to adhere to the observed trends in both microrheology and rheology and consistently showed similar physical properties regardless of the ratio between the components or the protein concentration. In chapters 3 and 4, I hypothesised that steric hindrances caused by the large ST-SasGlong-ST chains hinder the formation of dangling ends. Specifically, in equimolar combinations, I hypothesised that one ST-SasGlong-ST binds an SC3 array causing the expected strong but brittle hydrogels. However, increasing the quantities of ST-SasGlong-ST did not lead to increased crosslinking and the formation of dangling ends because the size of the
already bound ST-SasGlong-ST concealed the remaining SC3 binding sites to other crosslinkers. The steric hindrances translate into the constant physical properties exhibited by this combination. Clearly, the steric hindrances hypothesised here are not enough to hinder the formation of a hydrogel completely, as shown by Xu et al. for Nap based hydrogels [93]. However, steric hindrances have already been shown to influence the behaviour of protein based hydrogels, both covalently crosslinked hydrogels [210] and physically entangled systems [98]. In particular, Mulyasasmita et al. showed that strong physically entangled hydrogels were formed outside of the expected 1:1 ratio as some binding sites were obstructed and were not available to form productive interactions [98]. The authors showed that by balancing the ratio between the components they could increase the degree of crosslinking and, consequently, obtain strong hydrogels. This was not the case in the ST-SC protein hydrogels potentially due to the great mismatch between ST-SasGlong-ST (51.5 nm) and the shortest SC3 array (5 nm x 3 SC units) compared to the C7 and P9 protein polymers used by Mulyasasmita et al. Therefore, whilst the addition of extra ST units led to weakening of the resulting hydrogels in other combinations, the hypothesised steric hindrances caused by the mismatch between ST-SasGlong-ST and SC3 ensured a constant crosslinking density that translated into consistent viscoelastic properties.

Potential supporting evidence of the presence of steric hinderances in the combination of ST-SasGlong-ST and SC3 could be observed in its microrheological behaviour. In chapter 3, I showed that this combination exhibited partial or incomplete gelation, where the slope of the MSD never quite reached 0, but for the highest ST concentrations (3.5 and 4 mM). These findings could suggest that there are pockets of material with unbound ST-SasGlong-ST within the hydrogel network that allow free flow of the tracer beads and, consequently, appear as incomplete gelation. Moreover, it is possible that high ST concentrations were viscous enough to stop the free flow of the tracer beads, hence appearing as gelled as the crosslinked hydrogel. Further research is required to accurately determine the crosslinking density of ST-SasGlong-ST and SC3 and verify the presence of steric hindrances.
Interestingly, changes in the viscoelastic properties of protein hydrogels reported in the literature span both physically entangled systems and chemically crosslinked hydrogels as well as different crosslinking mechanisms. This would suggest that the underlying mechanisms regulating protein hydrogel formation are somewhat independent from the methods exploited, but rather dependant on the crosslinking or entanglements of the proteins themselves. Therefore, accurately determining the density of crosslinking between ST and SC is key to understand the underlying mechanisms of hydrogel formation and viscoelasticity of the protein hydrogels, and the process could be potentially generalised to more protein-based systems.

During my PhD, I attempted to determine the degree of crosslinking between ST and SC, but this investigation is not trivial, especially due to the unregulated and spontaneous gelation of the ST - SC system. For example, I attempted to determine the amount of ST bound to the SC arrays by progressively increasing the molar concentration of ST reacting with SC on an SDS-PAGE gel. However, the results were ambiguous and inconclusive as quantification of the protein bands on an SDS-PAGE gel is riddled with potential artefacts. Attempts to determine the crosslinking density via size exclusion chromatography (SEC) were also inconclusive due to technical issues linked to protein aggregation and ambiguities in the quantification of the results. Future attempts to determine the crosslinking density of the ST-SC protein hydrogels should focus on both physical characterisations, such as SANS, combined with computational approaches based on cluster aggregation kinetics models. Small angle scattering techniques, such as SANS, probe the material’s structure on the nanometer to micrometer scale. These techniques are based on the elastic scattering of radiation by a sample that provides information about the size, shape, and orientation of the sample’s components [148, 149]. ST-SC hydrogels could be probed via SANS to understand the behaviour of the folded proteins composing the hydrogels and potentially create an estimate of the crosslinking density. However, as the most abundant components will dominate the resulting elastic scattering, it is unlikely that smaller features, such as the hypothesised dangling ends, will be visible using this technique. Nevertheless, SANS could help shed light on the microstructure of the ST-SC protein hydrogels.
Furthermore, in this thesis I have shown that the ST-SC protein hydrogels are biocompatible. Specifically, I showed that the ST-SC protein hydrogels can be used as 2D structural support for liver cells as well as a 3D matrix able to encapsulate the cells for up to 4 days without loss of cell viability. My results are in agreement with plenty of other findings in the literature that demonstrated the suitability of protein-based hydrogels for tissue engineering applications (some detailed in Chapter 1). Notably, I was unable to accurately determine the porosity of the protein hydrogels, but, given the success of the encapsulation experiments, I inferred that the porosity was adequate for the exchange of nutrients and waste between the embedded cells and the surrounding medium. Because of the diverse characteristics, properties, and fabrication methods of hydrogels, the average pore size ranges from 5 nm to 500 µm [283, 284]. However, because the 1 µm diameter tracer beads used in the microrheological characterisation were able to accurately determine the gelation speed of the hydrogels whilst remaining embedded into the gelling system rather than sinking to the bottom of the glass slide, I deduced that the average pore size of the protein hydrogels was < 1 µm. Therefore, it is likely that the average pore size of the ST-SC protein hydrogels is in the nm scale. It would be interesting to determine the actual porosity of the hydrogels, and, most importantly, if the porosity changes in response to increases in ST units or protein concentration. Because porosity and viscoelastic properties of hydrogels are intrinsically interconnected [284], one would assume that porosity does change, but accurate experiments should be carried out to determine if that is indeed the case.

An elegant method developed by Burdick et al. quantified the porosity, pore area, and number of pores in granular hydrogels using a simple protocol based on the analysis of z-stacks collected via confocal microscopy scanned via a built-in function of the commercially available software ImageJ [285]. Briefly, Burdick et al. binarised the z-stack of hydrogels via a built-in threshold function using the Huang or Otsu algorithms. The binarisation allows to convert the z-stacks into black areas (where the hydrogel is present) and white areas (where no hydrogel is present - the pores). Using the analyse particle function, quantitative values describing the number of pores and the porosity of each slice in the confocal stack can be determined. Burdick et al. also detailed ways to limit the presence of artefacts in their analysis by limiting the size range of pore analysed. Because z-stack
were already successfully collected in this project and ImageJ is a commercially available software requiring no licence, the Burdick method could be applied to the ST-SC protein hydrogels to determine their average porosity. Moreover, protein hydrogel porosity was also estimated via scanning electron microscopy (SEM) combined with liquid displacements methods, such as the quantification of absolute ethanol filling the pores of the structures [286, 287]. Future attempts to determine the porosity of the ST-SC protein hydrogels should focus on adapting the methods described by Burdick et al. combined with imaging methods that allow to capture the structure of the hydrogels.

The most interesting result obtained during the biocompatibility studies was the difference in the viability of cells seeded on hydrogels with different stiffnesses. Briefly, the soft hydrogels (\( G' = 1,000 \) Pa) showed \( \approx 90\% \) cell viability, whilst their stiffer counterparts (\( G' = 10,000 \) Pa) showed \( \approx 60\% \) cell viability. In chapter 5, I hypothesised that the biosignalling of the softer hydrogels more closely mimicked the viscoelasticity of decellularised liver [223], hence the softer hydrogels offered a more suitable environment for liver tissue regeneration. These results could suggest that further optimisation of the ST-SC protein hydrogels should focus on mimicking the characteristics of the decellularised target organ as the seeded cells will confer the remaining rigidity to the scaffold. Furthermore, given that I showed that the ST-SC hydrogels are biocompatible using the HepG2-GFP cell line, the hydrogels should be tested in regards to their ability to induce the differentiation of pluripotent stem cells, thereby moving the first steps towards the development of an ST-SC matrix able to control the fate of the seeded cells and, further along the line, develop bespoke new organs.

Moreover, thanks to the genetically encodable approach used in this project, the Regan lab is also working towards the development of ST-SC protein hydrogels with controllable degradation kinetics, via the precise addition of MMP cleavage sites in the protein building blocks. This new iteration of hydrogels should not only show excellent biocompatibility and similar physical properties to the ones detailed here given the small size of the additional MMP site, but also show improved cell adhesion and invasion.
One of the main disadvantages of protein-based hydrogels is their reduced adaptability to biofabrication techniques, often due to the proteins’ low mechanical properties or incompatible crosslinking methods. In this thesis, I adapted the ST-SC hydrogel design to 3D printing using a dual nozzle configuration, thereby increasing the sophistication of the protein-based hydrogels and decreasing the gap between synthetic biology and tissue engineering. Further research should focus on broadening the library of techniques used for the biofabrication of the ST-SC protein hydrogels, starting with the RIFLE method developed by the Davies lab [281]. Currently, the Regan and Davies labs have joined efforts to developed novel ST-SC protein hydrogels using the RIFLE system.

Other biofabrication techniques that could be suitable for the ST-SC protein hydrogel design are meltelectrowriting and electrospinning. Both techniques rely on a pneumatic or volumetric feed used to extrude a heated polymer through a nozzle in an electrified field [288]. The polymer droplet at the nozzle tip forms a conical shape, the Taylor cone, that is drawn to a collector by the electric field [288]. Changing the material’s extrusion volume and the collector’s speed leads to controllable changes in the material deposition and fibres alignment [288]. Adding an extra nozzle able to extrude the second component (i.e. SC) alongside the first one (i.e. ST) could be a straightforward method to adapt the ST-SC protein hydrogel design to either biofabrication technique. Moreover, there would be no need to preemptively heat the proteins to liquefy them or accurately maintain the temperature of the electrospinning chamber or meltelectrowriting platform as the ST-SC reaction is stable between 4 and 37 °C [171]. However, it would be very interesting to see how changes in ‘traditional’ biofabrication parameters, such as pressure or feed rate, speed of the moving elements like the collector and/or the print head, and distance between nozzle and collector [288], affect the resulting viscoelastic properties of the ST-SC protein hydrogels.
Overall, the core advantage of the ST-SC system presented here is that it exp-loits recombinantly produced proteins, hence it is fairly straightforward to geneti-cally encode new features to modulate mechanical properties, cell attachment, and stimuli-responsiveness of the hydrogels via the modification of the protein building blocks. Moreover, because the hydrogels are recombinantly produced, the system is inherently sustainable as opposed to chemically synthetised peptides, often pro-duced with considerable toxic by-products [86].

However, the bottleneck currently limiting the scale-up of the ST-SC protein hydrogels is the volume of recombinantly produced and purified proteins needed to develop self-sustaining hydrogels. The Regan lab is working on increasing the yields of the ST crosslinkers and SC arrays via the use of fermenters as well as improving the yields of purified proteins by using fast and automatic systems such as the ÄKTA. In the future one could imagine fields of bacteria feeding on waste, such as plastic [289], overexpressing the proteins of interest. Alternatively, an attractive solution could be the use of cell-free expression systems, where recombinant proteins are expressed *in vitro* without the use of bacteria cells [179]. There are still issues with cell-free systems, primarily due to their inherent batch-to-batch vari-ability and low yields, but they offer a potential solution to scale-up the protein production needed to develop the ST-SC protein hydrogels.

In conclusion, the results presented in this thesis have advanced our under-standing of the mechanisms underlying ST-SC hydrogels, thereby contributing to bridging synthetic biology and tissue engineering towards the increased sophistica-tion of protein-based materials and the auspicious development of patient-specific organs on demand.
Chapter 8

Materials and Methods

8.1 Bacterial materials and methods

8.1.1 Bacterial growth media

Table 8.1 lists the medium routinely used for growing bacterial cells. All media was sterilised by autoclaving before use.

Table 8.1: Media used for bacterial cell culture

<table>
<thead>
<tr>
<th>Media</th>
<th>Purpose</th>
<th>Ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysogeny Broth</td>
<td>growth in liquid culture</td>
<td>10 g/L peptone, 5 g/L NaCl, 5 g/L yeast extract</td>
</tr>
<tr>
<td>LB Agar</td>
<td>growth in solid media</td>
<td>10 g/L peptone, 5 g/L NaCl, 5 g/L yeast extract, 16 g/L agar</td>
</tr>
<tr>
<td>Terrific Broth</td>
<td>growth in liquid culture</td>
<td>4 g/L yeast extract, 20 g/L tryptone, 4 mL/L glycerol</td>
</tr>
</tbody>
</table>
8.1.2 Antibiotics

Table 8.2 lists all antibiotics used for routine bacterial selection. Stock solutions were prepared and filtered through a 0.22 µm filter before aliquoting and storing at -20 ºC.

Table 8.2: Antibiotic used for bacterial selection

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Solvent</th>
<th>Stock concentration</th>
<th>Working concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>ddH2O</td>
<td>50 mg/mL</td>
<td>50 µg/mL</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>ddH2O</td>
<td>50 mg/mL</td>
<td>50 µg/mL</td>
</tr>
</tbody>
</table>

8.1.3 Bacterial Strains

Table 8.3 lists all strain used in this study. The appropriate strain was selected depending on the required function.

Table 8.3: Bacterial cell strains

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Purpose</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOP10</td>
<td>DNA cloning</td>
<td>F- mcrA Δ(mrr-hsdRMS-mcrBC) φ 80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ-</td>
<td>Lab stock</td>
</tr>
<tr>
<td>BL21 Gold(DE3)</td>
<td>Protein EXP</td>
<td>F- ompT hsdSB(rB-mB-) dcm (TetR) gal λ(DE3) endA Hte</td>
<td>Lab stock</td>
</tr>
</tbody>
</table>
8.1.4 Buffers

Table 8.4 lists all buffers and their respective recipes used for His-tag bacterial cell purification. SpyTagged proteins were washed using wash buffer 1 (5 mM imidazole), whilst SpyCatcher arrays were washed using wash buffer 2 (20 mM imidazole). Both proteins were eluted using the elution buffer (200 mM imidazole).

Table 8.4: Buffers used for His-tag purification of proteins

<table>
<thead>
<tr>
<th>Name</th>
<th>Ingredients</th>
<th>Purpose</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer</td>
<td>50 mM Tris - HCl, 300 mM NaCl, 10% glycerol</td>
<td>Protein resuspension</td>
<td>QIexpressionist manual</td>
</tr>
<tr>
<td>Wash buffer 1</td>
<td>50 mM Tris - HCl, 300 mM NaCl, 5 mM imidazole</td>
<td>His-tag purification</td>
<td>QIexpressionist manual</td>
</tr>
<tr>
<td>Wash buffer 2</td>
<td>50 mM Tris - HCl, 300 mM NaCl, 20 mM imidazole</td>
<td>His-tag purification</td>
<td>QIexpressionist manual</td>
</tr>
<tr>
<td>Elution buffer</td>
<td>50 mM Tris - HCl, 300 mM NaCl, 200 mM imidazole</td>
<td>His-tag purification</td>
<td>QIexpressionist manual</td>
</tr>
</tbody>
</table>

8.1.5 *E. coli* standard methods

8.1.5.1 Overnight growth

*E. coli* cells were harvested from a single colony grown on a LB agar plate, inoculated into a 5 mL LB with the appropriate antibiotic in a universal tube, and grown overnight at 37 °C with continuous shaking (200 rpm).
8.1.5.2 Competent cells

A single colony was inoculated into 5 mL LB with no antibiotic in a universal tube and grown overnight at 37 °C with continuous shaking (200 rpm). The next day, 200 mL of LB were inoculated using 500 µL from the overnight culture, and grown at 37 °C with continuous shaking (200 rpm) until OD600 was between 0.3 - 0.4. Cells were transferred into pre-frozen tubes, incubated on ice for 20 minutes, and collected by centrifugation at 4,000 rpm for 10 minutes at 3 °C. The supernatant was discarded and the cells were gently resuspended in 40 mL pre-chilled 50 mM CaCl\textsubscript{2}. Cells were centrifuged and resuspended as before a second time. After the second wash, cells were incubated on ice for 30 minutes, centrifuged at 4,000 rpm for 8 minutes at 0 °C. The supernatant was discarded, the cells were gently resuspended in 8 mL pre-chilled 50 mM CaCl\textsubscript{2} + 15 % glycerol, and incubated on ice for 2 hours. 100 µL of competent cells were aliquoted into pre-frozen microtubes and immediately stored at – 80 °C until use.

8.1.5.3 Heat shock transformation

Miniprepped DNA was routinely transformed into \textit{E. coli} cells by heat shock. Competent cells were thawed on ice for 10 minutes. 50 µL of competent cells were gently mixed with 1 µL of DNA and incubated on ice for 30 minutes before cells were heat shocked using a dry heat block at 42 °C for 90 seconds. Following this, cells were incubated on ice for 3 minutes, resuspended in 500 µL LB, and allowed to recover at 37 °C for an hour with continuous shaking (200 rpm). After recovery, 100 µL of cells were plated evenly on a LB agar plate containing the appropriate antibiotic and incubated overnight at 37 °C in a static incubator.

8.1.6 Cloning via Gibson assembly

8.1.6.1 Phusion PCR

DNA fragments were amplified using Polymerase Chain reaction (PCR) in an Applied Biosystems Verti Thermal Cycler or ProFlex PCR system. DNA amplification was carried out using Phusion polymerase (NEB) according to the manufacturer’s instructions. Purified template DNA was added to each 50 µL reaction with a final concentration between 1 - 10 ng and run in the thermocycler using the
annealing temperatures corresponding to the primer pair used whilst the extension
time was determined based on the length of the transcript (30 s per kb). For se-
quencing of the Azami Green - ST construct, primers provided by the sequencing
company (DNA Sequences and Services - www.dnaseq.co.uk) were used, T7F and
T7R.

8.1.6.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments by size. 1
% agarose (w/v) gel was used to visualise DNA smaller than 1 kb in size whilst 0.8
% agarose (w/v) was used to visualise DNA larger than 1 kb. TBE (Tris-Borate-
EDTA) buffer was combined with agarose powder at the required ratio and heated
in a microwave until dissolved. Once cooled, SBYR® Safe DNA Gel Stain was
added to the flask and mixed thoroughly to the recommended final concentration.
The solution was poured into a gel cast with a gel comb in place and left to set
for half an hour. DNA samples were mixed with the appropriate concentration of
loading dye and loaded onto the set gel. Hyperladder was used as the molecular
weight marker. Gels were run in 1x TBE buffer at 120 mV for 30 minutes, or until
good DNA separation could be observed. The Bio- Rad Gel Doc XR+ system with
filter 1 and SYBR Safe mode was used to visualise the DNA bands on gels.

8.1.6.3 Gibson assembly

Cloning of the Azami Green - ST construct was carried out using standard
Gibson Assembly protocols. DNA fragments obtained by PCR and purified were
mixed with Gibson Master Mix (NEB) according to the manufacturer’s instructions.
The solution was first incubated at 50°C for 1 hour and then DpnI (New England
Biolabs) was added at 37°C to digest any original template plasmid. The solution
mixed with DpnI was incubated at 37°C either for 1 hour. 1 µL of the solution was
used to transform TOP10 cells following 8.1.5.3. The cells were plated onto an LB
agar plate with the appropriate antibiotic, colonies were cultured overnight 8.1.5.1,
the DNA was extracted the next day 8.1.6.4, and sent for sequencing 8.1.6.1.
8.1.6.4 DNA purification and quantification

Plasmids were purified using standard protocols as described by the QIAquick® Spin Miniprep Kit (Qiagen). Linear DNA was purified from PCR or agarose gels using the Promega Wizard® SV Gel and PCR Clean-up System following the manufacturer’s instructions. All purified DNA was eluted from the spin columns using 35 µL of ddH2O. Purified DNA solutions were quantified using a DeNovix DS-11+ spectrophotometer, before storage at -20°C.

8.1.7 Protein expression and purification

Proteins were routinely expressed in *E. coli* and purified via His-tag. Due to the large amount of protein needed for this project, SC3 and SC4 were expressed by myself in the Regan Lab, whilst the ST crosslinkers, characterised by lower yields, were expressed by both myself in 1 L flasks in the Regan Lab and by Dr John White in the Chemistry Fermentation Suite at the University of Edinburgh in 10 L fermenters. SC5 and SC3 RGD were expressed and purified by Regan lab member Fokhrul Islam. Of note, SC5 is only partially used in this project as Fokhrul cloned it in 2022. When the proteins were expressed in the Chemistry Fermentation Suite, I gave Dr White a transformant plate containing colonies of the *E. coli* strain harbouring the appropriate plasmid and I received back pelleted cultures after protein expression. Regardless of where the proteins were expressed, all proteins were expressed using the same methods and purified by myself, with the exception of SC5 and SC3 RGD, using the methods listed in section 8.1.7.2.

8.1.7.1 Bacterial growth and expression protocol

5 mL LB of overnight culture were used to inoculate 500 mL of LB containing the appropriate antibiotic and grown at 37 °C with continuous shaking (200 rpm) until OD600 reached 0.6 – 0.8. Protein expression was induced using 1 mM IPTG and growth was allowed to continue for a further 22 hours at 18 °C with continuous shaking (200 RPM). Cultures were pelleted by centrifugation at 10,000 rpm and pellets were stored at – 20 °C until use.
8.1.7.2 Cell lysis and purification via batch method

Frozen pellets were resuspended in lysis buffer with cOmplete Protease inhibitor Cocktail tables (4693132001, Sigma-Aldrich) at a standard ratio of 1:30 buffer to cell culture volume. Resuspended cells were broken open using a continuous flow cell disrupter (CF1, Constant Systems) set at a pressure of 30 psi. Cell lysates were clarified by centrifugation at 18,000 rpm for 30 minutes at 4 °C, then stored on ice. Clarified lysates were incubated with pre-equilibrated Ni – NTA agarose resin (30230, Qiagen) with gentle agitation for an hour at 4 °C to allow for binding. Flow through was collected and the resin washed twice with 6 times column volume (CV) wash buffer. Protein of interests was eluted using 2 CV elution buffer. All purification fractions were collected and analysed via SDS-PAGE. Pure protein elutions were pooled together and extensively dialysed against 4 L of purified water using SnakeSkin Dialysis Tubing (ThermoFisher, various sizes). To ensure complete buffer exchange, dialysis was allowed to proceed overnight at 4 °C with continuous stirring. Dialysed proteins were freeze dried for 48 hours using an Alpha 1-2 LD plus (Martin Christ, Germany) and the freeze-dried protein powders were stored at – 80 °C until use. Purified protein solutions were quantified using a DeNovix DS-11+ spectrophotometer. The yield of each protein expression was determined via a Bradford assay, a colorimetric protein assay frequently used to determine protein concentrations [290]. The Bradford assay was used according to the manufacturer’s instructions. On average, I obtained 30 – 35 mg of purified protein per L of culture for each SC array and 10 – 12 mg per L for each ST crosslinker. I developed and optimised the batch method to avoid using traditional Ni-NTA purification columns, where the protein fractions are slowly eluted by gravity. Given the amount of protein expressed and purified for the project, it would have been very time consuming to use traditional methods. I carried out several rounds of optimisation and developed the final protocol used throughout the project.
8.1.7.3 SDS-PAGE

Protein expression, purification, and activity was routinely checked by SDS-PAGE. Samples in 4x gel loading buffer were boiled at 100 °C for 12 minutes using a thermocycler and 5 µL of each sample was analysed on homemade SDS-PAGE gels using Precision Plus Protein Dual Xtra Prestained Protein Standards as a molecular marker. Homemade gels were prepared at 7.5 % according to the standard methods developed by Amersham Bioscience Hoefer system protocols. SDS-PAGE gels were run at 200 mV for 25 minutes or until proteins samples had fully separated. SDS-PAGE gels were stained using InstantBlue® Comassie Protein Stain according to the manufacturer’s instructions to visualise protein bands. SDS-PAGE gels were imaged using a Bio-Rad Gel DOC XR+ system equipped with a white light filter and in Comassie mode. Figure 8.1 shows an example of the purification of each ST crosslinker and SC array on SDS-PAGE gels. The lower yield of the ST crosslinkers is reflected in the SDS-PAGE gels where the respective bands are smaller. Moreover, the lower yield of the SasG crosslinkers is consistent with previous results from current and past Regan Lab members [190, 191]. Of note, the molecular weight of SasG is 30 kDa, but it always runs high on SDS-PAGE gels, closer to 37 kDa. This phenomenon is also consistent with previous results obtained in the Regan Lab [190, 191].
Figure 8.1: Example of the purification of each ST crosslinker (top) and SC array (bottom) on 7.5% SDS-page gels. A) Purification of ST-SasG-ST, 30 kDa; B) Purification of ST-SasGlong-ST, 86 kDa; C) Purification of SC3, 41 kDa; D) Purification of SC4, 54 kDa. T = 0: sample before IPTG protein induction, T = 20: sample after 20 hours of IPTG induction, lysate: whole cell lysate, FT: *E. coli* proteins flowing through after incubation with Ni-NTA, IW1: imidazole wash 1, 5 mM for ST crosslinkers and 20 mM for SC arrays, IW2: imidazole wash 2, 5 mM for ST crosslinkers and 20 mM for SC arrays, ELU1: elution of protein of interest 1, 200 mM imidazole, ELU2: elution of protein of interest 2, 200 mM imidazole, dialysis: protein of interest after dialysis against distilled water.
8.1.8 Hydrogel preparation

Freeze dried protein powder was resuspended in ultrapure water at the desired mM concentration. SpyTag components were mixed together manually with SpyCatchers components at the predetermined ratio in an Eppendorf tube to initiate spontaneous gelation at room temperature. Hydrogels were stable at room temperature until use. When needed, 1 mL of 8 M urea was added to 60 µL of hydrogel an incubated overnight. The hydrogels were washed with ultrapure water prior to further experiments.

8.2 Microrheology methods

The methods listed in section 8.2 were adapted from Larsen and Furst [207] and developed in collaboration with Dr Davide Michieletto.

8.2.1 Sample preparation

To determine the speed of gelation of the hydrogel system, polystyrene microparticles with diameter 1 ± 0.03 µm (Polysciences, Warrington, PA) were used as tracer particles. Prior to use, microparticles were washed in ultrapure water and then diluted to give a ‘bead slurry’ of approximately 0.25% solids (w/v) aqueous suspension. Masked glass slides were used as sample chambers. Masking was done manually by cutting a rectangular shape (1x1 cm) in double sticky tape and positioning it onto the glass slide. Masking using double sticky tape allowed to create a chamber on top of the glass slide where the sample was not trapped between glass and coverslip. 5 µL of SC protein at the predetermined mM concentration were mixed together with 0.5 µL of the tracer particles slurry and the mixture was pipetted in the centre of the chamber on the glass slide. The mixture was allowed to equilibrate for 1 minute to avoid excessive drift. Drift was monitored under microscope. 5 µL of ST protein at the predetermined mM concentration were pipetted onto the glass slide and manually mixed together with the SC protein containing the tracer microparticles to initiate spontaneous gelation. Immediately after, the chamber was delicately sealed using a coverslip.
8.2.2 Multiple particle tracking microrheology

Data collection started as soon as a suitable ROI (Region of Interest) was identified (i.e. an area containing an appropriate amount of beads \( \approx 50 \), far from the sample’s edges, and far from both coverslip and glass slide). A ROI was identified in less than 2 minutes after ST and SC were mixed together. It has to be noted that due to the spontaneous and unregulated gelation of ST and SC, gelation started when ST and SC were mixed \( (t = 0_{\text{TRUE}}) \) rather than when recording was started \( (t = 0) \). This discrepancy \( (t = 0_{\text{TRUE}} \text{ vs } t = 0) \) was minimised as much as possible but it is acknowledged as a limitation of the method used. The embedded tracer particles were imaged in brightfield at a magnification of 60x using a Nikon Eclipse Ti inverted microscope (numerical aperture 0.7, 60 x air immersion Extra Long Working Distance objective, Nikon, Japan). The Extra Long Working Distance objective allowed for imaging \textit{in vitro} through the coverslip without introduction of aberrations and subsequent deterioration of the image quality. The movement of roughly 50 in-frame particles was recorded for a total of 6000 frames per minute \( (100 \text{ frames per second, fps}) \) using a CMOS high speed camera (ORCA – Flash 4.0 V3, Hamamatsu) and the Micromanager V1.4.19 software [291]. Static errors in determining particle centroid due to intrinsic variations in the experimental set up were minimised by spreading the tracer beads over a sufficient number of pixels to represent the particle’s brightness distribution reasonably [98]. This was achieved by keeping the illumination levels near the maximum allowed by detector saturation. Dynamic errors due to mismatches between the movement of the particles and image acquisition speed were minimised by selecting a short exposure time \( (\sigma = 1000 \mu s) \) [218].

The samples were imaged in real time for a minimum of 45 minutes and a maximum of 110 minutes. Particle tracking was carried out using a Phyton script based on the weighted centroid method developed by Crocker and Grier [205] and the ensemble-averaged mean square displacement \( < \Delta r^2(\tau) > \) was calculated. Particle tracking data analysis was carried out using a customised Python script adapted and optimised from the script originally written by Dr Davide Michieletto.
8.3 Rheology methods

8.3.1 Sample preparation

Hydrogels were prepared as explained in section 8.1.8 the day before rheological measurements were carried out to ensure complete gelation of the system regardless of the specific speed of gelation determined during microrheological characterisation.

8.3.2 Dynamic shear rheology

Rheological measurements were carried out using a stress-controlled TA Instruments Discovery Hybrid Rheometer (DHR-2, TA Instruments, New Castle, DE) equipped with a standard steel parallel plate geometry (diameter 8 mm). A 0.15 mm gap was selected to suit both gelled and non-gelling solutions at very high angular velocities/shear rates. Hydrogels (≈60 µL) were delicately taken out of an Eppendorf tube and placed on a sandblasted bottom plate positioned on top of a Peltier plate maintained at 25 ºC. The top plate was lowered at slow speed to reduce any orientation or stress effects. The samples were allowed to rest for 5 minutes after lowering of the top plate. The 5 minutes conditioning ensured consistency with the conditions of operation throughout. Sample dehydration was not observed in the timeframe of the rheological measurements (approximately 40 minutes). The linear viscoelastic region (LVR) was determined using a strain sweep with strain amplitude increasing from 0.1 to 100 % and constant frequency \( \omega \) 100 rad/s. The rheological properties of the hydrogels were monitored using a frequency sweep. The frequency sweeps were carried out within the established LVR at 1 % strain and decreasing oscillatory frequency \( \omega \) from 100 to 0.1 rad/s. The storage (\( G' \)) and loss moduli (\( G'' \)) were determined as a function of \( \omega \) at 25 ºC. Three independent measurements were recorded and the mean is reported. The data was recorded using the TA Instruments TRIOS software (TA Instruments, New Castle, DE).
8.4 Mammalian cell culture methods

Biocompatibility experiments were carried out using the cell line HEK293, used as standard in the Rosser Lab, and a hepatocyte cell line, the HepG2 derived cell line HepG2-CYC1–GFP, in which a GFP reporter gene has been knocked-in into the cytochrome c1 (CYC1) gene, allowing monitoring of the cells by fluorescence. Human HepG2-CYC1–GFP were a gift from Prof. van de Water of LACDR, Leiden University, Netherlands. Prior to all experiments, the ST and SC component were sterilised under UV light for 30 minutes in a tissue culture hood.

8.4.1 Mammalian cell maintenance

The cells were maintained at 37°C with 5 % CO2 in DMEM (Gibco) supplemented with 10 % (vol/vol) FBS (Gibco), 1 % L-glutamine, and 1 % penicillin/streptomycin. Cells were passaged every 3 days with 0.5 mL of trypsin-EDTA (0.25 %) solution (Gibco) and plated onto cell culture-treated T25 flasks.

8.4.2 Mammalian cell imaging

To reduce background fluorescence, the cells were imaged in DMEM (Gibco) - no phenol red supplemented with 10 % (vol/vol) FBS (Gibco), 1 % L-glutamine, and 1 % penicillin/streptomycin.

8.4.3 Cell biocompatibility

Human HepG2-CYC1–GFP or HEK293 were seeded in a 96 well plate without poly-D lysine coating (Greiner Bio-one, Germany) at a density of $0.01 \times 10^6$ per well. After 24 hours, 15 µL of ST-SasG-ST at 4 mM were manually mixed together with 15 µL of SC3 or SC4 at 1 mM on a parafilm sheet. Spontaneous gelation begun upon mixing at room temperature. Once gelation was complete, the hydrogels were delicately put inside the well of a 96 well plate containing the HepG2-CYC1–GFP. The cells were incubated with the hydrogels for three days, monitored everyday, and imaged on day three. To determine cell viability, cells were stained with Trypan Blue solution (0.4%, Gibco) and imaged again. Imaging was carried out on a
Leica DMi8 microscope (Leica microsystems, UK) in brightfield and at 488 nm for GFP excitation at magnification 20x.

8.4.4 Cell attachment

20 µL of ST-SasG-ST at 4 mM were manually mixed together with 20 µL of SC3 at 1 mM or 20 µL of SC4 at 1 mM inside the wells of a 96 well plate without poly-D-lysine coating (Greiner Bio-one, Germany) and left to gel in situ. The entire bottom of the well was covered by an hydrogel. Human HepG2-CYC1–GFP were seeded on top of the hydrogels at a density of 0.01x10⁶ per well and incubated at 37°C with 5 % CO₂. After 24 hours, the medium was changed to remove unattached floating cells and the plate was further incubated for another 24 hours at 37°C with 5 % CO₂. After 48 hours, the wells were washed again, the cells were detached by gentle pipetting, stained with Trypan Blue solution (0.4%, Gibco), and counted using an automated cell counter (Countess II, Thermofisher Scientific). Three samples per well were taken with N of wells per condition = 3. Two tailed T tests were carried out using GraphPad Prism version 9.3.1 for Mac OS, GraphPad Software, San Diego, California USA.

8.4.4.1 Fluorescence microscopy imaging of cells

0.5 µL of ST-SasG-ST at 4 mM were manually mixed together with 0.5 µL of SC3 at 1 mM or 0.5 µL of SC4 at 1 mM to initiate spontaneous gelation. The hydrogels were manually spotted as dots of approximately 1 µL onto a Petri dish using a multichannel pipette and flooded with HepG2-CYC1–GFP (approximately 30,000 cells). The cell medium was removed and the hydrogels were washed three times with 1 mL of PBS to remove non attached cells. Fluorescence imaging was carried out on a Leica DMi8 microscope (Leica microsystems, UK) equipped with a 488 nm laser for GFP excitation at magnification 4x. Fluorescence intensity analysis was carried out in ImageJ. Two tailed T tests were carried out using GraphPad Prism version 9.3.1 for Mac OS, GraphPad Software, San Diego, California USA.

8.4.5 Cell encapsulation

Human HepG2-CYC1–GFP were seeded in a 96 well plate without poly-D lysine coating (Greiner Bio-one, Germany) at a density of 0.01x10⁶ per well. The
cells were manually mixed together with 20 µL of ST-SasG-ST at 4 mM and 20 µL of SC3 at 1 mM or SC4 at 1 mM to initiate spontaneous gelation and consequent encapsulation of the cells. The encapsulated cells were incubated at 37°C with 5 % CO₂ overnight.

8.4.5.1 Z-stacks of encapsulated cells

Cells incubated in 3D inside the hydrogels were imaged every day for up to 4 days to monitor viability and morphological changes. Fluorescence confocal imaging and Z-stacks were acquired on a Zeiss LSM880 microscope equipped with a 488 nm laser for GFP excitation at magnification 10x. Images were processed in ImageJ. To ensure the least amount of disturbance to the cells, during each imaging session, the imaging platform of the Zeiss LSM880 microscope was maintained at 37°C and, at the end of each imaging session, the plate was immediately put back into the incubator at 37°C with 5 % CO₂.

8.4.5.2 MTT assay

Cell viability was quantified via the commercially available MTT kit (SigmaAldrich, Germany) following the manufacturer’s instructions. 20 µL of ST-SasG-ST at 2 or 4 mM were manually mixed together with 20 µL of SC3 at 1 mM or 20 µL of SC4 at 1 mM inside the wells of a 96 well plate without poly-D-lysine coating (Greiner Bio-one, Germany) and left to gel in situ. Human HepG2-CYC1–GFP were seeded in 100 µL of cell culture medium on top of the hydrogels at a density of 5x10⁴ per well and incubated at 37°C with 5 % CO₂ for 24 hours. The next day, 10 µL of MTT labeling reagent were added to each well (final concentration 0.5 mg/ml) and incubated for four hours at 37°C with 5 % CO₂. After the incubation period, 100 µL of solubilisation solution were added to each well and incubated overnight at 37°C with 5 % CO₂. Absorbance levels were read at 570 nm using a POLARstar Omega plate reader (BMG Labtech, UK). The percentage of cell viability was calculated by subtracting the respective background from the individual samples and using the following equation: (background subtracted sample absorbance / background subtracted control absorbance)*100. Two tailed T tests were carried out using GraphPad Prism version 9.3.1 for Mac OS, GraphPad Software, San Diego, California USA.
Bibliography


226


[202] K. M. Schultz and E. M. Furst, “Microrheology of biomaterial hydrogela-


Appendix A

DNA and protein sequences

A.1 Plasmids used in this study

Each plasmid is detailed in the following pages, with both DNA coding sequence and protein amino acid sequence. All proteins are expressed via the pTrc promoter. Plasmids are used to express the following proteins:

- SpyTag-SasG-SpyTag
- SpyTag-SasGlong-SpyTag
- SpyCatcher – GSx3 array
- SpyCatcher – GSx4 array
- SpyCatcher - GGSx5 array
- SpyCatcher - GGSx3 array with RGD
Table A.1: Plasmids used in this study

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A.1.1 SpyTag-SasG-SpyTag

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Table A.2: SpyTag-SasG-SpyTag

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261
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A.1.2 SpyTag-SasGlong-SpyTag

SasGlong protein (GEG+3x(EG)) flanked by one SpyTag at the N and C termini. Cleavable N terminal His tag. Benchling: https://benchling.com/s/seq-S4r vBMTg4MwD94VIv7tx?m=slm-zfk0j2Ogdyo5kKHyvMHT

Table A.3: SpyTag-SasGlong-SpyTag

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A.1.3 SpyCatcher 3

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Benchling: https://benchling.com/s/seq-SqSqFQytCEdys80vaAYI?m=slm-0J7C0uJyMB3WBRx5ZwUq

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A.1.4 SpyCatcher 4

Four SpyCatcher units linked by GS flexible linker. Cleavable N terminal His tag.

Benchling: https://benchling.com/s/seq-R6ZITqWrQ8LEwOXN7iss?m=slm-18jeUcdgKHXQBq1rpS

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A.1.5 SpyCatcher 5

Five SpyCatcher units linked by GGS flexible linker. C terminal His tag.
Benchling: https://benchling.com/s/seq-jVsRAJ2cPMhigCYQVfHM?m=slm-q4sR0CAB8oOniPHUQZ4k

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A.1.6 **SpyCatcher 3 RGD**

Three SpyCatcher units linked by GGS flexible linker. C terminal His tag and RGD.

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<th>SC3 RGD</th>
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<td>Resistance</td>
<td>Ampicillin</td>
<td>total size plasmid = 3476 bp</td>
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<td>Parent Vector</td>
<td>pFLinkC-XE</td>
<td>Seq plasmids:T7 + pBRforEco</td>
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