Smart Nanomaterials from Repeat Proteins and Amyloid Fibrils

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This dissertation is submitted for the degree of Doctor of Philosophy

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Declaration

This dissertation describes my own work undertaken in the Departments of Chemistry and Pharmacology, University of Cambridge, between October 2013 and April 2017 and includes nothing which is the outcome of work done in collaboration except as specified in the text.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution.

This dissertation does not exceed 60,000 words.

Alexander P.M. Guttenplan

February 2018
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Abstract

Protein-based materials are an important area of research for various reasons. Natural protein materials such as spider silk have mechanical properties which compare favourably to artificial or inorganic materials, and in addition are biodegradable and can be produced from easily available feedstocks. It is also possible to produce materials that incorporate the functionality of a natural protein, such as ligand-binding or catalysis of reactions, thus allowing this functionality to be used in the solid rather than solution phase. Two particularly interesting components for protein-based materials are amyloid fibrils and tandem repeat proteins. Amyloid fibrils are exceptionally strong, tough, highly-ordered structures that self-assemble from a wide range of simple building blocks. Meanwhile, tandem repeat proteins are a class of proteins that act as scaffolds to mediate protein-protein interactions and are known to act as elastic springs. Unlike globular proteins, tandem repeat proteins can be designed to bind specific ligands, and their ligand-binding properties and stability can be tuned separately. This work details the synthesis and characterisation of repeat protein and amyloid fibril components for a smart hydrogel, the production of these gels, and their characterisation using a microfluidic method that I developed. Although amyloid fibrils have previously been decorated with functional proteins, hitherto, this has usually been done by assembling the fibrils from already-functionalised components. This approach limits the functionality to species that can survive the harsh conditions of amyloid aggregation and do not disturb fibril assembly. Therefore, a method was developed to produce amyloid fibrils that displayed an alkyne functionality on their surface to allow functional proteins or other species to be attached after assembly. This involved the design and synthesis (using solid-phase peptide chemistry) of a peptide based on the previously known TTR105-115 peptide (derived from the amyloidogenic Transthyretin protein). These fibrils were characterised by AFM and TEM and it was then shown that the assembled fibrils could be functionalised using an azide-alkyne click reaction. The reaction was shown to work with a variety of ligands including proteins, which were found to retain their structure and function after crosslinking to the fibril. The fibrils with ligands attached were characterised by a variety of methods including LCMS (liquid chromatography-mass spectrometry) and super-resolution optical microscopy. Next, repeat proteins were produced recombinantly containing non-natural azido amino acids at their termini. Incorporation of non-natural amino acids was carried out using a number of different methods including amber codon suppression and methionine replacement. Micron-sized hydrogels were then formed from microfluidic-generated droplets by covalently crosslinking the alkyne-functionalised fibrils with the azide-functionalised repeat proteins. The initial experiments to show proof of principle were carried out with consensus-designed repeat proteins, but repeat proteins based on natural sequences were also used to make hydrogels that could later be tested for potential uptake of peptides known to bind these proteins. These hydrogels could potentially be used for drug delivery or other applications in which a chemical response to a mechanical stimulus is desired. The mechanical properties of the hydrogels were measured using novel microfluidic devices, which were designed and fabricated using standard PDMS-based soft lithography techniques. These are based on existing glass-capillary-based micromechanical measurement techniques. However, the new devices significantly improve on them in terms of usability and reproducibility.
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List of Abbreviations

2TY  2x Yeast Tryptone
AFM  Atomic Force Microscope
ATP  Adenosine Triphosphate
BCA  Bicinchoninic Acid
Boc  tert-butyloxycarbonyl
bp   base pairs
COC  cyclic olefin copolymer
cp   centipoise
C-terminus  Carboxyl terminus
CTPR  Consensus Tetratricopeptide Repeat
DARPin Designed Ankyrin Repeat Protein
DBCO  dibenzocyclooctyne
DCM  Dichloromethane
DIEA  N,N-diisopropylethylamine
DMF  N,N-dimethylformamide
DMSO  Dimethyl sulfoxide
DNA  Deoxyribonucleic acid
DTT  dithiothreitol
E. coli  Escherichia coli
EDTA  Ethylenediaminetetraacetic acid
eGFP  Enhanced Green Fluorescent Protein
FAM-5  Fluorescein Amidite, 5-isomer
FEP  fluorinated ethylene propylene
FITC  fluorescein isothiocyanate
**Fmoc** Fluorenylmethoxycarbonyl

**Fmoc-OSu** Fmoc N-hydroxysuccinimide ester

**GST** Glutathione S-Transferase

**HBTU** 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyliuronium hexafluorophosphate

**HEPES** 4-(2-hydroxyethyl)-1-piperazineneethanesulfonic acid

**HPLC** High-Performance Liquid Chromatography

**IMAC** Immobilised Metal Affinity Chromatography

**IPTG** isopropyl $\beta$-D-thiogalactoside

**LCMS** Liquid Chromatography Mass Spectrometry

**M** Molar

$m/z$ Mass-to-charge ratio

**MOPS** 3-(N-morpholino)propanesulfonic acid

**MWCO** Molecular Weight Cut-Off

**N** Normality

**Ni$^{2+}$-NTA** Nickel-Nitrilotriacetic Acid

**N-terminus** Amino terminus

**OD** Optical Density

**PAGE** Polyacrylamide gel electrophoresis

**PBS** Phosphate Buffered Saline

**PCR** Polymerase Chain Reaction

**PDB** Protein Data Bank

**PDMS** Polydimethylsiloxane

**PFO** Perfluoroocctanol

**PGMEA** 1-methoxy-2-propyl acetate

**PMMA** polymethylmethacrylate

**PNK** Polynucleotide Kinase

**Q-TOF** Quadrupole Time-of-Flight

**RNA** Ribonucleic acid

**rpm** revolutions per minute

**SDS** Sodium dodecyl sulfate
**SOC**  Super Optimised broth with Catabolite repression

**TAE**  Tris-acetate-EDTA

**TCEP**  tris(2-carboxyethyl)phosphine

**TFA**  Trifluoroacetic acid

**THPTA**  Tris(3-hydroxypropyltriazolylmethyl)amine

**TIRF**  Total Internal Reflection Fluorescence

**TLC**  Thin-layer chromatography

**TNKS2ARC4**  Tankyrase 2 Ankyrin Repeat Complex 4

**TPR**  Tetratricopeptide Repeat

**Tris**  2-amino-2-(hydroxymethyl)-1,3-propanediol

**TTR**  Transthyretin
Chapter 1

Introduction

1.1 Protein Nanotechnology

Proteins are biological macromolecules consisting of chains of amino acids linked by peptide bonds (Sanger, 1952) that show a very large variety of structure and chemical functionality and have evolved for a wide range of functions. They are synthesised biologically from simple amino acid building blocks, and self-assemble into extremely complex structures. The structure of a protein is determined entirely by its sequence (Anfinsen, 1973), and proteins with designed sequences can be produced using standard molecular-biology techniques. Engineered protein-based materials combine the biological functionality of natural proteins with the design flexibility of synthetic polymers. Unlike synthetic polymers, proteins are monodisperse, and recombinant expression in bacteria yields less variability from batch to batch than harvesting from natural sources (DiMarco and Heilshorn, 2012). The synthesis of proteins also does not produce toxic byproducts (Heddle, 2008). These properties make designed proteins a promising area of nanotechnology research.

Although nucleic acids were the first biological molecules to be used for nanotechnology, as pioneered by Seeman (1982, 2010), there are still various technical issues with the field of nucleic-acid nanotechnology such as the high
cost of DNA and the propensity for errors in self-assembly (Pinheiro et al., 2011). As noted by Gradišar and Jerala (2014), the building blocks of self-assembled protein nanostructures do not need to be chemically synthesised which dramatically reduces the cost of these materials compared to DNA nanostructures, and the importance of multiple interactions rather than the single nucleic-acid base-pairing reduces the propensity for errors in self-assembly.

Protein-based materials show potentially useful mechanical properties. These have inspired scientists to attempt to replicate these properties in materials produced in vitro, such as the collagen-mimetic peptides developed by O’Leary et al. (2011) or the wide range of research into elastin-like peptides (reviewed by Almine et al. (2010)). Another example is the well-known strength of spider silk, a protein material that exhibits both a very high elastic modulus and a high degree of toughness, in addition to a non-linear response to force (Cranford et al., 2012). These properties come from the nanoscale structure of the material, which combines highly-ordered, tough crystalline domains with disordered regions that exhibit elasticity. (Vollrath and Porter, 2006).

In addition to their mechanical properties, proteins in biological systems self-assemble into a wide variety of shapes such as rods, sheets and capsules. This ability to self-assemble can be exploited to rationally design assemblies of proteins for nanotechnological applications (Howorka, 2011). Examples include the highly symmetrical multimers developed by King et al. (2014) and Gradišar et al. (2013) or the cages developed by Fletcher et al. (2013). These larger structures could allow for the possibility of functional nanoscale machinery made of several individual protein molecules, replicating the machinery in cells such as the bacterial flagellar motor (Sowa and Berry, 2008). For instance, Furuta et al. (2016) have developed molecular motors based on the motor protein dynein. The wide range of functions that proteins carry out also means that they can potentially be used for protein-based devices that duplicate the functions of inorganic electronic components, such as the holographic memory devices based on bacteriorhodopsin (Wagner et al., 2013).
Protein-based hydrogels are one interesting class of protein-based materials (Schultz et al., 2009). These materials exhibit tunable mechanical properties (Fang and Li, 2012; Kim et al., 2013) and can be designed for a range of functions which exploit the properties of the protein components, including as cell-culture scaffolds (Petkau-Milroy and Brunsveld, 2013) and for drug delivery (Banta et al., 2010). A protein-hydrogel “smart” drug-delivery system could be used to deliver a drug in response to an internal or external stimulus (Alvarez-Lorenzo and Concheiro, 2014), using the intrinsic capability of its components to bind ligands and respond to stimuli with conformational changes. In principle, such materials also have the advantage that their components are biocompatible (Woolfson and Mahmoud, 2010).

1.2 Tandem Repeat Proteins

Proteins containing a repetitive amino acid sequence are extremely common in biological systems (Kajava, 2012). According to the census carried out by Marcotte et al. (1999), 14% of all known proteins contain repetitive sequences, as do a third of human proteins as repetitive sequences are more common in eukaryotes than in prokaryotes. One particular class of repeat proteins is the solenoid tandem repeats, consisting of units of between 5 and 42 amino acids which stack to form a stable superhelical structure (Kobe and Kajava, 2000; Kajava, 2011). These individual units do not independently fold, but when combined they will fold due to stacking interactions between them. A wide variety of solenoid tandem repeat proteins exists, including tetratricopeptide repeats, HEAT repeats and ankyrin repeats (Main et al., 2003a).

In biological systems, repeat proteins mainly act as scaffolds mediating protein-protein interactions. The inner surface of the superhelix has variable curvature and surface-exposed residues, and the binding surfaces of repeat proteins have evolved to bind a wide variety of ligands from short peptides to entire globular proteins (Kobe and Kajava, 2000). Some repeat proteins also bind non-
Figure 1.1: A variety of solenoid tandem repeat proteins bound to ligands (in grey)

Designed proteins:  
a) CTPR390 binding terminal peptide of HSP90 (PDB 3KD7),  
b) DARPin in complex with MAP kinase ERK2 (PDB 3ZU7),  
c) TAL effector dHax3 binding methylated dsDNA (PDB 4GJR).

Natural proteins:  
d) Gankyrin (Ankyrin repeat) in complex with S6 ATPase (PDB 2DVW),  
e) β-catenin (ARM repeat) in complex with E-cadherin (PDB 1I7X),  
f) PUF domain recognising cytosine (PDB 2YJY).

Images rendered in PyMOL (Schrodinger LLC, 2015)
protein ligands, such as DNA in the case of TAL effectors (Boch et al., 2009) or even ice in the case of ice-binding and ice-nucleation proteins (Davies, 2014). A variety of repeat proteins bound to peptide, protein and nucleic acid ligands is shown in Figure 1.1.

The architecture of solenoid repeat proteins is much simpler than that of globular proteins, as can be seen from a comparison of their respective contact maps (Figure 1.2). In the native state of a globular protein, residues can be in contact with other residues from anywhere in the sequence, but in a repeat protein they only contact residues that are in the same repeat unit or adjacent repeat units and therefore close to them in the sequence (Main et al., 2003a, 2005).

![Figure 1.2: Comparison of the contact maps of a repeat protein (left) and a globular protein (right), adapted from Main et al. (2003a)](image)

This lack of contact with residues in other repeat units distant in the sequence causes solenoid repeat proteins to behave as elastic springs on stretching, as measured by Lee et al. (2006), as repeat units unfold sequentially (Serquera et al., 2010; Lee et al., 2010) in a manner affected by ligand binding (Settanni et al., 2013).

The structure of tandem repeat proteins makes them more amenable to rational design than globular proteins are. Although in principle it should be
possible to produce a protein with arbitrary structure by designing the appropriate amino acid sequence, in practice simulations are not advanced enough to solve the general problem of designed protein folds (Huang et al., 2016). However, the relatively simple architecture and topology of repeat proteins simplifies the problem. Alignments of repeat protein sequences have been used to create consensus-designed sequences Mosavi et al. (2002) that give extremely thermodynamically stable structures (Kohl et al. (2003); Main et al. (2003b), reviewed in Javadi and Itzhaki (2013)), and more recently the Baker lab (Brunette et al., 2015) have designed new classes of repeat proteins that are not related to any existing protein.

In addition, the residues on the binding surface of a repeat protein are not involved in the inter-repeat interactions that give it structural stability. Therefore, they can be tuned to bind a specific ligand without affecting the protein’s stability— and, conversely, the protein’s stability can be tuned (Kohl et al., 2003; Main et al., 2003b) without affecting its ligand-binding ability. Combined with their modular nature which allows arbitrary numbers of different repeats of the same class to be assembled in any order, designed repeat proteins form a large space of rationally designed proteins.

1.2.1 Ankyrin Repeat Proteins

The 33-residue ankyrin repeat motif, consisting of a beta-turn followed by two antiparallel alpha-helices, was first discovered in yeast cell cycle regulatory proteins and Drosophila signalling proteins (Breeden and Nasmyth, 1987) and was later named after the human protein ankyrin, which contains 24 such repeats, by Lux et al. (1990). The ankyrin repeat is one of the most commonly occurring tandem repeat motifs (Mosavi et al., 2002) appears in more than 2,000 natural proteins across all three domains of life (Jernigan and Bordenstein, 2014) all of which are involved in protein-protein interactions (Li et al., 2006).

Designed ankyrin repeat proteins, or DARPin, consist of a variable number of consensus-designed ankyrin repeat units (Wetzel et al., 2008) flanked by N-
and C-terminal capping repeats as shown in Figure 1.3. The latter differ in sequence from the internal repeats, having hydrophilic residues on their external surfaces while the internal repeats have hydrophobic repeats on both sides. The internal repeats have seven variable amino acids which are part of the protein’s binding surface and therefore can be used to tune its ligand-binding properties (Binz et al., 2003). Full-consensus DARPinS are extremely thermally stable—those of sufficient length are impossible to fully unfold even with the combination of heating and high concentrations of chemical denaturants (Wetzel et al., 2008)—but their stability can be tuned by replacing some of the consensus residues. DARPinS are expressible in very good yield and are devoid of cysteines, which is beneficial both in terms of ease of expression and purification and when considering cysteine-based bioconjugation methods. They also contain only one internal methionine residue, in the N-terminal capping repeat, which can be replaced by a leucine as exists in the analogous position in the internal repeats with little or no effect on stability. This property was exploited by Simon et al. (2012) to conjugate them to other molecules using both cysteine and unnatural amino acids incorporated in the place of methionine.
Some ankyrin repeat proteins or domains found in nature also have some of these desirable properties. For instance, TNKS2ARC4, one of the ankyrin repeat domains of the human disease-associated protein tankyrase, does contain four cysteines but does not contain any methionines (Guettler et al., 2011).

1.2.2 Tetratricopeptide Repeat Proteins

The tetratricopeptide repeat, or TPR, is a 34-residue helix-turn-helix motif (Sikorski et al., 1990). As with ankyrin repeat proteins, a consensus sequence (giving a protein with structure as shown in Figure 1.4) has been identified, in this case by Main et al. (2003b), which again is devoid of cysteines and methionines and has the added advantage, compared to DARPins, of containing a highly conserved tryptophan which makes measurement of the protein’s concentration by absorption of 280nm UV light (Edelhoch, 1967) significantly easier.

![Figure 1.4: Structure of a full-consensus TPR, rendered in PyMOL (Schrodinger LLC, 2015) from PDB file 2FO7 (Kajander et al., 2007)](image)

Due to the modular nature of CTPRs, there is much interest in using them for materials. Grove et al. (2012) developed peptide-binding smart gels based on the evolved peptide binding ability of natural TPR domains (Brinker
et al., 2002), while other ordered CTPR-based nanostructures include the fibrinous structures developed by Phillips et al. (2012) and the films developed by Grove et al. (2013). Speltz et al. (2015) have also shown that sets of TPRs can be designed with orthogonal peptide binding ability, making them potential building blocks for more complex assemblies of proteins.

1.3 Amyloid Fibrils

Amyloid fibrils are highly ordered and extremely stable self-assembled protein structures consisting of long, rigid filaments. They can be formed by the aggregation of any of a wide variety of proteins and peptides (reviewed in Knowles et al. (2014)). The misfolding of proteins to form these fibrils has been implicated in diseases such as Alzheimer’s and Parkinson’s, though the toxic agents are probably intermediates in fibril formation rather than the fibrils themselves (Bucciantini et al., 2002). There has been cases where, rather than representing a disease state, amyloid fibrils have been shown to have biological function (Chapman et al., 2002), including in humans where they are involved in melanin synthesis (McGlinchey et al., 2011).

Amyloid fibrils share a common “cross-beta” core structure consisting of parallel beta sheets (Sunde et al., 1997). Due to this structure, they are some of the mechanically strongest biological systems known (Knowles et al., 2010; Ruggeri et al., 2015) with an elastic modulus similar to that of steel or dragline spider silk. In addition, the fibrils are extremely rigid, with persistence lengths on the micron scale, equivalent to hundreds of thousands of individual monomer units (Knowles et al., 2007).

1.3.1 Amyloid Fibrils in Nanotechnology

The properties of amyloid fibrils, and the fact they self-assemble, have made them attractive for use in nanotechnology, as reviewed by Hauser et al. (2014). For instance, Knowles et al. (2010) developed self-organised films of amyloid fib-
rils which were free-standing structures which could be used to organise smaller molecules and had Young moduli similar to those of their fibril constituents. In comparison, similar films made from other filament-like nanostructures such as carbon nanotubes have Young moduli significantly less than those of their components. Byrne et al. (2011) also used amyloid fibrils as a the fibre component of a fibre-reinforced composite material.

Beyond their mechanical properties, amyloid fibrils have been studied for use in carbon capture (Li et al., 2014), as drug carriers (Shimanovich et al., 2015), or as underwater adhesives (Zhong et al., 2014). They also have the ability to display functional proteins (Baxa et al., 2002), which has been used by Forman et al. (2012, 2013) for research into protein-based electronics in combination with biological electron-transfer mechanisms. Amyloid-forming peptides can also be designed de novo (Makin et al., 2005), and fibrils formed from such peptides have been used to template silica nanowires (Al-Garawi et al., 2015) which are both chemically and thermally stable (Al-Garawi et al., 2016). Other de novo-designed peptides have been shown to form aggregates with biological activity, templating the amyloid aggregation of a normally non-amyloidogenic protein (Gallardo et al., 2016).

1.3.2 Fibrils Based on the TTR105-115 Peptide

Transthyretin (TTR), a protein found in human blood, can misfold and form amyloid fibrils which are implicated in diseases including familial amyloid polyneuropathy and familial amyloid cardiomyopathy (Johnson et al., 2012). The eleven-residue fragment consisting of residues 105-115 from TTR, with the sequence YTIAALLSPYS, was discovered by Gustavsson et al. (1991) to also form fibrils. Due to the ease of synthesis of this short peptide, TTR105-115 has become a popular model system for the study of amyloid. The structure of TTR105-115 fibrils has been determined to atomic resolution by Fitzpatrick et al. (2013)—Figure 1.5. Fitzpatrick et al. (2015) also determined the mechanical properties of these fibrils. In addition, TTR105-115 and related peptides
have been used as a model system for amyloid-based nanotechnology since the work of MacPhee and Dobson (2000). TTR105-115 fibrils have also been shown to display functionality, such as cell adhesion sequences, added at the C-terminal end of the peptide on the exterior of the fibril (Gras et al., 2008; Bongiovanni et al., 2012; Bongiovanni and Gras, 2015).

Figure 1.5: Structure of a TTR105-115 fibril, from Fitzpatrick et al. (2015)

1.4 Bioconjugation and Bioorthogonal Chemistry

Proteins found in nature include a wide range of chemical functionality beyond that available from the twenty genetically-encoded amino acids. A number of chemical methods have been developed to add still more different groups to proteins, either by the incorporation of non-canonical amino acids during biosynthesis (Link et al., 2003; Johnson et al., 2010) or by chemical modification of an
existing protein molecule as reviewed by Boutureira and Bernardes (2015). This is done for various reasons, including to incorporate bioorthogonal reactivity. If a bioorthogonally reactive species— in other words, one which reacts with the desired partner but not with any other chemical species that can be found in a cell— is introduced, this can be used to allow chemical reactions within the cell to be investigated.

1.4.1 Incorporation of Non-canonical Amino Acids in Expressed Proteins

The first non-canonical amino acid to be incorporated into a protein expressed in vivo was selenomethionine, by Cowie and Cohen (1957). The greater atomic mass of selenium compared to sulfur later made this method popular for the expression of proteins for X-ray crystallography, as developed by Hendrickson et al. (1990). Therefore, systems for the incorporation of selenomethionine in place of methionine became commercially available. This can be accomplished either using an auxotrophic strain of E. coli such as the B834(DE3) strain developed by Wood (1966) and first used by Leahy et al. (1992) or by suppressing the biosynthesis of methionine in a non-auxotrophic strain as pioneered by Van Duyne et al. (1993). Either way, in the absence of methionine, cells incorporate selenomethionine in its place. As this affects the function of proteins, cells are cultured in conditions where methionine is available so that it is incorporated into endogenous proteins. Conditions are then changed to either remove methionine from the media (for auxotrophic cells) or suppress its biosynthesis (for other strains) before over-expression of the desired protein under the control of an inducible promoter. These systems have since been repurposed for the incorporation of other non-canonical amino acids which, like selenomethionine, are potential substrates for the E. coli methionyl-tRNA synthetase in the absence of methionine (Hest et al., 2000). In particular, Kiick et al. (2001) demonstrated the incorporation of azide functionality, and alkynes have also been incorporated by this method (Simon et al., 2012).
One main disadvantage of methionine replacement is that it is residue-specific rather than site-specific. All methionine residues in the sequence are replaced by the non-canonical amino acid, which limits the applications of this method to proteins that do not contain any methionine residues that cannot be replaced by mutation. In addition, as Kiück et al. (2001) and Hest et al. (2000) have shown, it is limited to certain amino acids, and according to Wang et al. (2008) a non-canonical amino acid in the N-terminal position can be removed by the same cellular processing machinery that removes the N-terminal methionine (Hirel et al., 1989). Lang et al. (2012); Sachdeva et al. (2014); Chin (2014) and co-workers have developed a method that overcomes these limitations, though it results in low yields of protein.

### 1.4.2 Bioorthogonal Reactions and Click Chemistry

As stated earlier in this section, a bioorthogonal reaction is one which can take place within a living system without interacting or interfering with it (Sletten and Bertozzi, 2011). These reactions are useful for linking molecules together within living cells or cell lysates, as pioneered by Saxon and Bertozzi (2000) with the Staudinger ligation reaction between an azide and a triarylphosphine. In order to be of use for bioorthogonal chemistry, a reaction must be able to take place in water at physiologically relevant temperature and pH. In addition, while not necessary (and not true of the Staudinger ligation) it is desirable that a reaction not produce any major by-products. Taken together, these are the criteria outlined by Kolb et al. (2001) for a ‘click’ chemical reaction.

Another ‘click’ reaction which has been widely used (Speers and Cravatt, 2004) for bioorthogonal ligation is the Huisgen 1,3-dipolar cycloaddition between an azide and an alkyne, catalysed by copper (I), which was developed by Rostovtsev et al. (2002). This reaction produces a stable triazole linkage with no by-products, goes to completion at 37°C in water, and has no potential for cross-reactivity with other biological molecules as neither alkynes nor azides exist in biological systems. For these reasons, it has become a standard method
for bioconjugation (Presolski et al., 2011).

The main problem with this reaction is the use of potentially-toxic copper salts as the catalyst. An alternative reaction was developed by Agard et al. (2004) that proceeds without a catalyst, replacing the alkyne with a cyclooctyne whose ring strain promotes the reaction. A wide range of cyclooctynes have been developed with modifications that increase the rate of the reaction (Beatty et al., 2010). The main disadvantages of this method are that cyclooctynes are time-consuming to produce (Dommerholt et al., 2010) and require genetic encoding with evolved orthogonal tRNA/ami

1.5 Microfluidics

Microfluidic techniques involve the flow of fluids through narrow channels with dimensions measured in microns. At these length scales, there are important differences in the behaviour of fluids compared with longer length scales (Purcell, 1977; Squires and Quake, 2005). The dimensionless Reynolds number, which describes the ratio of inertial to viscous effects (Atencia and Beebe, 2005), is extremely low at microfluidic length scales, meaning that inertial effects such as turbulence are almost entirely absent. For instance, flow is entirely laminar since the transition to turbulent flow occurs at Reynolds numbers of 2000–3000 (Squires and Quake, 2005) while the Reynolds number in micron-scale channels is often significantly less than 1. In addition, other dimensionless numbers such as the Péclet number describing the ratio between convection and diffusion and the capillary number describing the ratio between interfacial and viscous forces are important and have very different values in microscale compared to macroscale systems. As turbulent and convective mixing are absent in microfluidic systems, mixing takes place only by diffusion (Janasek et al., 2006). Both because of these differences in behaviour and because much smaller quantities of reagents are required, microfluidic techniques have become popular.
in the chemical and biological sciences (Whitesides, 2006). They have been used for a wide range of experiments, including protein characterisation by electrophoresis (Herling et al., 2013), cell culture for ‘organs-on-a-chip’ (Huh et al., 2010), and optimisation of organic synthesis reactions (Elvira et al., 2013).

The first reference to a device relying on the non-mixing of liquids at low Reynolds number is the electrophoresis device developed by Philpot (1940). Devices more similar to the modern concept of a microfluidic device were initially fabricated from silicon or glass using techniques developed for the semiconductor or microelectromechanical systems (MEMS) industry, as in the work of Manz et al. (1990) or Woolley and Mathies (1994). However, microfluidics became much more accessible, and therefore more popular, after the development of techniques for fabricating the devices from the flexible polymer PDMS (Polydimethylsiloxane) as described by Ng et al. (2002). Photolithography is used to create a reusable positive-relief ‘master’ from which PDMS channels can be cast, and the channels are then irreversibly bonded to a glass slide using plasma oxidation (Figure 1.6). These techniques allow devices to be designed and fabricated in less than 24 hours without the use of a clean room or chemical etching reagents such as hydrofluoric acid (Duffy et al., 1998). The masters are also reusable—large numbers of identical replicate devices can be cast from the same master.

1.5.1 Droplet Microfluidics

One popular application of microfluidics is the generation of large numbers of monodisperse droplets at a T-junction using devices based on the initial design of Thorsen et al. (2001); Anna et al. (2003). These droplets can act as miniature reaction vessels allowing the generation of very large numbers of discrete repeats of a reaction (Teh et al., 2008). As well as chemistry, microdroplets have also been used in recent years for high-throughput biological experiments (Kaminski et al., 2016). The small size of microdroplets has long been known as a method of studying single cells (see the studies of cells encapsulated in droplets by
Figure 1.6: Schematic of PDMS microfluidic device fabrication. A silicon wafer coated in photoresist (a) is exposed to UV light through a mask (b) in areas corresponding to the desired channels, crosslinking the photoresist in these areas. Uncrosslinked photoresist is washed away by a developer solution, leaving a positive-relief master (c). This master is used to cast PDMS (d) which is then removed from the master and bonded to a glass slide to give the finished device (e).
Lederberg (1954); Nossal and Lederberg (1958)) or single enzyme molecules (Rotman, 1961). More recently, Knowles et al. (2011) have used microdroplets to study single nucleation events leading to protein aggregation.

Reactions in microdroplets have several advantages beyond the ability to study single molecules or events. In addition to the obvious economic and environmental benefits of using very small (nanolitre to picolitre) volumes of precious reagents, and the potential for massively parallel experiments such as the droplet PCR sequencing pioneered by Tewhey et al. (2009), the screening of enzymes by Romero et al. (2015) or the directed-evolution experiments of Gielen et al. (2016), a range of reactions have been shown to be accelerated under microdroplet conditions Yan et al. (2016). For example, Fallah-Araghi et al. (2014) demonstrated an increase in the rate of synthesis of an imine by the condensation of an aldehyde and an amine when the reaction was carried out in microdroplets due to the presence of the oil-water interface.

1.5.2 Microgels

The monodispersity of microfluidically generated droplets means that they can be converted into equally monodisperse gels or other mesoscale particles. The use of microfluidics to form hydrogels gives both reproducibility and a high degree of control over the size and composition and hence properties of the particle (Heida et al., 2016). These microgels have been made using a wide variety of constituents including photopolymerisable monomers (Nie et al., 2006), alginate polysaccharides (Huang et al., 2006) and amyloid-forming peptides (Shimanovich et al., 2015), for a range of applications including drug delivery (Lu et al., 2016), cell culture (Jiang et al., 2014) and protein detection using DNA aptamers (Srinivas et al., 2011). Microgels have also been used as reaction vessels for the directed evolution of enzymes, allowing successful plasmids to be recovered by automated sorting (Fischlechner et al., 2014).

For many of these applications, and for many of the various applications of microgels produced by other methods such as emulsification, the mechanical
properties of the microgel are important. Therefore, various methods have been developed to quantify these. However, all of these methods present practical problems (Guo and Wyss, 2011). For instance, micropipette aspiration (Kwok and Evans, 1981) or AFM using a tipless cantilever (Kumachev et al., 2013) require the difficult and time-consuming microscopic localisation of individual microgel particles.

In order to avoid this problem, Wyss et al. (2010) developed the method of capillary micromechanics which calculates the properties of the microgel based on its deformation under a known pressure as it is forced by hydrostatic pressure into a tapering capillary. The efficacy of this method was demonstrated by Voudouris et al. (2013), and the glass capillaries were replaced by PDMS in the work of Li et al. (2015), which also further expands on the physical principles involved. However, all capillary micromechanics methods to date use a single capillary, which can be blocked and causes problems if an insufficiently dilute suspension of microgels is being measured. Li et al. (2015) partially solve this problem with a valve introduced before the device.

1.6 Aims of Project

The main aim of this project is to synthesise the first in a new class of nanomaterials, in which repeat proteins are used to covalently link amyloid fibrils together. This material will take the form of a hydrogel, but with the ligand-binding ability of tandem repeat proteins. Although both repeat proteins (Grove et al., 2012) and amyloid fibrils (Shimanovich et al., 2015) have been used to form hydrogels, the combination of the two is interesting as it combines the very different mechanical properties of the two classes of protein- the elastic, spring-like behaviour of repeat proteins and the toughness and rigidity of amyloid fibrils. In addition, functional repeat proteins could be incorporated in a much more rigid material thanks to the incorporation of amyloid fibrils.

Initially, protein and fibril components will be synthesised containing func-
Figure 1.7: Schematic of the production and structure of the desired material. Synthetic alkyne-containing peptides self-assemble (a) to form amyloid fibrils displaying alkyne functionality on their surface. These alkynes react (b) in a bioorthogonal 'click' reaction with repeat proteins that have been expressed with azides at their N- and C-termini, to give a material that consists of a network of amyloid fibrils linked together by repeat proteins. When force is applied to this material (c) the repeat proteins unfold repeat by repeat (red stars) in response.
tional groups that allow them to be bioorthogonally linked together. This link needs to be a covalent bond due to the great strength of the β-sheet interactions in amyloid fibrils so that the link between repeat protein and fibril is not the ‘weak link’ in the overall structure. While bioorthogonal labelling of repeat proteins had previously been demonstrated by Simon et al. (2012), and therefore this portion of the project can be based initially on known methods, a new method will have to be developed to functionalise amyloid fibrils. This will be based on the work of Gras et al. (2008) in displaying desired functionalities on the surface of the fibrils.

Once the material is formed as microgels using droplet microfluidics, it will be characterised. This will involve developing new methods for mechanical testing based on the work of Wyss et al. (2010), as capillary micromechanics is the most suitable existing method but still presents practical difficulties. The mechanical response of the microgels to temperature changes will also be measured.

### 1.6.1 Motivation and applicability

The desired material will be a “smart” material capable of producing a chemical response to a mechanical stimulus. Repeat proteins can bind ligands on their inner convex surface (Forrer et al., 2003), and unfold in response to force (Lee et al., 2006). Such unfolding and loss of secondary structure obviously entails the loss of the ligand-binding surface. Therefore, when the repeat protein component of a material unfolds in response to force, the ligand-binding surface is lost and the ligand is released.

The amyloid fibril component of the material acts to link the repeat proteins together and provide a tough “framework” for the material. Films of amyloid fibrils are already known for their mechanical strength (Knowles et al., 2010), and therefore have been studied for their potential as scaffolds displaying other, functional proteins (Forman et al., 2012, 2013). However, in this case the amyloid fibril component acts not just as a scaffold but also as a “handle” to allow
force to be applied to the repeat protein component, bringing the known functions of repeat proteins from the solution phase to a bulk hydrogel phase.

Such a material has potential applications in fields such as drug delivery (Alvarez-Lorenzo and Concheiro, 2014) for instance in a joint implant which releases a drug when the joint it is implanted in comes under strain, a force sensor which releases a reporter molecule when it comes under load, or a component of a self-healing material that releases a molecule required for repair.

Additionally, it is known that ligand binding by repeat proteins changes their mechanical properties (Settanni et al., 2013). A change to the mechanical properties of the repeat protein component of the material produced in this work should change the properties of the material as a whole. Therefore, such a material could also exhibit a mechanical response to a chemical stimulus as ligand binding changed its mechanical properties. This would allow the material to act as a chemical sensor, either via a change in shape or via constant measurement of its mechanical properties.
Chapter 2

Materials and Methods

2.1 Reagents

Unless otherwise stated, all reagents, enzymes and kits were obtained from commercial sources. C41 competent cells were obtained from the Itzhaki lab culture, Alpha select competent cells from Bioline (London, UK), and B834 (DE3) methionine-auxotrophic competent cells from Novagen (Millipore UK, Watford, UK). Recipes for general buffers and media are as follows. MilliQ standard purified water was used for making up buffers, and distilled water used for organic reactions.

Non-natural amino acids: Azidohomoalanine and α-propargylserine were initially synthesised but for later experiments were obtained from IRIS Biotechnology (Marktredwitz, Germany). Propargyllysine was obtained from IRIS Biotechnology.

Ampicillin stock: 50 mg/mL in water, sterile filtered
IPTG (isopropyl β-D-thiogalactoside) stock: 1M in water, sterile filtered
K-MOPS mix for M9 Minimal Media: Prepared as a 10x stock consisting of:

- 800 mL of a solution of 1.0 M MOPS and 0.1 M Tricine adjusted to pH 8.0 with potassium hydroxide
• 10 mL of 1 mM Fe(II)Cl₂
• 10 mL of 1 M CaCl₂
• 10 mL of 1 M MgSO₄
• 10 mL trace metals mix:
  - 20 mM H₃BO₃
  - 5 mM CoCl₂
  - 2 mM CuCl₂
  - 10 mM MnCl₂
  - 2 mM ZnCl₂
  - 2 mM Na₂MoO₄
• 125 mL of 4M NaCl

LB-Amp Agar plates: 1.5% (w/v) agar, 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl in water, autoclaved. 0.1% ampicillin stock was added to the solution after it cooled but before plates were cast.

M9 Minimal Media: Consists of 10% (v/v) 10x K-MOPS stock (see above), 20 mM NH₄Cl, 4 mM KPi pH 8.0, 0.4% (w/v) glucose, 0.1% (v/v) vitamin mix (see below), made up to volume with water.

Phosphate Buffered Saline: 8 g/l NaCl, 0.2 g/l KCl, 1.15g/l Na₂HPO₄, 0.2 g/l KH₂PO₄ in water, pH 7.3.

2TY (2x Yeast Tryptone) Media: Made up using capsules (MP Biomedical) or powder (Formedium) according to manufacturer’s instructions.

Vitamin Mix for M9 Minimal Media: 1000x stock consists of 1 g/l each of riboflavin, niacinamide, pyridoxal and thiamine, dissolved in water and sterile filtered.

Protease Inhibitor Tablets: Roche cOmplete EDTA-free or SigmaFAST, one tablet per 50 mL cell lysate (Sigma-Aldrich)
TAE (Tris-acetate-EDTA) 50x stock: 242 g Trizma Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol) base, 57.1 mL glacial acetic acid and 18.6g EDTA (Ethylene-diaminetetraacetic acid) were diluted to a final volume of 1L in water. 1x TAE was used for making up gels for DNA (Deoxyribonucleic acid) agarose gel electrophoresis and as a running buffer.

DNA Gels: Made up in 1x TAE with agarose (1% w/v) and GelRed (Biotium, 0.01% v/v) or SYBR Safe (Life Technologies, 0.01% v/v)

SDS-PAGE buffer: NuPAGE MES-SDS running buffer (Thermo Fisher) or Run-Blue Teo-Tricine running buffer (Expeideon), diluted with water according to manufacturer’s instructions.

Clark & Lubs Buffer: 50 mM in H$_3$BO$_4$ and KCl, pH adjusted to desired value with NaOH (Clark and Lubs, 1916)

2.2 Molecular Biology

2.2.1 Plasmids

Unless otherwise stated, all vectors confer ampicillin resistance. Sequences of constructs and primers are indicated in Appendix I.

DARPins

DARPins were designed based on the full consensus sequence (Wetzel et al., 2008) with an N-terminal hexahistidine tag and either 4 or 8 internal repeats for a total of 6 or 10 ankyrin repeats respectively. For clickable DARPins, the only changes to this sequence were the addition of a C-terminal methionine and the replacement of the internal methionine in the N-capping repeat with a leucine, giving a sequence which was devoid of internal methionines and had a methionine at each end.

In addition, DARPins, also with a total of 6 and 10 ankyrin repeats, were designed with cysteines in the 3rd and terminal positions for thiol-based cross-linking methods, though these were not expressed and no further work has been...
done on them. These sequences were codon optimised for better expression in *E. coli* and supplied as synthetic clones in the pQE30 expression vector by Life Technologies.

**CTPRs**

A plasmid containing a 6-repeat full-consensus TPR in the pRSETa vector was obtained from Elin Sivertsson. Conventional site-directed mutagenesis (see 2.2.2) was used to add a C-terminal methionine. Presumably due to internal recombination at some point in this process, two repeats were lost leaving a 4-repeat CTPR, but expression and testing continued with this sequence rather than continuing with molecular biology.

**TNKS2ARC4**

A plasmid containing Ankyrin Repeat Complex 4 of the human poly(ADP-ribose) polymerase Tankyrase 2 (TNKS2ARC4, Guettler *et al.* (2011)) in the pOWEN vector with a C-terminal His-tag was obtained from Rohan Eapen. It was necessary to add a C-terminal methionine and to convert the alanine in position 2 to an arginine. This mutation reduces cleavage of the N-terminal amino acid (Hirel *et al.*, 1989; Wang *et al.*, 2008). After both site-directed mutagenesis (2.2.2) and Round-the-Horn PCR (2.2.2) were unsuccessful, the construct was copied by PCR with primers designed to include the desired mutations (2.2.2) and cloned into the pQE30 vector.

**PR65**

A plasmid containing the PR65 subunit of protein phosphatase 2A was obtained from Marie Synakewicz. Due to this protein’s large number of internal methionines, methionine substitution could not be used to incorporate non-natural amino acids. Instead, propargyllysine was incorporated by amber codon suppression (Lang *et al.*, 2012; Sachdeva *et al.*, 2014; Chin, 2014). Amber stop codons at the desired locations were already in place when the plasmid was
received, so no further molecular biology was necessary. The plasmid contained
PR65 with a C-terminal His-tag and thrombin-cleavable N-terminal GST-tag
expressed under the control of a constitutive promoter, and an orthogonal ribo-
some expressed under the control of an IPTG-inducible promoter, as well as a
gene for kanamycin resistance.

2.2.2 Primer Design and PCR

Site-Directed Mutagenesis

Primers were designed using PrimerX (http://bioinformatics.org/primerx)
using the default parameters: melting point between 75 °C and 85 °C, GC
content between 40% and 60%, total primer length between 25 and 45 bp and
5' and 3' flanking regions between 11 and 21 bp.

Template DNA (50 µg/mL, 10 µL, 500 ng) was mixed with Phusion HF x5
Buffer (10 µL), forward and reverse primers (0.25 µL each, 100 µM), dNTPs (2
µL, 10 mM) and water for a total volume of 49 µL. Phusion polymerase (2,000
units/mL, 1 µL) was added last, and PCR was performed on a TC-512 thermal
cycler (Techne) using the following programme: Initial denaturation at 95 °C
for 30 s; 18 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30s
and extension at 72 °C for 4 min; followed by a final extension step of 10 min at
72 °C. The reaction mixture was then digested with DpnI at 37 °C for at least
one hour to remove residual template DNA, before transformation into α Select
Silver cells.

Round-the-Horn PCR

Round-the-Horn PCR is an alternative method for introducing changes to a
plasmid that some members of the group have found more reliable. Whereas
in conventional PCR the primers overlap to create a nicked circular product,
in RTH PCR they anneal back to back to give a linear product. Primers with
phosphorylated ends are used, so that the product can be circularised by liga-
Primers were designed manually using the SnapGene Viewer software (GSL Biotech, Chicago, USA) with a length of about 20 bp, and ending in either G or C. For phosphorylation, the primers (5 µL, 100 µM) were added to 10x PNK buffer (5 µL), ATP (1 µL, 100 mM) and water (38 µL) for a total of 49 µL. T4 PNK (1 µL) was added last and the mixture incubated at 37 °C for 45 minutes then heated to 65 °C for 20 minutes to inactivate the enzyme.

Phosphorylated primers (2.5 µL of each) were added to 5x Phusion HF buffer (10 µL), template DNA (0.5 µL, 54 ng), dNTPs (1 µL, 10 mM) and water (33 µL). Phusion polymerase (2,000 units/mL, 0.5 µL) was added last and PCR performed on a TC-512 thermal cycler (Techne) using the following cycle: Initial denaturation at 98°C for 2 minutes; 30 cycles of denaturation at 98 °C for 2 minutes, annealing at 65 °C for 30 seconds and extension at 72 °C for 7 minutes; followed by a final extension step of 10 minutes at 72 °C.

The reaction mixture was digested with DpnI at 37 °C for 3 hours, then purified by gel electrophoresis on a 1% Agarose-TAE-GelRed gel. The band was excised and purified using a GeneJet Gel Extraction Kit according to the manufacturers instructions. The purified linear plasmid DNA (1 µL, ca. 20 ng) was mixed with water (13 µL), 4x QuickStick Ligation buffer (5 µL) and QuickStick Ligase (1 µL) and incubated at room temperature for 15 minutes. The ligation product (5 µL) was transformed into αSelect Bronze cells as detailed in 2.2.3.

PCR and subcloning

As the repeat proteins being studied in this project have non-natural amino acids included at the N- and C-termini, it was often desirable to introduce mutations towards the ends of the gene. In this case, the gene could be copied by PCR with primers that contained the desired mutation(s). Primers were designed manually using SnapGene Viewer, and PCR carried out as in 2.2.2 with the exception that the extension time could be shorter (30s to 1 minute)
as only a relatively small section of DNA was being copied.

Meanwhile, the desired vector was digested with either HF (NEB) or Fast-Digest (Thermo) restriction enzymes in the appropriate reaction buffer for at least 3 hours at 37 °C and the digested vector purified by gel electrophoresis on a 1% Agarose-TAE-GelRed gel. The band was excised and purified using a GeneJet Gel Extraction Kit according to the manufacturers instructions. Uncut and single-cut vectors were also run on the gel as controls to ensure that the DNA had been cut at both restriction sites- if it had not, sequential rather than simultaneous digestion was used. The purified linearised vector was treated with 1 U alkaline phosphatase at 37°C for 30 minutes to dephosphorylate DNA ends and increase the efficiency of ligation, then heated to 65 °C for 5 minutes to inactivate the enzyme. The PCR product was digested and purified in a similar way but was not treated with alkaline phosphatase. As the PCR product was significantly smaller than the templated DNA, they were easily separated on an agarose gel so DpnI digestion to remove template DNA was not necessary.

The insert was then ligated into the vector using either Quick-Stick Ligase (Bioline) or Anza T4 DNA Ligase (Thermo Scientific) at room temperature for 15 or 5 minutes respectively. A 3:1 molar excess of insert to vector was used. The ligation reaction mixture was transformed into αSelect Gold cells as detailed in 2.2.3. A 'control ligation' was also carried out in which the insert was omitted- the ligation was considered to have been successful and colonies picked if the plate with the insert contained significantly more colonies than the control ligation plate.

**PCR Screening**

When a ligation produces a very large number of colonies many of which contain re-ligated vector rather than the desired plasmid, picking, growing and sequencing the DNA of colonies until the desired plasmid is found can be unfeasibly time- and resource-intensive. PCR colony screening is a method by which the presence of an insert can be determined more rapidly and cheaply.
A PCR master mix was prepared with primers that will amplify the desired insert—often the promoter and terminator sequences of the vector are used. 500 µL of this master mix contains 100 µL 5x PCR buffer, 12.5 µL dNTPs, 5 µL of each primer (at 50 µM) and 5 µL polymerase, made up to 500 µL with water.

The master mix is split into 20 µL aliquots, and individual colonies were picked, dipped in an aliquot of the master mix and streaked on an LB-Agar plate supplemented with the desired antibiotic. PCR was performed using the following cycle: Initial denaturation at 98 °C for 3 minutes; 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds and extension at 72 °C for 30 seconds; followed by a final extension step of 5 minutes at 72 °C. Note that there is no need to extract DNA from the cells, as the initial high-temperature denaturation step breaks them open and releases the plasmid into the reaction mixture where it can be amplified.

The reaction mixtures were then run on an agarose gel, together with control reactions on a plasmid containing an insert of similar size to the desired one (as a positive control) and on empty vector (as a negative control). If a band was visible on the gel at the molecular weight of the desired insert, a colony was picked from the corresponding region of the streak plate, grown and the plasmid DNA extracted and sequenced with appropriate primers.

2.2.3 Transformation and Extraction of DNA

Plasmid DNA (1 µL unless stated) was added to an appropriate grade of competent cells and left on ice for 30 minutes. The cells were then heat-shocked at 42 °C for 45 seconds and placed back on ice to recover for 2-5 minutes. Sterile 2xYT medium (400 µL) was added and the cells shaken at 37 °C for 60 minutes before being plated out onto LB-Amp agar plates and incubated at 37 °C overnight.

Colonies were picked from each plate and inoculated into 2xYT medium (5 mL) supplemented with ampicillin (50 µg/mL). These cultures were shaken at 37 °C overnight, harvested by centrifugation at 4000x g for 5 min, and DNA
extracted using a GeneJet Plasmid Miniprep Kit according to the manufacturers instructions. Concentration of the DNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific) and sequences were determined by Source BioScience or EuroFins MWG.

**Removal of His-Tag from DARPins**

Due to an oversight, the protein as encoded in the plasmid designed in 2.2.1 contained two His-tags in sequence, one from the vector and one from the sequence designed to be cloned into it. As this meant that the protein contained an additional methionine (which would be replaced by azidohomoalanine in the clickable protein) it was potentially problematic for use in the eventual nano-material. Therefore, site-directed mutagenesis was used to delete the second His-tag. Primers were designed with 26 bases around the region to be deleted, ending ideally in 2 or more GC bases.

**Repair of Ribosome Binding Site**

The construct with the second His-tag removed in the PCR above proved not to be able to express protein. On inspection of the sequence, it became apparent that a point mutation had been introduced in the ribosome binding site 10 bp upstream of the initial ATG codon (G mutated to A). Primers were designed to repair this mutation by site directed mutagenesis, again with a total length of 52 bp and beginning and ending in C or G.

Using the same protocol above, the DNA sequence could not be repaired—the PCR gave the template sequence. Different polymerases (Q5 Hi-Fidelity, Q5 HotStart, PfuTurbo) were tried, with reaction conditions optimised for the specific polymerase, but were similarly unsuccessful. This may have been due to the polymerase’s error correction mechanisms treating the desired single point mutation as an error. Therefore, Round-the-Horn PCR was used instead. This was also unsuccessful, so the gene from the originally-ordered plasmid was copied by PCR with the initial BamHI restriction site moved to after the first His-tag.
This PCR product was then cloned into the pQE30 vector.

2.3 Protein Expression and Purification

2.3.1 Standard Method for DARPin expression

The plasmid containing the DARPin construct was transformed into chemically competent *E. coli* B834 (DE3) cells (Novagen) using the heat shock method as above. SOC (Super Optimised broth with Catabolite repression) medium (250 µL) was added and the cells shaken at 37°C for 60 minutes before being plated onto LB-Amp plates and grown at 37 °C. Colonies were picked and grown overnight in 2xYT media (2x 100 mL) supplemented with ampicillin (100 µg/mL) and glucose (1% w/v). These starter cultures were shaken overnight at 37 °C before being added to 2xYT media (2x 1 L) supplemented with ampicillin (50 µg/mL) and glucose (1% w/v). Cultures were grown at 37 °C, 220 rpm until optical density (OD) at 600 nm reached 0.6, then induced with IPTG (1 mM) for 4 hours at 30 °C.

Cultures were pelleted by centrifugation (5000x g, 4 °C, 7 minutes) and re-suspended in ice-cold DARPin Lysis Buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 tablet of protease inhibitor cocktail per 50 mL solution) before being lysed on an Emulsiflex c5 homogeniser at 10,000 psi or by sonicating (probe sonicator, 6x 15 seconds). The lysate was centrifuged (35,000x g, 4 °C, 60 minutes) to pellet cell debris, and the supernatant bound onto 1 mL per litre cell culture of Ni²⁺-NTA (Nickel-Nitrilotriacetic Acid)-Agarose beads (QIAGen or Expreeze) at 4 °C for 60 minutes. The resin was washed 3 times with DARPin Lysis Buffer then the protein was eluted 3 times with PBS_E (PBS, 300 mM imidazole). The protein was purified by size-exclusion gel filtration chromatography using a HiLoad 26/60 Superdex 75 column (GE Healthcare, Amersham, UK) using PBS as the eluting solvent. Purity was checked on a NuPage 4-12% Bis-Tris gel or Expreeze RunBlue SDS 4-20% gel, and concentrations determined by a BCA assay using a Pierce BCA Protein Assay Kit (Thermo Fisher).
Fractions were flash-frozen as 500 µL aliquots and stored at -80 °C until further use.

2.3.2 Modified Protocol for Clickable DARPins

6x0.5L flasks of E.coli B834 (DE3) culture were grown as in 2.3.1. Before induction, the cells were pelleted by centrifugation (4000x g, 4 °C, 7 minutes) and washed 3 times by resuspending them in PBS at 4 °C. The cells were then resuspended in 6x0.5 L of minimal medium consisting of SelenoMet Medium Base and SelenoMet Nutrient Mix (Molecular Dimensions, Newmarket, UK) prepared according to the manufacturer's instructions and supplemented with 50 µg/mL ampicillin and 40 µg/mL of either methionine (for control experiments) or azidohomoalanine (to give clickable proteins). The cultures were then shaken for 15 minutes at 30 °C to deplete intracellular methionine pools before being induced with 1 mM IPTG for 4 hours at the same temperature. The protein was purified as in 2.3.1. Incorporation of non-natural amino acids was checked by amino-acid analysis at the PNAC service (Department of Biochemistry, University of Cambridge)

2.3.3 Expression of Clickable TPR

This protein was expressed and purified as in 2.3.2, except that the quantity grown was 8 x 0.5L and that the buffer used to elute the protein from the beads was 50 mM Tris pH 7.5, 500 mM NaCl, 500 mM imidazole.

2.3.4 Expression of Clickable TNKS2ARC4

This protein was expressed in C41 competent cells using a method based on that of Van Duyne et al. (1993). The plasmid was transformed into the cells by heat shock as in 2.2.3 and the cells plated onto LB-Amp agar plates and grown at 37 °C overnight. A single colony was picked and grown for 8 hours at 37 °C in 2xTY media (5 mL) supplemented with ampicillin (50 µg/mL).
This culture was inoculated (1:1000 ratio) into M9 minimal media (100 mL) supplemented with ampicillin (50 µg/mL) and grown overnight at 37 °C. The culture was then inoculated (1:100 ratio) into M9 media (8x 0.5L) supplemented with ampicillin (50 µg/mL) and grown at 37 °C until the desired optical density for induction was reached.

To stop biosynthesis of methionine, 0.5 g/l of a solid mixture of lysine, threonine, phenylalanine (20 wt% each), leucine, isoleucine, valine and azido-homoalanine (10 wt% each) was added to the culture. The flasks were shaken for 15 minutes at 37 °C before induction with 1 mM IPTG for 4 hours at 30 °C.

Cultures were pelleted by centrifugation (5000x g, 4 °C, 7 minutes) and re-suspended in ice-cold His-Tankyrase Resuspension Buffer (50 mM Tris-HCl pH 8, 500 mM NaCl, 2 mM DTT, 10 mM imidazole, 1 tablet of protease inhibitor cocktail per 50 mL solution) before being lysed on an Emulsiflex c5 homogeniser at 10,000 psi. The lysate was centrifuged (35,000x g, 4 °C, 35 minutes) to pellet cell debris, and the supernatant bound onto 1 mL per litre cell culture of Ni²⁺-NTA-Agarose beads (QIAGen or Expedeon) at 4 °C for 60 minutes. The resin was washed 3 times with 50 mL His-Tankyrase Wash Buffer (as Resuspension Buffer without the protease inhibitor) and the protein eluted 3 times with 1 mL per litre cell culture of His-Tankyrase Elution Buffer (50 mM Tris-HCl pH 8, 500 mM NaCl, 500 mM imidazole, 2 mM DTT). The protein was purified by size-exclusion gel filtration chromatography using a HiLoad 26/60 Superdex G75 column (GE Healthcare, Amersham, UK) and His-Tankyrase Loading Buffer (50 mM HEPES pH 7.5, 300 mM NaCl, 2 mM DTT) as the eluting solvent. Purity was checked on an Expedeon RunBlue SDS 4-20% gel, and concentrations determined from UV absorption at 280 nm using the extinction coefficient calculated by the ProtParam tool (Gasteiger et al., 2005). Fractions were flash-frozen in liquid nitrogen as 100 µL aliquots and stored at -80°C until further use.
2.3.5 Expression of Clickable PR65

The PR65 plasmid detailed in 2.2.1, and the pKW1 plasmid (a gift from Kaihang Wang, Chin lab, LMB) containing a tRNA\textsubscript{CUA} and aminoacyl-RNA synthetase pair evolved to incorporate propargyllysine (Sachdeva et al., 2014) plus a gene for spectinomycin resistance, were simultaneously transformed into \textit{E.coli} MDS42 electrocompetent cells by electroporation. The cells were grown overnight at 37 °C on LB-Agar plates supplemented with 25 µg/mL kanamycin and 37.5 µg/mL spectinomycin.

The plates were used to inoculate 100 mL of 2TY media supplemented with 25 µg/mL kanamycin and 37.5 µg/mL spectinomycin, and the culture shaken at 200 rpm and 37 °C overnight. This starter culture was then diluted into 6x 0.5 L of the same media, and shaken at 37 °C until the OD had reached 0.5. IPTG and propargyllysine were then added to give final concentrations of 1 mM and 2 mM respectively, and the culture shaken at 37 °C for a further 5 hours before being harvested by centrifugation at 4,000x g for 7 minutes.

The cell pellet was resuspended in PR65 Lysis buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 1 mM DTT) with a protease inhibitor tablet and lysed on an Emulsiflex c5 homogenizer before being centrifuged at 35,000x g for 35 minutes. GST beads (Expedeon, 1 mL per litre cell culture) were then added and the protein allowed to bind onto the beads for 2 hours at 4 °C before being washed twice with 25 mL PR65 Lysis buffer and once with 40mL GST Pull-down buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM DTT). The beads were resuspended in 8 mL GST Pull-down buffer, thrombin (25 U/litre cell culture) was added and the protein cleaved overnight at 4 °C before being eluted with 3x 5 mL GST Pull-down buffer. Protein expression was checked by SDS-PAGE on a RunBlue SDS 4-20% gel (Expedeon).

The combined elutions were bound onto Ni\textsuperscript{2+}-NTA beads (1 mL/litre cell culture) for 2 hours at 4 °C. The beads were washed three times with IMAC Binding Buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 20 mM imidazole, 1 mM DTT) before the proteins were eluted with 3x 3 mL IMAC Elution Buffer(100
mM Tris-HCl pH 8, 2 mM EDTA, 200 mM imidazole, 2 mM DTT). Purity was checked by SDS-PAGE, and the fractions containing >95% purity of the target protein were pooled, concentrated (Vivaspin 30,000 kDa MWCO, Generon) and buffer exchanged (ZEBAspin 2 mL, Thermo Fisher) into PBS with 1 mM DTT before being flash-frozen in liquid nitrogen and stored at -80 °C.

2.3.6 Azide-functionalised eGFP

A construct of eGFP in the pOPIN-J vector was a gift from Owen Burbidge. The plasmid was transformed into C41 competent cells by heat shock and grown overnight on LB-Amp agar plates. Colonies were picked, inoculated into 3x 1 L 2 supplemented with 50 µg/mL ampicillin, and grown at 37 °C until the OD reached 0.6 before being induced with 1 mM IPTG and shaken at 18 °C overnight.

The cells were harvested by centrifugation (5,000x g, 35 minutes) and the visibly green pellet resuspended in Lysis/Wash Buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 30 mM imidazole) and lysed on an Emulsiflex c5 homogenizer before being centrifuged at 35,000x g for 35 minutes. Ni-NTA beads (4 mL) were added and the protein allowed to bind onto the beads for 1 hour before being washed with 3x 50 mL Lysis/Wash Buffer and eluted with 3x 4 mL Elution buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 500 mM imidazole). The protein was purified by size-exclusion chromatography on a HiLoad 26/60 Superdex G75 column (GE Healthcare, Amersham, UK), flash-frozen in liquid nitrogen and stored at -80 °C.

The N-terminal amine of the eGFP was converted to an azide using the protocol of Schoffelen et al. (2011). This method reliably converts N-terminal amines to azides without modifying lysine residues. The protein was exchanged into 50 mM Clark & Lubs buffer at pH 8.9 using a NAP-5 column (GE Healthcare) to give a 71.8 µM solution. 240 µL of this was added to 227 µL of 50 mM Clark & Lubs buffer at pH 8.9 and 33 µL of a 2 mg/mL solution of imidazolyl-1-sulfonyl azide (synthesized by Yu Heng Lau, University of Cambridge, according
to the method of Goddard-Borger and Stick (2007)). The mixture was shaken at 37°C overnight, and the extent of diazotation checked by mass spectrometry (PNAC service, Department of Biochemistry).

### 2.3.7 Small-Scale Protein Expression Testing

When problems were encountered with proteins not expressing, for instance after the removal of the additional His-tag and repair of the ribosome binding sequence, the expression was repeated on a small scale to test whether proteins were being expressed insolubly or not expressed at all, and to investigate the effect of induction conditions on protein expression.

A single colony of B834 (DE3) E.coli transformed with the relevant plasmid as above was picked and grown overnight at 37°C and 220 rpm in 5 mL 2TY medium supplemented with 100 µg/mL ampicillin and 1% glucose. The next day, 500 µL aliquots of this culture were inoculated into 2x 5 mL 2TY medium supplemented with 50 µg/mL ampicillin and 1% glucose, and grown at 37°C, 220 rpm until an OD of 0.6 was reached. Both of these cultures were then induced with 1 mM IPTG and shaken at either 30°C for 4 hours or 18°C for 16-20 hours. Cells were then harvested by centrifugation at 4,000xg for 10 minutes.

The cell pellets were resuspended in 300 µL BugBuster Protein Extraction Reagent Master Mix (Merck) and incubated for 10-20 minutes at room temperature with shaking until the cell extract was no longer viscous. A 12 µL aliquot of this was saved as the total cell protein, and the remainder was centrifuged at 13,000xg for 10 minutes.

The supernatant was kept as the total soluble protein. The pellet was resuspended in 1 mL of a 1:10 BugBuster:water mixture, vortexed to ensure complete resuspension, and centrifuged at 13,000xg for 10 minutes. This was repeated, and the final pellet resuspended in 200 µL of 1:10 BugBuster:water as the purified inclusion bodies. The total cell protein, total soluble protein and purified inclusion bodies were run on a NuPAGE 4-12% Bis-Tris PAGE gel.
2.3.8 FITC Labelling of Proteins

Proteins were labelled with FITC (fluorescein isothiocyanate) using a standard protocol. Briefly, starting with a 500 μL aliquot of protein in 150mM PBS, the ionic strength was adjusted to 1M by adding 100 μL 4 M NaCl and the pH adjusted to between 9 and 10 by adding 2 μL KOH. A fivefold molar excess of FITC was added as a 100 mM stock in DMSO, and the solution shaken at 37 °C in the dark overnight. The next day, the protein was desalted and separated from unreacted dye on a NAP-5 desalting column (GE Healthcare), eluting in PBS. Fractions were checked for the presence of protein by UV-visible spectrophotometry and SDS-PAGE.

2.3.9 Circular Dichroism Spectroscopy

Thermal unfolding measurements of both the azidohomoalanine-containing and methionine-containing versions of the repeat proteins CTPR4 and Ank6 were performed in PBS on a Chirascan circular dichroism spectrometer (Applied Photophysics, Leatherhead, UK) over a temperature range from 20 to 90 (for CTPR4) or 94 (for Ank6) °C in smooth ramp mode at a ramp rate of 2 °C/minute. Three repeat measurements were taken.

2.4 Synthesis

2.4.1 Non-Natural Amino Acids

\textit{O}-propargylserine

\begin{center}
\begin{tikzpicture}
\end{tikzpicture}
\end{center}

Figure 2.1: Reaction scheme for synthesis of propargylserine
See Figure 2.1 for scheme.

Boc-L-Serine (2.0 g, 9.7 mmol) was dissolved in DMF (N,N-dimethylformamide) (100 mL) and cooled on ice. Sodium hydride (60% dispersion in mineral oil, 935 mg, 23.4 mmol) was added. After evolution of hydrogen gas stopped (about 20 minutes), propargyl bromide (80 wt% in toluene, 1.52 mL, 13.6 mmol) was added dropwise and the mixture stirred on ice for 15 minutes.

The mixture was allowed to warm to room temperature and stirred for 2 hours, then water added to destroy excess sodium hydride. The mixture was evaporated to dryness in vacuo and the residue dissolved in water. The aqueous layer was washed with ether and acidified with 1N potassium hydrogen sulfate. The acidic aqueous phase was extracted with ethyl acetate (3x 80 mL), washed with water (80 mL) and brine (80 mL) and dried over Na$_2$SO$_4$. The solvent was removed under reduced pressure, and the product purified by flash column chromatography on silica with DCM (Dichloromethane): 8% methanol: 1% acetic acid as the eluting solvent. Boc-O-propargyl serine (1.84 g including some residual solvent) was obtained as an orange oil.

The product of the previous reaction (1.84 g) was dissolved in the minimum volume of diethyl ether. HCl (2.0 M solution in diethyl ether, 10 mL) was added and the mixture stirred at room temperature for 2 hours. The progress of the reaction was followed by TLC.

On consumption of the starting material, the reaction mixture was filtered using a glass sinter funnel. O-propargyl serine hydrochloride (350 mg) was collected as an off-white precipitate, washed with ether, and dried in a vacuum desiccator.

O-propargyl serine hydrochloride (350 mg, 1.95 mmol) was dissolved in water (3 mL) and triethylamine (550 µL, 4 mmol). Separately, Fmoc-OSu (691.7 mg, 2.05 mmol) was dissolved in acetonitrile (2.2 mL) with gentle heating. The solutions were mixed and the pH raised to 9.0 with triethylamine. The solution was stirred for 30 minutes at room temperature, then filtered and the solvent removed under reduced pressure. 20% citric acid was added to the resulting
sticky oil. The precipitate formed was filtered, dissolved in ethyl acetate, washed with 20% citric acid, water and brine, and dried (MgSO₄). The solvent was removed under reduced pressure to give Fmoc-O-propargyl serine (446 mg, 1.22 mmol, 62.6% yield) as an off-white solid. As this was pure by TLC, further purification was not necessary.

**Azidohomoalanine**

Fmoc-glutamine (3.14 g, 8.53 mmol) was added to a solution of [bis(trifluoroacetoxy)iodo]benzene (PIFA, 6.01 g, 14.0 mmol) in DMF:water (2:1, 66 mL). After 15 minutes, pyridine (1.60 mL, 19.9 mmol) was added and the mixture stirred at room temperature for 19 hours.

The solvent was removed under reduced pressure, and the oily residue dissolved in water (50 mL). The solution was acidified (1 mL conc. HCl) and washed with ether (4 x 30 mL). The aqueous phase was adjusted to pH 6 with 2 M NaOH solution, and the resulting precipitate filtered, washed with water (5 x 30 mL), ice-cold ethanol (10 mL) and ether (10 x 10 mL) and dried in vacuo. Fmoc-diaminobutyric acid (1.78 g, 5.20 mmol, 61% yield) was obtained as an off-white solid. See Figure 2.2 for scheme.

![Figure 2.2: Reaction scheme for synthesis of Fmoc-diaminobutyric acid](image)

Fmoc-diaminobutyric acid (1.03 g, 3.01 mmol) was dissolved in a biphasic mixture of water (15 mL), methanol (30 mL) and DCM (25 mL). CuSO₄·5H₂O (5 mg, 0.02 mmol) and imidazole-1-sulfonyl azide hydrochloride (2.01 g, 9.59 mmol, synthesised by Yu Heng Lau according to the method of Goddard-Borger and Stick (2007)) were added and the mixture adjusted to pH 9 with aqueous
K₂CO₃ solution and stirred vigorously for 18 hours.

The reaction mixture was diluted with DCM (30 mL) and the aqueous phase isolated. The organic phase was extracted with saturated aqueous Na₂CO₃ (2 x 50 mL). The combined aqueous extracts were washed with ether (2 x 50 mL), acidified to pH 2 with concentrated HCl, and extracted with ether (3 x 60 mL). The organic extracts were dried (MgSO₄) and concentrated in vacuo. See Figure 2.3 for scheme.

Figure 2.3: Reaction scheme for synthesis of Fmoc-azidohomoalanine

Fmoc-azidohomoalanine (300 mg) was dissolved in a solution of 20% piperidine in DMF (5 mL) and stirred at room temperature. The progress of the reaction was followed by TLC and on completion (30 minutes) ether was added to precipitate the product and the precipitate allowed to settle.

The reaction mixture was centrifuged and the supernatant poured off. The precipitate was resuspended in ether, centrifuged and the ether poured off. This washing step was repeated. The precipitate was then dissolved in water and freeze-dried. Azidohomoalanine (57 mg) was obtained as a white solid.

2.4.2 Solid-Phase Peptide Synthesis of Amyloid-Forming Peptides

Peptides shown in Table 2.1 were synthesised on a CEM Liberty or Liberty Blue peptide synthesiser (CEM Microwave Technology Ltd., Buckingham, UK) using standard solid-phase techniques (Amblard et al., 2006) on Rink Amide resin (Millipore UK, Watford, UK) with HBTU/DIEA as the activator. The resin was washed with DCM and dried under vacuum. The peptide was cleaved
from the resin by shaking with 1 mL per 100 mg resin of a 95:2.5:2.5 mixture of TFA:water:triisopropylsilane for 2 hours, followed by elution with 8 mL TFA. The solvent was removed under nitrogen, and the peptide triturated with ether. It was then isolated by centrifugation, washed with ether (5x 7 mL), freeze-dried and purified by HPLC (High-Performance Liquid Chromatography). Purity was checked by LCMS (Liquid Chromatography Mass Spectrometry), and peptides were stored as lyophilised solid at -20°C.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTR105-115</td>
<td>YTIAALLSPYS</td>
<td>Wild-type</td>
</tr>
<tr>
<td>TTR-A1</td>
<td>YTIAALLSPYSGGGX</td>
<td>Alkyne-functionalised, X=O-propargylserine</td>
</tr>
<tr>
<td>TTR-Z1</td>
<td>YTIAALLSPYSGGGAha</td>
<td>Azide-functionalised, Aha=azidohomoalanine</td>
</tr>
</tbody>
</table>

Table 2.1: Sequences of Synthesised Peptides

2.5 Amyloid Fibrils

2.5.1 Formation and Characterisation

Fibrils were formed according to the method used by Gras et al. (2008). Amyloid-forming peptides (see 2.4.2) were dissolved in water with 10% v/v acetonitrile at a concentration of 10 mg/mL. The solution was shaken at 37°C for 24 hours, then stored at -20°C.

An aliquot of the 10 mg/mL suspension of amyloid fibrils synthesised as above was diluted 1000-fold in water. A 300 µL drop of this dilute suspension was placed on a freshly cleaved mica cover slip, allowed to dry and imaged in air using tapping mode on a JPK NanoWizard III AFM (atomic force microscope, JPK Instruments AG, Berlin, Germany). Images were flattened and processed using standard techniques and the Gwyddion software (Nečas and Klapetek, 2012).
2.5.2 Click Reactions with Amyloid Fibrils

Reaction with Gold Nanoparticles

Fibrils were synthesised as above from TTR105-115 peptide both with and without the addition of 5 wt% TTR-A1 peptide. Fibrils (20 µL of 10 mg/mL suspension), a premixed catalyst consisting of 0.4 mM CuSO$_4$, 0.4 mM THPTA (Tris(3-hydroxypropyltriazolylmethyl)amine) and 1.2 mM sodium ascorbate (20 µL) and a suspension of azide-functionalised 10 nm diameter gold nanoparticles (Nanocs, California, USA, 0.05 wt% Au in water, 40 µL) were added to water (320 µL) and shaken at room temperature for 16 hours.

An aliquot of the reaction mixture was imaged by AFM as above. In addition, aliquots of the reaction mixture were adsorbed onto 400 mesh copper grids with a continuous carbon support film (EM Resolutions Ltd, Saffron Walden, UK) for 60 seconds then imaged without further staining on a Tecnai G2 TEM (transmission electron microscope, FEI, Hillsboro, OR, USA) operated at 200 kV. Images were captured on an AMT 60B camera (Advanced Microscopy Techniques, Woburn, MA, USA) with Deben software (Deben, Bury St. Edmunds, UK).

Reaction with Azidohomoalanine

Fibrils formed from the TTR-A1 peptide were reacted with azidohomoalanine using an adapted version of the method of Presolski et al. (2011). Azidohomoalanine hydrochloride (1.4 mM), TTR-A1 fibrils (1 mg/mL, 700 µM monomer equivalent), copper (II) sulfate (0.2 mM), THPTA (1 mM) and sodium ascorbate (10 mM) were dissolved in water (0.5 mL). All reagents were added as concentrated stock solutions in water, the copper (II) sulfate and THPTA were mixed before being added, and the sodium ascorbate added last. The reaction mixture was placed in a closed tube and shaken at room temperature for 24 hours. A 200 µL aliquot of the reaction mixture was ultracentrifuged at 313,000 xg for 30 minutes using a TLA-100 rotor (Beckman Coulter, High Wycombe, UK).
to isolate the fibrils. The supernatant, containing any unaggregated monomer, was removed by pipetting and the fibrils resuspended in 200 µL water. The resuspended fibrils were analyzed by LCMS on a Waters Xevo G2-S instrument (Waters, Manchester, UK) using a C18 column. MS experiments were performed at a capillary voltage of 1900 V, cone voltage of 80 V and source offset voltage of 80 V. Spectra were processed using MassLynx V4.1 software (Waters).

Reaction with Fluorescein

Fibrils formed from the TTR-A1 peptide were reacted with 5-fluorescein amidite azide (FAM-5 azide, Lumiprobe, Hannover, Germany) (50 µM) using the same method as above. Again, a 200 µL aliquot of the reaction mixture was ultracentrifuged to give a visibly yellow pellet of fibrils. These were resuspended in 200 µL water before being diluted for imaging by TIRF (Total Internal Reflection Fluorescence) microscopy (see 2.5.2).

Reaction with eGFP

Fibrils formed from the TTR-A1 peptide were reacted with eGFP using a similar method to that detailed above for azidohomoalanine by adding fibrils and catalyst to the solution of azide-functionalized eGFP prepared in 2.3.6. The reaction mixture contained 26.5 µM eGFP in Clark & Lubs buffer (pH 8.9). A control reaction was also carried out in the absence of the click catalysts. Again, after the reaction the fibrils were pelleted by ultracentrifugation and resuspended in water. As an additional control, the reaction was also carried out with unaggregated lyophilized peptide (10 mg/mL) instead of fibrils, with the addition of 10% acetonitrile at 37°C. As the click reaction is faster than amyloid fibril formation, taking place over a timescale of tens of minutes (Hong et al., 2009) rather than hours (Bongiovanni et al., 2012), the intention of this control was to attempt to form fibrils after the peptide had been conjugated to eGFP. The eGFP-functionalized fibrils were imaged by TIRF microscopy using the
method in 2.5.2 In addition, the fluorescence polarization of free eGFP and of eGFP clicked onto fibrils was measured using an LS-55 luminescence spectrometer (Perkin-Elmer, Beaconsfield, UK). Either a 7.18 μM solution of eGFP in Clark & Lubs buffer or a tenfold dilution of the pelleted and resuspended fibrils from the click reaction mixture were measured at 25°C in a QS-284 quartz glass cuvette (Hellma Analytics, Müllheim, Germany) with an excitation path length of 10 mm and an emission path length of 2 mm, using an excitation wavelength of 488 nm and an emission wavelength of 509 nm with both excitation and emission slits set to 5 nm and the photomultiplier tube voltage at 600 V. Quoted figures are an average of 10 measurements.

**Total Internal Reflection Fluorescence Microscopy**

*TIRF images were taken by Laurence Young, Department of Chemical Engineering and Biotechnology, University of Cambridge*

Fibrils were diluted 10-fold and a 5μL aliquot was deposited onto chambered coverglass (80827, µ-Slide, ibidi). Fibrils were allowed to settle for 10 minutes before adding 200 μL PBS. Prior to imaging, the coverglass was cleaned using 1 M potassium hydroxide for 30 minutes before washing 3 times with ultrapure water. Fibril samples were imaged using a 100x/1.49NA objective (UAPON100XOTIRF, Olympus, Southend-on-Sea, UK) on an Olympus IX71 inverted microscope with custom built TIRF illumination. The incident angle for TIRF was controlled by laterally translating the laser focus in the back focal plane of the microscope objective. EGFP was excited using a 488 nm diode laser (iBeam SMART, Toptica, Gräfelfing, Germany), with detection via a 525/30 nm bandpass filter (FF01-525/30, Semrock, Rochester, NY, USA) onto an sCMOS camera (ORCA Flash 4.0, Hamamatsu, Welwyn Garden City, UK). Image acquisition was controlled using Micro-Manager software (Edelstein *et al.*, 2010) with 100-200ms exposure times.
2.6 Click Reaction Testing

2.6.1 Click Reactions With Repeat Proteins

Click reactions were carried out between repeat proteins and alkyne- or azide-functionalised dyes in order to show that the proteins would undergo a click reaction. In one example, azide-functionalised CTPR4 (48 µM) was reacted with FAM-5-alkyne (Lumiprobe, 34.2 µM) in water using a protocol adapted from that of Presolski et al. (2011). The Cu (I) catalyst was produced in situ from copper (II) sulfate (1 mM), THPTA (5 mM) as a stabilising ligand and hydroxylamine (10 mM) as the reducing agent. The reaction was carried out in a closed tube for 3 hours at 37 °C. Hydroxylamine was used because the protein had been seen to degrade in the presence of ascorbate (Chiou, 1983).

The reaction mixture was run on a 4-20% SDS-PAGE gel (Expedeon, Swavesey, UK) and visualised under UV light to determine whether the dye had been successfully conjugated to the protein.

2.6.2 Click Reactions Between Repeat Proteins and Amyloid Fibrils

Click-functionalised repeat proteins and amyloid fibrils were reacted together in order to test their mutual activity. Two different reaction protocols were tested on a 20 µL scale, one based on the method of Presolski et al. (2011) and the other based on that of Sachdeva et al. (2014). The first method was as in 2.6.1 with 24 µM protein and 1 mg/mL fibrils. For the second method, the same quantities of protein and fibrils were used, and the catalyst was 2 µL of a “click-mix” consisting of 1 µL copper (II) sulfate (100 mM), 5 µL THPTA (100 mM) and 2.5 µL sodium ascorbate (1 M) in water (total 100 µL) - protein, fibrils and catalyst were mixed in PBS and left to stand at room temperature for 2 hours. In addition, “control” reactions were carried out where one component (catalyst, protein or fibrils) was omitted.

The reaction mixtures and samples of unreacted protein and fibrils were run
on a NativePAGE gel (Expedeon) for 100 min at 120 V in D/N Running Buffer (Expedeon). The gel was stained overnight with Coomassie Blue G250 stain and then destained in 20% methanol and 10% acetic acid.

2.7 Microfluidics

2.7.1 Fabrication of PDMS devices

Microfluidic devices were made from PDMS using standard soft lithography techniques (Duffy et al., 1998; Whitesides, 2006). Briefly, a 50 \( \mu \text{m} \) layer of SU-8 3050 photoresist (Kayaku Microchem, Westborough, MA, USA) was spin-coated onto silicon wafers and a mask used to expose only the area of the desired channels to UV light before PGMEA (1-methoxy-2-propyl acetate) was used to wash away uncrosslinked photoresist. The devices were fabricated from Sylgard 184 photoresist (Dow Corning, Midland, MI, USA) and cured at 65°C for 3 hours. Devices were cut out, inlet and outlet holes punched using an 0.75mm biopsy punch, and devices bonded to glass slides using a Femto plasma bonder (Diener Electronic, Ebhausen, Germany).

Syringes were connected using polyethylene tubing and flow rates regulated using Nemesys syringe pumps (Cetoni, Korbüßen, Germany). While being operated, microfluidic devices were viewed using a Zeiss Axio Observer inverted light microscope (Carl Zeiss Ltd, Cambridge, UK) fitted with a Mikrotron MotionBLITZ EoSens mini (Mikrotron, Unterschleißheim, Germany) or Photometrics CoolSNAP MYO (Photometrics, Tucson, AZ, USA) camera. Images were processed using ImageJ (Schneider et al., 2012)

2.7.2 Surfactant for Droplet Microfluidics

AZ-900, a perfluorinated polyether-polyethylene glycol-perfluorinated polyether (PFPE-PEG-PFPE) triblock copolymer surfactant, was synthesised based on the method of Holtze et al. (2008) and Zinchenko et al. (2014). Krytox 157 FSH (17.1 g, 3.0 mmol; DuPont, Stevenage, UK) in HFE7500 (FluoroChem,
Hadfield, UK; 1 mL) was stirred at room temperature under a nitrogen atmosphere. Thionyl chloride (0.44 mL) was added and the mixture stirred at room temperature for 1 hour then spun at maximum speed on a rotary evaporator at atmospheric pressure before volatile compounds were removed in vacuo. The resulting oil was resuspended in HFE7500 (5 mL) and Jeffamine ED-900 (1.35 g, 1.5 mmol; a gift from Huntsman Performance Products, Everberg, Belgium) was added. The resulting white and very viscous reaction mixture was spun on a rotary evaporator at room temperature and atmospheric pressure for 18 hours at maximum speed.

A small amount of HFE7500 (ca. 2 mL) was added to reduce the viscosity of the mixture before water (50-100 mL) was added and the biphasic mixture stirred vigorously before the aqueous layer was discarded. This wash procedure was repeated two more times before the oil phase and any remaining aqueous phase were centrifuged for 15 minutes to obtain complete phase separation. The oil phase was carefully removed and filtered through a 0.45 µm syringe filter. The solvent was removed under reduced pressure (rotary evaporator at 65 °C for one hour followed by high vacuum at room temperature overnight) and stored at 4 °C.

![Reaction scheme for synthesis of AZ-900 surfactant](image)

Figure 2.4: Reaction scheme for synthesis of AZ-900 surfactant
2.7.3 Droplet Making

Single-junction droplet making devices were fabricated using standard designs and the method detailed in 2.7.1. Before use, the devices were treated with Aquapel (PPG Industries, Pittsburgh, PA, USA) and flushed with air to ensure that the device walls would be wetted with the fluorinated oil continuous phase and not the aqueous phase. FC-40 fluorinated oil with the addition of either 2-4% w/v AZ-900 surfactant (synthesised as detailed in 2.7.2) or 1% w/v 008-FluoroSurfactant (RAN Biotechnologies, Beverly, MA, USA) was used as the continuous phase. Oil and aqueous phases were flowed into the droplet makers with flow rates adjusted to obtain monodisperse droplets in large quantities.

Click Reactions in Droplets

Repeat proteins and amyloid fibrils were reacted in droplets to form microgels. The method of Presolski et al. (2011) was used here, as it requires the reaction to take place at 37 °C rather than at room temperature. Before the reaction, repeat proteins were exchanged from purification buffer into PBS using either Zeba Spin desalting columns (Thermo Scientific, Loughborough, UK) or CentriPure P5 desalting columns (EMP Biotech, Berlin, Germany). Droplets were formed as detailed in 2.7.3 from a solution containing 1 mg/mL alkyne-functionalised fibrils, 0.3 mg/mL protein, 1 mM CuSO$_4$, 5 mM THPTA and 10mM hydroxylamine as reducing agent. The components of this solution were mixed together on ice and the injection syringe kept cold in order to prevent the reaction taking place and the solution gelling before the droplets were formed. The emulsion of droplets in FC-40 oil was then incubated for 3 h at 37 °C to allow the crosslinking ‘click’ reaction to take place.

Alginate Microgels

Alginate microgels were formed using a method based on the work of Chen et al. (2016). Water-in-oil droplets were formed using the method described in 2.7.3 with the addition of 0.5% v/v acetic acid to the oil phase. The aqueous phase
consisted of 2% w/v alginate and 100 mM calcium-EDTA. The emulsion was left to gel for 30 minutes at room temperature. Alginate microgels were either used in oil or washed with PFO (Perfluorooctanol) in order to transfer them into water.

2.7.4 Mechanical Testing of Microgels

Capillary Micromechanics

Tapered pipettes were fabricated from Intracel glass capillaries (Intracel, Royston, UK) with an internal diameter of 0.5mm and external diameter of 1.0mm using a Sutter P-2000 pipette puller (Sutter Instrument, Novato, CA, USA). The non-tapered end was connected to a syringe via polyethylene tubing, and a dilute suspension of microgels introduced using a syringe pump.

PDMS-Based Mechanical Testing Devices

Mechanical testing devices were designed (in collaboration with Kadi Liis Saar) consisting of a wide measurement chamber with a series of pillars across it, shaped such that there were several tapering channels between them as in Figure 2.5. These channels were 200 µm long and had an inlet width of either 50 µm or 90 µm and an exit width of either 10 µm or 15 µm. Device height was approximately 50 µm. There were also two wider channels on the outside of the pillars. The devices had two inlet channels to allow a suspension of microgels and pure continuous phase to be flowed in and their flow rates controlled separately without having to disconnect and reconnect syringes—these channels combined in a Y-shaped junction before the inlet chamber.

Figure 2.5: Design of a PDMS mechanical testing device
Devices were fabricated using standard techniques as described in 2.7.1. Surface treatment of the device varied depending on the properties of the material to be measured and the continuous phase it was suspended in. If a hydrophilic surface was desired, the device was either subjected to a longer period of plasma treatment after bonding (Bodas and Khan-Malek, 2006) or simply filled with water after bonding to preserve the hydrophilic silanol groups on the channel surface. Conversely, if a hydrophobic surface was desired, the device was filled immediately after bonding with a 1% w/v solution of $1H,1H,2H,2H$-perfluorododecyltrichlorosilane in FC-40 fluorinated oil. This was left to react for approximately 2 minutes before the channels were rinsed and filled with FC-40 for storage.
Chapter 3

Functional Amyloid Fibrils

In this chapter, the development of a new method for functionalising amyloid fibrils after they have assembled is detailed. This method is based on a short peptide consisting of a total of 15 amino acids—11 from a natural amyloid-forming peptide, a three-glycine linker, and a non-canonical alkyne-containing amino acid—meaning that synthesis of the peptide by standard chemical techniques, which allow the incorporation of arbitrary amino acids, is straightforward. The resulting fibrils have an alkyne functional group displayed on their surface, which can react with an azide on the desired cargo molecule in a copper-catalysed ‘click’ reaction. Cargo molecules tested included non-natural amino acids, dyes and functional proteins.

As briefly reviewed in 1.3.1, there have been several studies aimed at making amyloid fibrils functional. For example, Forman et al. (2012, 2013) were able to display cytochrome proteins on the surface of amyloid in order to use the fibrils as electrically conducting nanowires. However, in most previous work on functional fibrils the desired functionality was attached to the peptide monomers before they were assembled into the fibrils. Pre-assembly functionalisation limits the available choice of functional species to those that do not unduly perturb assembly and whose functionality can survive the conditions of assembly (Woolfson and Mahmoud, 2010). This newly-developed method allows the fibrils to
instead be functionalised after they are assembled. As the conditions for this reaction are significantly milder, they are tolerated by a much wider range of substrates and do not affect the structure of the fibrils themselves.

The use of the ‘click’ reaction also allows proteins to be attached to fibrils if they have been expressed with a non-natural amino acid which contains an azide moiety. As discussed in 1.4.1 there are a wide range of methods for incorporating these amino acids into proteins, some of which are site-specific and/or capable of incorporating multiple molecules of the same non-natural amino acid at different positions within the same protein. Therefore, proteins can be attached to the fibrils at different positions rather than simply via a terminus as in the work of Hudalla et al. (2014).

In previous work, the ‘click’ reaction of azides and alkynes has been used to functionalise fibril-forming peptides with cargo species such as oligonucleotides (Humenik and Scheibel, 2014) and polymers (Dehn et al., 2012) prior to aggregation. However, this again means that the cargo species must be compatible with the conditions of fibril formation. The approach detailed in this chapter allows species incompatible with these conditions to be attached to fibrils.

Finally, while there as not sufficient time to explore this here, one further advantage of this approach is that it uses peptides produced by solid-phase peptide synthesis, which is a chemical rather than biological method so in principle allows almost any chemical functionality to be displayed on the fibril. Therefore, the fibrils could in future be functionalised by means of a wide range of reactions, such as strain-promoted azide-alkyne cycloaddition (Ornelas et al., 2010) or the reaction of tetrazines with trans-cyclooctenes (Blackman et al., 2008) or norbornenes (Han et al., 2010).

3.1 Design of Amyloid System

The system used, named TTR-A1, was based on the eleven-residue TTR105-115 peptide, an amyloid-forming fragment of the human protein transthyretin,
as discussed in 1.3.2. As detailed by Gras et al. (2008), residues added to the C-terminus of this peptide will be displayed on the outer surface of the fibrils it assembles into under relatively simple conditions (10 mg/mL peptide dissolved in water with 10% acetonitrile will form fibrils within 24 hours).

The alkyne functionality of the peptide came from the non-natural amino acid O-propargylserine, which was chosen for ease of synthesis (see 2.4.1). This amino acid was connected to the N-terminal serine of TTR105-115 via a three-glycine linker to increase the flexibility and accessibility of the alkyne. The TTR-A1 peptides were synthesised as detailed in 2.4.2 and fibrils formed and their morphology checked by AFM as detailed in 2.5.1, giving micron-sized straight fibrils as shown in Figure 3.1.

Figure 3.1: Atomic Force Micrograph of Alkyne-Functionalised Amyloid Fibrils. Fibrils were formed from 10 mg/mL 100% TTR-A1 peptide in water with 10% acetonitrile, shaken at 37 °C for 24 hours. This solution was diluted 1000-fold in water before imaging.
3.2 Reaction with Gold Nanoparticles

Initially, it was thought that fibrils could be reactive if only a small quantity of alkyne-functionalised peptide was doped into an otherwise unfunctionalised fibril. Reactions were carried out as detailed in 2.5.2. AFM imaging of the unreacted fibrils (Figure 3.2) showed some differences between the doped and undoped fibrils. Doped fibrils were shorter, and more likely to have broken up into short segments. As these differences were not apparent in the TEM images (see Figure 3.3), they are probably due to damage from the AFM tip—TEM samples are also dried, so damage from the drying process is less likely to have caused these changes. Note, however, that the fibrils here are longer than the TTR-A1 fibrils used in other sections—potentially the functionalised peptide slows down fibril elongation.

TEM images were taken of the fibrils after reaction with gold nanoparticles as detailed in 2.5.2. No staining of the TEM samples was required as the fibrils are dense enough to be visible even unstained, as extended grey structures. Therefore, both for ease of sample preparation and to make the gold nanoparticles more visible, samples were not stained. While the gold nanoparticles are visible in association with the fibrils (the black object, darker due to their greater density), they appear in association with both functionalised and unfunctionalised fibrils. In addition, the fibrils are not nearly as densely covered as might be expected by comparison with the results obtained for immunogold labelling of fibrils by Gras et al. (2008). Therefore, this may well be a non-specific association between the amyloid fibrils and the PEG coating of the nanoparticles.

This reaction was not successful, probably due to insufficient concentration of either accessible alkyne groups or catalyst. Due to other methods being developed which were more straightforward or more relevant for the desired application of the functional fibrils, it was not repeated with 100% TTR-A1 fibrils.
Figure 3.2: AFM images of TTR105-115 fibrils (top) and TTR105-115 fibrils doped with 5 wt% TTR-A1 (bottom). Fibrils were formed from 10 mg/mL peptide in water with 10% acetonitrile, shaken at 37 °C for 24 hours. This solution was diluted 1000-fold in water before imaging.
Figure 3.3: TEM images of TTR105-115 fibrils (top) and TTR105-115 fibrils doped with 5 wt% TTR-A1 (bottom) after attempted reaction with azide-functionalised gold nanoparticles. Scale bars are 400 µm.
3.3 Reaction with Azidohomoalanine

As detailed in 2.5.2, 100% TTR-A1 fibrils were initially reacted with azidohomoalanine to test the reactivity of the alkyne. The fibrils were then isolated from soluble material by ultracentrifugation, denatured and the released peptides subjected to mass spectrometry analysis as outlined in Figure 3.4.

![Figure 3.4: Scheme of mass-spectrometry analysis strategy](image)

The mass spectrum of the fibrils (Figure 3.5) has a peak with an $m/z$ value of 1637.87, which corresponds to the calculated mass of the ‘clicked’ adduct with azidohomoalanine. The major peak at $m/z=1515.79$ corresponds to this adduct with the loss of the N-terminal tyrosine, and the peak at 1531.76 corresponds to it with the loss of the tyrosine sidechain. The peak at 1493.80 corresponds to unreacted peptide, either due to the reaction not going to completion or because the alkyne moieties in some of the peptide molecules were buried in the fibril and unable to react.

By contrast, fibrils made from the unfunctionalised amide-terminated TTR105-115 peptide did not react, and the major peak in their mass spectrum is at $m/z=1197.68$, corresponding to unreacted peptide. Note that the difference in $m/z$ value for the peaks in the two experiments is due to the unfunctionalised peptide lacking the unreacted amino acid and the triglycine linker.

3.4 Reaction with Fluorescein

The functionalization process was next repeated with 100% TTR-A1 fibrils and a fluorescein analogue containing an azide group, as detailed in 2.5.2. The visible yellow colour of the pellet after ultracentrifugation of the fibrils suggested that
Figure 3.5: Mass spectrum of TTR-A1 fibrils reacted with azidohomoalanine they had reacted to form a fluorescein adduct. TIRF microscopy as detailed in 2.5.2 showed fluorescent fibrils (Figure 3.6). This confirms that fluorescein has been localised to the fibril surface by a ‘click’ reaction between the azide on the dye and the alkynes on the fibril.

Figure 3.6: Total Internal Reflection Fluorescence Micrograph of Fluorescein-Functionalised 100% TTR-A1 Fibrils. Image captured by Laurence Young, Department of Chemical Engineering and Biotechnology, University of Cambridge
3.5 Reaction with eGFP

Figure 3.7: Reaction mixtures where eGFP (26.5 µM in Clark & Lubs Buffer, pH 8.9) was reacted with TTR-A1 before aggregation (left, 10 mg/mL peptide and 10% v/v acetonitrile) and after aggregation (right, 1 mg/mL fibrils, 700 µM monomer equivalent). Both reactions also contained copper (II) sulfate (0.2 mM), THPTA (1 mM) and sodium ascorbate (10 mM). Reactions were carried out at 37 °C (left) or room temperature (right) for 16 hours.

As a final demonstration of the generality of this approach for functionalising 100% TTR-A1 amyloid fibrils, an entire functional protein—namely eGFP—was attached to 100% TTR-A1 fibrils using the methods described in 2.5.2. There was visibly more eGFP in the supernatant of the control sample without ‘click’ catalyst than in that of the ‘clicked’ fibrils, suggesting that eGFP had successfully been conjugated to the fibrils. In addition, the sample where aggregation took place after conjugation of eGFP to peptide monomer contained a visible precipitate. This reaction mixture no longer exhibited fluorescence on exposure to UV light, whereas the ‘clicked’ fibrils did (Figure 3.7)
This suggests that eGFP does not retain its fluorescence when exposed to the conditions of TTR-A1 aggregation, possibly because it has itself aggregated. TIRF micrographs of the fibrils reacted with eGFP (Figure 3.8) also show fluorescent fibrils, which are again absent in the control reaction carried out in the absence of catalyst.

![Figure 3.8: Total Internal Reflection Fluorescence Micrograph of eGFP-Functionalised Fibrils, formed by reaction between TTR-A1 fibrils (1 mg/mL, 700 µM monomer equivalent) and eGFP (26.5 µM) in Clark & Lubs buffer (pH 8.9) with copper (II) sulfate (0.2 mM), THPTA (1 mM) and sodium ascorbate (10 mM) as catalyst. False-colour image- colours correspond to intensity not colour of fluorescence Image captured by Laurence Young, Department of Chemical Engineering and Biotechnology, University of Cambridge](image)

Finally, the fluorescence polarisation of eGFP bound to fibrils is significantly greater than that of free eGFP (Figure 3.9). This difference is smaller than might be expected given the large size of the fibrils, but this can be explained by the fact that the bound GFP has some freedom of movement relative to the fibril due to the 23-amino-acid flexible ‘tether’ (consisting of the triglycine linker in the TTR-A1 peptide, the amino acids forming the covalent link, and the purification tag on the eGFP) between the eGFP and the fibril, and the flexibility of the N-terminal region of eGFP (Arpino et al., 2012).

Further proof of the binding of eGFP to fibrils could be provided by nano-IR or conventional FTIR spectroscopy to detect the vibrations of bonds in the
Figure 3.9: Fluorescence Polarisation of Fibril-Bound (product of reaction between TTR-A1 fibrils (1 mg/mL, 700 µM monomer equivalent) and eGFP (26.5 µM) in Clark & Lubs buffer (pH 8.9) with copper (II) sulfate (0.2 mM), THPTA (1 mM) and sodium ascorbate (10 mM) as catalyst) and Free (7.18 µM in Clark & Lubs buffer at pH 8.9) eGFP, measured with an excitation wavelength of 488 nm and an emission wavelength of 509 nm at 25 °C.

A triazole ring produced by the “click” reaction. Alternatively, mass spectrometry of the denatured eGFP-bound fibrils would show a peak corresponding to the mass of the eGFP-peptide adduct. The size of the eGFP monomer is such that it can be visualised by AFM (Collins et al., 2004) or TEM (Kim et al., 2015) so these methods could be used to visualise the decorated fibrils. Perhaps most usefully, the TIRF and fluorescence polarisation experiments above could be repeated with a more stringent wash step to eliminate the possibility of non-specific non-covalent binding of eGFP to fibrils.
3.6 Conclusions

The approach demonstrated in this chapter is a novel strategy to form one-dimensional nanoscale scaffolds that can be functionalised via a ‘click’ reaction after they have self-assembled. This approach, in contrast to other approaches for organising molecules via amyloid self-assembly, allows a wide variety of molecular species to be used including those that are not compatible with the conditions of self-assembly.

The TTR-A1 peptide was designed based on the TTR105-115 amyloid-forming peptide with the addition of a C-terminal alkyne, and successfully self-assembled into micron-sized fibrils. These fibrils were shown to be capable of undergoing a ‘click’ reaction with various azide-containing molecules, including proteins which did not retain their functionality under the conditions of fibril assembly. This opens up the possibility of using amyloid fibrils as scaffolds to organise a wide variety of cargo components from small molecules to proteins.

In the case of azidohomoalanine, the mass spectrum in Figure 3.5 shows that the majority of peptides in the fibrils have the cargo species attached. Of course, this proportion will vary depending on a number of factors such as the stoichiometry of the conjugation reaction and the steric bulk of the cargo species— in the case of eGFP, which is relatively large and was used in a much lower stoichiometric ratio, most peptides in the fibrils will not have an eGFP molecule conjugated to them and their alkyne groups will remain unreacted.

In addition, it is of course possible to express a protein containing multiple azide groups at different locations in its sequence- for example, one at each terminus. In this case, the protein can react with alkyne groups on different fibrils, linking them together to form a hydrogel network. The formation and properties of such a material are described in Chapter 6.
Chapter 4

Click Reactions on Proteins

In this chapter, the expression and characterisation of repeat proteins containing non-natural amino acids for “click” reactions is detailed. Although similar experiments have been carried out in the past, such as the PEGylation of DARPsins detailed in Simon et al. (2012), it did not prove possible to simply replicate them so some optimisation of the protocol was required in order to obtain proteins with the desired level of non-canonical amino acid incorporation and to successfully carry out a “click” reaction. The protocol published by Presolski et al. (2011) provided a great deal of useful information for the optimisation of “click” reactions on proteins. As well as a full-consensus DARPin (Wetzel et al., 2008) and CTPR (Main et al., 2003b), the natural ankyrin repeat protein TNKS2ARC4 (Guettler et al., 2011) was expressed with the incorporation of the non-canonical amino acid azidohomoalanine. This protein was chosen because plasmids coding for it were available in the Itzhaki lab and required minimal modification to incorporate azidohomoalanine in the desired positions. In addition, fluorescent peptides known to bind TNKS2ARC4 (Xu et al., 2017) were available in the lab and could be used to explore this protein’s ligand-binding properties. PR65 (Groves et al., 1999) was expressed with the incorporation of the alkyne-containing non-canonical amino acid propargyllysine to explore a potential ‘reverse’ method where alkyne-containing proteins are conjugated to
fibrils displaying azide functionality. This protein was chosen as the plasmids required to express it with propargyllysine incorporated through amber codon suppression were already available in the Itzhaki lab. Both tankyrase (Xu et al., 2017) and PR65 (Tsytlonok and Itzhaki, 2012; Tsytlonok et al., 2013) had already been thoroughly characterised in the Itzhaki lab.

4.1 Expression of Azide- or Alkyne-Containing Proteins

4.1.1 Expression of Azide-Containing DARPin

Azide-containing DARPin were initially expressed using the plasmid ordered in 2.2.1, even though as detailed in 2.2.3 this plasmid coded for an additional hexa-His tag and with it an additional methionine which would be replaced by azidohomoalanine. However, while a protein with three rather than two azide groups would be problematic for the synthesis of a material, it could still be of use for testing the conditions for a ‘click’ reaction. Therefore, it was expressed using the method detailed in 2.3.2.

Amino acid analyses of the expressed proteins (performed by the PNAC facility, Department of Biochemistry, University of Cambridge) showed that the conditions of induction and protein expression are very important in order to obtain the desired level of substitution of non-canonical amino acids for methionine when expressing proteins according to the method of Kiick et al. (2001). In this method, methionine-auxotrophic cells are grown in media containing methionine before being transferred to methionine-free media for induction of protein expression under the control of the lac operon. This is necessary because methionine, and not a substitute such as azidohomoalanine, is required for the cells to grow. Before induction, the cells are shaken for 15 minutes in the methionine-free media to allow the depletion of intracellular methionine pools (Tamaskovic et al., 2012).
However, even with this precaution if the protein is expressed overnight then there is a significant level of incorporation of methionine. This is probably because some methionine must be present in the cells in endogenous proteins that incorporate it. Degradation of these proteins as part of proteostasis releases methionine, which can then be recycled by incorporation into the expressed protein. This can be reduced by limiting the expression time such that there is less time for proteins to degrade and release their methionine. It was found that limiting expression to 4 hours at 30°C as in the method of Tamaskovic et al. (2012) reduces methionine incorporation to an acceptable level of less than 20%.

4.1.2 Expression of Other Azide-Containing Proteins

Azide-containing CTPR4 (see 2.2.1) was expressed using the same method as the azide-containing DARPin. However, a different method was used for azide-containing TNKS2ARC4. This is due both to the less-stable natural repeat domain requiring a different set of buffers, and due to the methionine-auxotrophic B834(DE3) cells not being available. Therefore an alternative method was used which relied on using the cells' internal regulatory machinery to stop biosynthesis of methionine. In both cases, proteins were expressed with a satisfactory level of azidohomosalanine incorporation as determined by amino acid analysis.

4.1.3 Expression of Alkyne-Containing PR65

Alkyne-containing PR65 was expressed according to the method detailed in 2.3.5. As propargyllysine converts to lysine under the acid hydrolysis conditions of amino acid analysis, incorporation levels were instead determined by mass spectrometry (PNAC facility).

4.2 Circular Dichroism Spectroscopy

As shown by Wetzel et al. (2008) for ankyrin repeat proteins and Main et al. (2003b) for tetratricopeptide repeat proteins, full-consensus tandem repeat pro-
proteins are extremely stable, and often cannot be thermally denatured. In addition, the architecture of a repeat protein suggests that alteration of residues at the N- and C-termini, and therefore on the solvent-exposed phases of the capping repeats, to include bioorthogonal reactivity should not affect many intramolecular contacts and therefore should not significantly destabilise the protein. However, while repeat proteins with bioorthogonally reactive non-canonical amino acids in these positions have been produced by Simon et al. (2012), it has not yet been shown that this change does not destabilise them.

The thermal stability of both azide-containing and control versions CTPR4 and Ank6 proteins with sequences as in Table A.1 was therefore measured by circular dichroism spectroscopy as detailed in 2.3.9. Note that even the control versions in this case differ from the full consensus sequence by the addition of an N-terminal hexahistidine purification tag with an uncleaved initiator methionine and unused thrombin cleavage site, and of a C-terminal methionine. In the case of Ank6 the sole internal methionine, in the N-capping repeat, was replaced by a leucine chosen by analogy to the internal repeats.

For each protein, the circular dichroism spectrum shown in Figure 4.1 is the average from three repeat measurements at 20 °C. In addition, the normalised ellipticity at 223 nm, corresponding to one of the characteristic bands for an α-helix, was plotted against temperature for each protein in Figure 4.2.

4.3 Test Click Reactions on Proteins

4.3.1 Test Click Reactions on DARPins

Initially, in an attempt to replicate the work of Simon et al. (2012), reactions were carried out between the azide-functionalised DARPins and alkyne-functionalised PEG. However, these were unsuccessful- on an SDS-PAGE gel of the reaction mixture, no band was visible for either protein-PEG conjugate or unreacted protein. Tests omitting different constituents of the reaction mixture (Figure 4.3) determined that the combination of copper and ascorbate was
Figure 4.1: Circular Dichroism Spectra of Azide-Containing and Control CTPR4 and Ank6. All measurements were taken in 150 mM PBS at 20 °C. Protein concentrations are 4 µM (Ank6-Met), 5 µM (CTPR4-Met), 5.3 µM (Ank6-Aha), 5.2 µM (CTPR4-Aha)
Figure 4.2: Circular Dichroism Melting Point Curves of CTPR4 and Ank6. All measurements were taken in 150 mM PBS. Protein concentrations are 4 µM (Ank6-Met), 5 µM (CTPR4-Met), 5.3 µM (Ank6-Aha), 5.2 µM (CTPR4-Aha).
<table>
<thead>
<tr>
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<th>PEG</th>
<th>Copper</th>
<th>Ascorbate</th>
<th>Plastic</th>
</tr>
</thead>
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<tr>
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<td>-</td>
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<td>+</td>
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</tbody>
</table>

Table 4.1: Conditions for testing reaction conditions for protein cleavage. Components are 6-repeat DARPin with ‘extra’ His-tag (12 μM), 5kDa PEG-alkyne (40 μM), CuSO₄ (0.1 mM), THPTA (0.5 mM), sodium ascorbate (5 mM). Reactions in absence of ‘copper’ also do not include THPTA. Reaction in absence of ‘plastic’ was carried out in a glass vial to exclude the possibility of reaction with the plastic tube. Reactions were shaken for 2 hours at room temperature, then quenched with 0.2 mg/mL EDTA.

Further testing (not shown) showed no difference between reactions carried out in standard deionised water or milliQ pure water. The stability of a range of proteins was then tested in 0.1mM CuSO₄ and 5mM sodium ascorbate. In addition to ‘control’ preparations (containing methionine) of the 4-repeat TPR and 6-repeat DARPin constructs, samples of PR65 repeats 1-9.5, β-catenin (an responsible for the loss of protein, probably due to cleavage by the radical mechanism outlined by Chiou (1983).

Figure 4.3: SDS-PAGE gel of reactions testing DARPin cleavage by ascorbate. Lane numbers refer to Table 4.1.
armadillo-repeat protein), E-cadherin (an intrinsically-disordered protein) and eGFP (a β-barrel globular protein) were tested, all at 4 µM protein. All of the proteins except for eGFP were noticeably degraded as shown in Figure 4.4.

Figure 4.4: SDS-PAGE gel of reactions testing protein cleavage by ascorbate. “+” and “−” refer to reactions in the presence and absence of copper (0.1 mM as CuSO₄) and ascorbate (5 mM as sodium ascorbate). Concentration of proteins was 4 µM.

Therefore, a different reducing agent was needed. After unsuccessful trials with TCEP (tris(2-carboxyethyl)phosphine), which is used by Simon et al. (2012) but is stated by Presolski et al. (2011) to interfere with the reaction, hydroxylamine was used for future experiments. In addition, the concentration of catalyst was increased. The protein was also reacted with a DBCO (dibenzocyclooctyne)-functionalised dye in a strain-promoted copper-free click reaction in order to check that the azide was present and accessible.

Under these new conditions, the 6-repeat azide-containing DARPin was reacted with an alkyne-functionalised dye as detailed in 2.6.1. After SDS-PAGE, the band for the protein was fluorescent under UV light, showing that the dye
had been successfully conjugated to the protein (Figure 4.5).

![Fluorescent band on SDS-PAGE gel from azide-containing DARPin conjugated to fluorescein](image)

**Figure 4.5:** Fluorescent band on SDS-PAGE gel from azide-containing DARPin conjugated to fluorescein

### 4.3.2 Test Click Reactions on Other Proteins

The four-repeat CTPR expressed in 4.1.2 was successfully reacted with an alkyne-functionalised fluorescein dye in a similar manner to above, using the method detailed in 2.6.1. Again, a fluorescent band corresponding to the molecular weight of the protein was visible on an SDS-PAGE gel under UV light.

Alkyne-functionalised PR65 as expressed in 4.1.3 was reacted with azide-functionalised fluorescein using a method based on that of Sachdeva et al. (2014), but without success. Therefore, a new method more similar to the desired microgel-forming reaction was developed to test the reactivity of proteins.

### 4.4 Click Reactions Between Proteins and Fibris

The production of the intended material required a successful ‘click’ reaction to take place between the azide on a repeat protein and the alkyne on an amyloid
fibril. One way to determine if this reaction has taken place without making or characterising a material is to simply run a PAGE gel of the reaction mixture under native (non-denaturing) conditions. As amyloid fibrils are too large to enter the gel, proteins conjugated to them will also not enter it, so a band corresponding to the protein will not be seen. This method is detailed in 2.6.2.

This method was first tested with PR65 and azide-functionalised fibrils formed from the TTR-Z1 peptide (see Table 2.1). Although some difference was seen between “clicked” and “unclicked” lanes, the difference was not particularly pronounced. At this point, due to the limited success of reactions with PR65 and the successful preparation of other proteins, work on PR65 was stopped.

The method was then used to test the reactivity of the alkyne-functionalised TPR4 with alkyne-functionalised TTR-A1 fibrils, which were already known to form microgels together.
Table 4.2: Conditions for testing reaction between protein (CTPR4, 24 µM) and fibrils (TTR-A1, 1 mg/mL). Cat (P) and Cat (S) refer to catalyst mixtures described by Presolski et al. (2011) and Sachdeva et al. (2014) respectively.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Protein</th>
<th>Fibrils</th>
<th>Cat (P)</th>
<th>Cat (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>2</td>
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</tbody>
</table>

Figure 4.6: NativePAGE gel of proteins conjugated to fibrils. See Table 4.2 for reaction conditions.
It can be seen in Figure 4.6 that the bands corresponding to the protein in Lanes 1 and 2— the lanes which contained a full reaction with protein, fibrils and catalyst— are very much weaker than those in the lanes which did not contain fibrils or catalyst. This confirms that the protein is conjugating to the fibrils. If the protein were simply associating with fibrils in a non-specific manner we would expect to also see weaker bands in lanes with fibrils but without catalyst, while if the catalyst were degrading the protein we would expect to see weaker bands in lanes with catalyst but without fibrils.

4.5 Conclusions

The introduction of azides to proteins, including repeat proteins, and their use for bioorthogonal conjugation have been demonstrated in the past. However, the reaction does require optimisation for the specific proteins being used. In this chapter, the reaction was successfully optimised such that a ‘click’ reaction took place between repeat proteins and amyloid fibrils. Therefore, both components of the intended material have been synthesised and shown to react with each other in the desired manner. That they form a material, and the properties of that material, are shown in a later chapter.

As well as the native gel method detailed in 4.4, other methods could have been used to further demonstrate the ”click” reaction between repeat proteins and amyloid fibrils. Possibly the most effective would have been methods based on analytical ultracentrifugation under mildly denaturing conditions (to prevent most non-covalent binding without affecting the integrity of the extremely stable amyloid fibrils) to separate species bound to fibrils from those that are free in solution. The fibrils, with attached repeat proteins but in the absence of any repeat proteins that had not become ’clicked’ to fibrils, could then be analysed in a variety of ways.

Denaturation followed by mass spectrometry, as used in Chapter 3, is one possibility, as the mass of the peptide-repeat protein adduct would be detected.
Another is circular dichroism spectroscopy in folding conditions- in the presence of fibrils decorated with repeat proteins, negative bands at 222 and 208 nm would be seen for the $\alpha$ helices of the repeat protein as well as the characteristic 218 nm negative band of the $\beta$ sheets in amyloid fibrils (Greenfield, 2006).

A final, more direct means of proving the functionalisation of fibrils would be immunogold labelling of the repeat proteins, making them visible when the fibrils were imaged by AFM or TEM.

Due to limitations in time and availability of reagents and equipment, these experiments could not be carried out within the timescale of the project.
Chapter 5

Mechanical Testing of Microgels

5.1 Introduction

The protein-based material being produced as part of this work takes the form of microgels—soft particles with dimensions of the order of tens of microns. Artificial materials of this class have a wide range of current applications, including both for drug delivery (Malmsten, 2006) and in various consumer products including food, cosmetics and household products (Stokes and Frith, 2008). In addition, living cells can also be considered as soft objects that exist on this length scale, and the mechanical properties of cells are physiologically important (Kasza et al., 2007). For these reasons, it is desirable to develop a method to measure the mechanical properties of this class of particle.

Current methods all suffer from significant limitations. For instance, atomic force microscopy (Wiedemair et al., 2007) and micropipette aspiration (Kwok and Evans, 1981), although well developed, require the difficult and time-consuming microscopic localisation of an individual particle, and only measure the properties of a small region rather than of the particle as a whole. The technique
of capillary micromechanics developed recently by Wyss et al. (2010) addresses these issues, and was attempted as a method for characterising the new materials in this work, but suffers from problems with the reliability and reproducibility of fabricating the glass capillaries it uses, which also requires special equipment. The technique was recently translated to PDMS-based devices by Li et al. (2015), but this does not solve the additional issue that a single microgel must be reliably introduced into the measurement capillary to give a good measurement. Other PDMS-based techniques also suffer from various limitations. For instance, the real-time deformability measurements developed by Mietke et al. (2015); Otto et al. (2015) only operate over a relatively limited modulus range, while the beam-bending methods of Duprat et al. (2014) require a particular anisotropic particle shape. In this chapter, I outline the development of a new method for mechanical testing of soft mesoscale objects which can be carried out on several objects in parallel using PDMS devices which can be reproducibly fabricated using standard techniques.

5.2 Standard Capillary Micromechanics

Initially, an attempt was made to characterise protein microgels using the capillary micromechanics methods developed by Wyss et al. (2010). However, various problems were encountered. Even with the use of a laser pipette puller to fabricate micropipettes, fabrication was not reproducible— the pipette did not always have a hole in the tip to allow continuous phase to escape, and even those pipettes which were successfully made were not reproducibly identical in shape. In addition, the cylindrical shape of the micropipette made imaging difficult— it was hard to find the correct focal plane and ascertain where the boundaries of the glass were, as seen in Figure 5.1. Finally, this method involves flowing a suspension of microgels into the pipette. If more than one particle enters the micropipette and they “stack” in the tip, no data can be acquired and the experiment needs to be started again. For all these reasons, work was stopped.
on this method in favour of the newly-developed devices detailed below.

Figure 5.1: Image of an attempt at producing a micropipette for capillary micromechanics

5.3 Design of PDMS-based Devices for Mechanical Testing

Devices were designed that contained several tapered chambers in parallel, each of which fulfils the same function as the tapering tip of a micropipette. However, there are several differences in operation between the new devices and micropipettes used for capillary micromechanics. Due to the nature of PDMS-based fabrication of microfluidic devices, the cross-section of each chamber is a rectangle of constant height and steadily decreasing width rather than a circle of decreasing radius—standard device fabrication methods are essentially two-dimensional, and while Li et al. (2015) were able to produce a single PDMS channel with a circular cross-section for their devices it was felt that the parallel nature and ease of fabrication of these devices outweighed the potential inaccuracies introduced by a rectangular cross-section. The difference in cross-section shape is accounted for in the mathematical analysis of the images obtained in testing.

The devices also have large side channels which are significantly wider than the tapering channels. These side channels allow debris and the majority of microgels to pass through the device without becoming trapped in or blocking one of the tapering measurement channels. The flow rate of the suspension of
gels is varied to make sure that some gels become trapped in the measurement channels, before pure continuous phase is flowed in to remove any gels left in the side channels and start the measurement.

Another difference is the presence of multiple parallel chambers in the same device. This means that even if one chamber is not giving usable results due to particles “stacking” within the chamber, non-particle debris, or device fabrication errors, it will still be possible to obtain results from other chambers. Even if the entire device is inoperable, the presence of multiple (usually 9 or 12) identical devices on the same chip allows experiments to continue without having to fabricate a new chip, which makes the workflow more efficient. Also, if enough chambers are working well, repeat experiments can be carried out simultaneously by simply imaging several gels in adjacent chambers.

Finally, the method by which the pressure differential is applied to the particle being measured is different. In a micropipette-based capillary micromechanics measurement, flow through the micropipette stops when a particle enters the tip. Hydrostatic pressure is applied and measured either using a commercial pressure regulator or by increasing the height of the fluid reservoir. However, in these devices flow continues through the wider side channels even if all of the tapering measurement channels have been blocked by particles. In fact, even in the absence of particles, the vast majority of flow is through the side channels due to their larger cross-sectional area. The pressure differential is determined by the fluidic resistance of the side channels and the flow rate of continuous phase, which is controlled using a syringe pump.

Initial designs had a single inlet. However, this caused problems in the transition from loading of gels into the device to measuring their mechanical properties. If the flow rate of the suspension of gels was used to vary the pressure, large numbers of additional gels entered the device and made the measurements meaningless. Replacing the syringe loaded with gel suspension with one loaded with pure continuous phase often resulted in air bubbles being introduced into the device. While normally it would be possible to reverse the
flow by connecting a syringe to the outlet in order to give a droplet of liquid at the inlet, allowing a droplet-to-droplet connection without air bubbles, in this case reversing the flow causes the particles to leave the test chambers.

5.4 Fabrication of Teflon devices

This section details work done in collaboration with Yingbo Zhang

Fabricating the mechanical test devices out of fluoropolymers such as Teflon is an alternative to PDMS fabrication that presents many potential advantages. These materials are extremely fluorophilic and both hydrophobic and lipophobic, so biological molecules are less able to adsorb onto their surface. In addition, unlike PDMS they do not absorb small molecules from solution or leach residual precursor molecules into the channel, and are compatible with a much wider range of solvents than PDMS (Ren et al., 2011). Finally, Teflon is much stiffer than PDMS (Grover et al., 2008). For these reasons, Zhang (2016) developed a method based on the work of Ren et al. (2011) to fabricate microfluidic devices from fluoropolymers.

This method relies on a PDMS master— in other words, a piece of PDMS with raised features corresponding to the channels in the desired Teflon device. A slide-sized section of Teflon FEP (fluorinated ethylene propylene) (1 mm thickness, Foshan Yongmeijia Plastic Material Co., China) was heated to 275 °C on a hotplate. At this temperature, the Teflon sheet softens to the point where it deforms easily even when under pressure from the relatively soft PDMS master. A metal clamp (custom fabricated by the Department of Chemistry mechanical workshop) was then used to press the PDMS master into the soft Teflon for approximately 10 seconds before the entire assembly (master, Teflon device and clamp) was allowed to cool. PDMS masters used in this process could be re-used.

There were two methods used to produce the PDMS masters. The first was to use the same direct photolithography techniques used to produce SU-8 masters to create a reversed (positive) SU-8 master with grooves in the photoresist
corresponding to the channels in the eventual device. This obviously required a specialised photomask to be produced with the transparent and black areas reversed compared to the photomasks used for fabrication of masters for PDMS devices (see Figure 1.6 and subsection 2.7.1).

However, this method of fabrication presented problems when used for the fabrication of high-aspect-ratio channels (with a width small compared to their height). This includes the narrower sections of the measurement channels in the mechanical testing devices used in this work, which had a height of 50 µm but were only 10–15 µm wide. Often these channels were not correctly formed in the finished PDMS devices, probably due to the difficulty of patterning narrow grooves in SU-8 photoresist. The PGMEA developer would not fully wash undeveloped photoresist out of these grooves, meaning that the corresponding positive-relief features on the PDMS master would not form as seen in Figure 5.2.

![Figure 5.2: A PDMS master for producing Teflon devices in which the narrow channels have failed to form](image)

Therefore, a second method was used to produce the PDMS master. This method involved starting with a negative-relief PDMS master, which could be a set of conventional PDMS devices that had not had inlet and outlet holes punched or been bonded to glass. This master was oxidised in a plasma oven for 100 seconds then treated with trichloro(1H,1H,2H,2H-perfluorooctyl)silane
(10 \mu L, vapour diffusion) in vacuum for 5 minutes. This silane treatment gave an extremely hydrophobic surface such that when this master was used to cast a positive-relief PDMS master as in 2.7.1, the positive-relief master could easily be peeled away from the negative-relief master after curing. This method was capable of producing the high-aspect-ratio channels needed for the microfluidic mechanical testing devices, as shown in Figure 5.3.

Figure 5.3: Successfully fabricated features in a Teflon microfluidic device, master fabricated from a negative-relief PDMS master

Once the Teflon device was produced, inlet and outlet holes were drilled using an electric drill (TBM 220, Proxxon GmbH, Germany) with an 0.8 mm bit and connected to blunt-ended metal dispensing tips (1/2 inch length, 0.6 mm internal diameter, Intertronics, UK). A sheet of COC (cyclic olefin copolymer) film was used to close the channels, and the device as a whole, sandwiched between two layers of PDMS or Parafilm as “cushions” to spread compressive forces, was clamped together using a polycarbonate clamp (designed by Lianne Roode). This clamp was fitted with 5 screws on each side to allow even compression.

However, it proved to be difficult to reliably connect the metal inlet and outlet tips to the device. In addition, perfluoroalkylsilane treatment proved to be able to make PDMS devices sufficiently hydrophobic, and as found in 5.7.1 PDMS proved to be stiff enough for this application. Therefore, Teflon devices
were not used in this work.

5.5 Measurement of Mechanical Properties Using PDMS Devices

Devices were tested with a variety of particles in the 50 µm size range, in suspension in either water or FC-40 fluorinated oil. The surface treatment depended largely on the continuous phase. For aqueous continuous phases, extended plasma treatment followed by storage filled with water was used to make the devices hydrophilic, while for fluorinated oil continuous phases devices were treated with a perfluoroalkylsilane to make the device surfaces hydrophobic and fluorophilic. Although initially Aquapel (PPG Industries, Pittsburgh, PA, USA) was used as the surface treatment, this tended to cause fouling as it is simply a physical coating rather than chemically reacted with the surface as silanes are.

To use the device, the syringe loaded with continuous phase was connected first and used to ‘prime’ the device so it was filled with continuous phase, removing any air bubbles and leaving a droplet of continuous phase at the microgel inlet. This allowed the second syringe, containing a suspension of microgels or other particles in the same continuous phase, to be connected in a ’droplet-to-droplet’ manner without introducing any air bubbles. Initially, the suspension of microgels was flowed in slowly until one or more gels became trapped in the tapering channels. At this point, its flow was stopped and the flow of continuous phase started, in order to allow pressure to be applied to the microgels without the risk of introducing more microgels which would ‘stack’ on top of them. The flow rate was varied to give a range of pressures- after each change in flow rate, gels were allowed to stabilise in their new shape before an image was captured. Gels were measured both as they deformed in response to increasing pressure and as they returned to their original shape as the pressure was reduced. It was also sometimes found to be necessary to maintain a slow flow rate in the
gel-suspension channel (less than 10% of that in the continuous-phase channel) to prevent backflow. As the two channels join before the measurement area, this can simply be added to the continuous-phase flow rate to give the total rate for calculation of pressure drop.

Deformation of the microgel was measured using ImageJ (Schneider et al., 2012). From these values, elastic and bulk modulus were calculated using a method based on the work of Wyss et al. (2010) with modifications to account for the different method used to apply pressure and the cross-section of the channel being rectangular as opposed to circular.

While previous work used either a pressure regulator or gravity to directly set the hydrostatic pressure used to deform the microgels, in this method the flow rate of continuous phase is controlled instead. The pressure difference between the fluid behind and in front of the trapped particle was calculated based on the flow rate using calculations based on the analogy between electrical and fluidic circuits (Kim et al., 2006). The fluidic analogue of Ohm’s Law \( V = IR \) is \( \Delta P = RQ \), where \( \Delta P \) is the pressure difference between two points (in analogy to electrical potential difference), \( R \) is the hydraulic (as opposed to electrical) resistance and \( Q \) is the flow rate. As the fluid is considered to be incompressible, it does not matter whether mass or volumetric flow rate is used as the two are equivalent. Similarly, operating in an effectively non-compliant device at low Reynolds number means that compliance and inertial effects, the fluidic equivalents of capacitance and inductance, can safely be ignored.

The ends of the tapering measurement channels have a much smaller cross-sectional area than the larger side channels. This means that their fluidic resistance is much higher and flow through the measurement channels can be neglected when calculating the pressure difference. The dramatically simplifies the calculation, especially since it is not longer necessary to consider whether each measurement channel is blocked by a gel particle.

For the side channels, due to the high aspect ratio the fluidic resistance can be calculated using the following approximation based on Mortensen et al.
(2005):

\[ R_{h, \text{side}} = \frac{12\eta L}{wh^3(1 - 0.63\frac{h}{w})} \]  

(5.1)

where \( L, w \) and \( h \) are the channel length, width and height, and \( \eta \) is the viscosity of the fluid. As there are two side channels, the overall pressure drop is calculated by treating them as resistances in parallel:

\[ \frac{1}{R_{\text{side,total}}} = \frac{1}{R_{\text{side,1}}} + \frac{1}{R_{\text{side,2}}} \]  

(5.2)

where \( R_{\text{side,1}} \) and \( R_{\text{side,2}} \) are the resistances of the two paths from the entrance to the exit of the test chamber via the side channels, calculated using Equation 5.1. Note that the effective side channel length for this purpose varies depending on which of the test chambers contains the gel particle—this variation needs to be taken into account in the following calculations.

Figure 5.4: Schematic of a PDMS mechanical testing device labelled for resistance calculations

In the case of the devices used for testing, \( w \) and \( h \) are both 50 \( \mu \)m. The value
of $\eta$ for FC-40 according to the manufacturer’s specification is 4 cp (centipoise) = $0.004 \text{ kg m}^{-1} \text{s}^{-1}$. The shortest effective length of a side channel—from A to $A'$ in Figure 5.4—is 510 $\mu$m. The gap between chamber entrances, such as from A to B or $A'$ to $B'$ in Figure 5.4, is 145 $\mu$m, so twice this amount is added onto the effective length. The pressure drop for each measurement channel, as labelled in Figure 5.4, is calculated from the following resistance values for FC40:

<table>
<thead>
<tr>
<th>Path</th>
<th>Resistance/kg m$^{-4}$ s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A to A</td>
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</tr>
<tr>
<td>B to B'</td>
<td>$1.22 \times 10^{13}$</td>
</tr>
<tr>
<td>C to C'</td>
<td>$1.45 \times 10^{13}$</td>
</tr>
<tr>
<td>D to D'</td>
<td>$1.57 \times 10^{13}$</td>
</tr>
</tbody>
</table>

Table 5.1: Fluidic resistance values calculated for side channels in FC-40 as shown in Figure 5.4 based on Equations 5.1 and 5.2

From here, the pressure drop between the two labelled points, which is considered to be equal to the pressure drop across the particle, can be calculated using the following approximation:

$$\Delta P \approx R_{\text{side, total}} Q_{\text{cont}}$$  \hspace{1cm} (5.3)

Where $Q_{\text{cont}}$ is the flow rate of continuous phase.

The relation of pressure from the capillary walls to applied fluid pressure $p$ in a circular capillary is

$$p_{\text{wall}} = \frac{p}{\sin \alpha} \frac{R_{\text{wall}}}{2L_{\text{wall}}}$$  \hspace{1cm} (5.4)

where $p_{\text{wall}}$ is the force per unit area acting on the contact surface between the particle and the capillary, $R_{\text{wall}}$ and $L_{\text{wall}}$ are the average radius and length of the contact area, and $\alpha$ is the taper angle (Voudouris et al., 2013). This is obtained from the balance of the external forces acting on the particle in a direction parallel to the capillary. Frictional forces on hydrogels are very low (Guo and Wyss, 2011; Tominaga et al., 2008) and have therefore been ignored in this analysis, as in the work of Wyss et al. (2010).
In the case of the PDMS devices, \( p_{wall} \) can only act through the side walls of the channel and not through the floor or ceiling. This is because the floor and ceiling are parallel to the direction of flow, so pressure exerted by them can have no component in a direction parallel to the capillary. Therefore, a different relation between the pressure exerted by the walls and the fluid pressure drop is needed.

Starting with the fact that, in the absence of static friction, if the particle is stationary the force \( F_{hyd} \) from the difference in fluid pressure must be balanced by the longitudinal component of the contact force \( F_{wall} \) between the particle and the wall:

\[
F_{hyd} = F_{wall,\parallel} \quad (5.5)
\]

We then integrate each force over the surface on which it acts:

\[
\int_{A_{gel}} \Delta P \cdot \hat{n} \, dA = \int_{A_{wall}} p_{wall} \cdot \hat{n} \, dA \quad (5.6)
\]

From Equation 5.6, integrating the fluid pressure over the cross-sectional area of the channel where it is blocked by the particle and the wall pressure over the area of sidewall in contact with the particle, we obtain:

\[
\Delta P_{h_{\text{max}}} = 2hL_{\text{band}}p_{wall} \sin \alpha \quad (5.7)
\]

where \( w_{\text{max}} \) is the width of the particle at its widest point that touches the channel walls, \( L_{\text{band}} \) is the total length of channel wall in contact with the particle, \( h \) is the channel height and \( \alpha \) the taper angle. While \( w_{\text{max}} \) and \( L_{\text{band}} \) are obtained from analysing the image of the particle in the channel (see Figure 5.5), \( \alpha \) is obtained from the device design and \( h \) estimated from the characteristics of the photoresist used to fabricate the device master. While in principle a more accurate value of \( h \) could be obtained by direct measurement using a profilometer, this was not available at the time.
Figure 5.5: Image of a microgel in a mechanical testing device defining the measurements taken. Both $r_{\text{band}}$ and $r'$ are averages of the measured value of $w_{\text{min}}$ and $w_{\text{max}}$. 
Equation 5.7 can be rearranged to give:

\[ p_{\text{wall}} = \frac{1}{2 \sin \alpha} \frac{w_{\text{max}}}{L_{\text{band}}} \Delta P \]  

(5.8)

Having calculated \( p_{\text{wall}} \) using Equation 5.8, the compressive modulus \( K \) and shear modulus \( G \) can be calculated from \( p_{\text{wall}} \), \( p \) and the measured values of the longitudinal strain \( \varepsilon_z \) and radial strain \( \varepsilon_r \), defined relative to the dimensions of the particle at the smallest pressure used:

\[
K = \frac{1}{3} \left( \frac{2p_{\text{wall}} + p}{2\varepsilon_r + \varepsilon_z} \right) 
\]

(5.9)

\[
G = \frac{1}{2} \left( \frac{p_{\text{wall}} - p}{\varepsilon_r - \varepsilon_z} \right) 
\]

(5.10)

Note that the value of the radial strain \( \varepsilon_r \) is negative, as the width of the particle is decreasing as it is forced into the tapering channel.

### 5.6 Error Analysis

#### 5.6.1 Propagation of Errors

Notation: \( \alpha \) is the uncertainty in \( x \)

**Error in Pressure Calculations**

The values of \( \frac{2p_{\text{wall}} + p}{3} \) and \( \frac{p_{\text{wall}} - p}{2} \) are important for the calculation of \( K \) and \( G \).

As

\[
p_{\text{wall}} = p \frac{r_{\text{band}}}{2L_{\text{band}}} 
\]

(5.11)

\[
\frac{2p_{\text{wall}} + p}{3} = \frac{2 \left( \frac{r_{\text{band}}}{2L_{\text{band}}} \right) + 1}{3} p 
\]

(5.12)
\[
\left( \frac{\alpha^2 p_{wall} + P}{3 \rho_{wall} + P} \right)^2 = \left( \frac{\alpha P}{P} \right)^2 + \left( \frac{\alpha r_{band}}{r_{band}} \right)^2 + \left( \frac{\alpha L_{band}}{L_{band}} \right)^2
\]  

(5.13)

The value of the relative error in \( \frac{p_{wall} - P}{2} \) is the same.

**Error in Volume Change**

Volume change \( \frac{\Delta V}{V} \) can be expressed in terms of measurements as follows:

\[
V = \frac{4}{3} \pi r'^2 L' = \frac{2}{3} \pi r'^2 L'
\]  

(5.14)

Where \( r' \) and \( L' \) are the measured average radius and length of the particle (see diagram). As

\[
\frac{\Delta V}{V} = \frac{V_0 - V}{V}
\]  

(5.15)

where \( V_0 \) is the volume at the lowest flow rate used.

Cancelling the factor of \( \frac{2\pi}{3} \),

\[
\frac{\Delta V}{V} = \frac{r'^2 L' - r'^2 L'_{0}}{r'^2 L'} = \frac{r'^2 L'_{0}}{r'^2 L'} - 1
\]  

(5.16)

where \( r'_{0} \) and \( L'_{0} \) are the radius and length of the particle measured at the lowest flow rate used.

The error in this quantity,

\[
(\alpha \Delta V)^2 = \left( \frac{2\alpha r_{0}^2}{r_{0}} \right)^2 + \left( \frac{2\alpha r'}{r'} \right)^2 + \left( \frac{\alpha L_{0}'}{L_{0}'} \right)^2 + \left( \frac{\alpha L'}{L'} \right)^2
\]  

(5.17)

**Error in Strain Values**

The value \( \varepsilon_r - \varepsilon_z \) can be expressed simply in terms of measured quantities as follows:

\[
\varepsilon_r - \varepsilon_z = \left( \frac{r' - r'_{0}}{r'_{0}} \right) - \left( \frac{L' - L'_{0}}{L'_{0}} \right) = \left( \frac{r'_{0}}{r'_{0}} - 1 \right) - \left( \frac{L'_{0}}{L'_{0}} - 1 \right) = \frac{r'}{r'_{0}} - \frac{L'}{L'_{0}}
\]  

(5.18)

The most straightforward way to calculate the errors in this case is:
\[
\left( \frac{\alpha r'}{r_0} \right)^2 = \left( \frac{\alpha r'}{r'} \right)^2 + \left( \frac{\alpha r'}{r'_0} \right)^2
\] (5.19)

Similarly,
\[
\left( \frac{\alpha L'}{L_0} \right)^2 = \left( \frac{\alpha L'}{L'} \right)^2 + \left( \frac{\alpha L'}{L'_0} \right)^2
\] (5.20)

From the values of the error calculated in Equations 5.19 and 5.20, the error in \( \varepsilon_r - \varepsilon_z \) can be calculated as follows:
\[
\alpha_{\varepsilon_r - \varepsilon_z} = \sqrt{\alpha_{r_0}^2 + \alpha_{r_0'}^2}
\] (5.21)

### 5.6.2 Value of Errors

It is assumed that any errors in the value of \( p \) due to variations in the flow rate are negligible compared to other errors. The main source of error in \( p \) is therefore flow through the remaining measurement channels. However, as this is systematic rather than random error, it is not considered in this analysis. Similarly, the value of the hydrostatic resistance is dependent on the dimensions of the channels. Due to the nature of the lithographic methods used to fabricate microfluidic devices, it is assumed that the length and width of the channel are accurately reproduced from the design with negligible error (Lawes, 2005). While there is some error in the feature height of the master, and therefore the channel height, this does not vary between devices produced from the same master and is therefore, again, a systematic error. Therefore, the only source of random error is error in the measurement of distances on the images. It is estimated that these values can be measured, using ImageJ, to around \( \pm 1 \mu m \).

However, as the values of \( r_{band} \) and \( r' \) are derived from the average of the measured values of \( w_{min} \) and \( w_{max} \), the errors in the measurement of \( w_{min} \) and \( w_{max} \) are added in quadrature to give the errors in \( r_{band} \) and \( r' \).
5.7 Testing of PDMS Devices on Known Materials

5.7.1 Testing with PMMA Microspheres

As a control to determine the behaviour of the mechanical testing devices when used to test very rigid undeformable particles, devices were tested using a suspension of 50.3 µm diameter PMMA (polymethylmethacrylate) microspheres in water (Research Particles GmbH, Berlin, Germany, a gift from Dr. Saji Eapen and Dr. Robert Davies, SPD, Cambridge). As the compressive modulus of PMMA is several orders of magnitude greater than that of PDMS (Armani et al., 1999; Kim et al., 2004), this experiment tested what would happen if particles were tested that were significantly stiffer than the device itself.

![PMMA microparticles in a mechanical testing device under water flow of 1000 µL/h](image)

While the hydrostatic pressure drop across the testing chamber was not sufficient to visibly deform the particles, as seen in Figure 5.6, the device also did not visibly deform. This result means that deflection of the test chamber walls under testing conditions, especially with the very much less stiff particles this method is intended to measure, is small enough to be negligible so does not need to be accounted for in the analysis of the data.
5.7.2 Attempted Testing with PDMS Microspheres

An attempt was made to synthesise PDMS microspheres, based on the work of Di Benedetto et al. (2013), in order to determine how the devices would behave when used to test particles of a similar stiffness to PDMS. Uncured PDMS was used as the discontinuous phase in a single-junction droplet maker, with water containing 2% either AZ-900 or Tween 20 surfactant as the continuous phase. However, probably due to the properties of the surfactant used, droplets did not form- the stream of PDMS did not break up into droplets at the junction, and the output was instead a continuous stream of PDMS.

5.7.3 Testing with Alginate Microgels

The devices were tested with alginate microgels synthesised as detailed in 2.7.3. Initially, they were tested in water in a hydrophilically treated device. However, the process of transferring the alginate microgels from oil into water using PFO was not reliable- often the gels would break down or otherwise be lost. Therefore, in order to save time and materials, these experiments were only carried out in oil.

The values of \(w_{\text{max}}\) and \(L_{\text{band}}\) were measured from the images shown in Figure 5.8 using ImageJ (Schneider et al., 2012). The length of the gel \(L\) was also measured, as was the minimum width \(w_{\text{min}}\) — the radius \(r\) of the gel was taken to be the average of \(w_{\text{min}}\) and \(w_{\text{max}}\). These were used to determine the values of \(\varepsilon_r\) and \(\varepsilon_z\). As can be seen in Figure 5.7, one of the side channels became blocked with debris, so flow through this channel was taken to be zero. Therefore, the resistance values in Table 5.2 were used instead. From left to right, the three microgels in Figure 5.8 are in the channels lettered BB’, CC’ and DD’ in Figure 5.4.

From these experiments, graphs of volumetric strain \(\Delta V/V\) against volumetric stress \(\frac{2p_{\text{wall}}}{3}+p\) were drawn with error bars determined as detailed in 5.6 to give a line whose slope corresponds to the compressive modulus \(K\) of the
Figure 5.7: Alginate microgels in a mechanical testing device at oil flow of 10 µL/h. Note debris blocking right side channel.

<table>
<thead>
<tr>
<th>Path</th>
<th>Resistance/kg m⁻⁴ s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>A to A'</td>
<td>1.06 × 10¹³</td>
</tr>
<tr>
<td>B to B'</td>
<td>1.66 × 10¹³</td>
</tr>
<tr>
<td>C to C'</td>
<td>2.26 × 10¹³</td>
</tr>
<tr>
<td>D to D'</td>
<td>2.86 × 10¹³</td>
</tr>
</tbody>
</table>

Table 5.2: Fluidic resistance values calculated for side channels in FC-40 as shown in Figure 5.4 based on Equations 5.1 and 5.2, with the “bottom” side channel, representing the longer flow path, blocked.

As the extent of gelation is not necessarily uniform between microgels, it is not surprising that the results (while similar in the cases of those from channels CC' and DD') are not identical. The blocked side channel, and the debris visible in test channel BB', may also have had some effect. However, these values are broadly similar to the literature value for 2% alginate hydrogels of 8-12 kPa determined by LeRoux et al. (1999) using a parallel plate rheometer, if higher than that of 6 kPa determined for the same material by Markert et al. (2013) using AFM.
5.7.4 Testing with Soy Protein Microgels

The devices were also tested using 35 mg/mL soy protein isolate micro-hydrogels in oil (a kind gift from Dr. Marc Rodriguez-Garcia, Knowles Group, University of Cambridge). While a method does exist to transfer these hydrogels into an aqueous continuous phase, Li et al. (2015) have shown that water will flow through a hydrogel confined in a narrow PDMS capillary as well as deforming it. To avoid having to account for this effect, the experiments were only performed in oil. Again, the values of $w_{\text{max}}$, $w_{\text{min}}$, $L_{\text{band}}$ and the length of the gel $L_l$ were measured from the images shown in Figure 5.10 using ImageJ (Schneider et al., 2012).

As shown in Figure 5.11, the compressive modulus of these microgels is within a believable range for soy protein hydrogels, as seen in for example the work of Kisiday et al. (2002) or Snyders et al. (2007) both of which give values of around 1 kPa.
Figure 5.9: Determination of compressive modulus $K$ for alginate microgels

Figure 5.10: Soy protein isolate microgels in a mechanical testing device at varying oil flow rates. Numbers refer to oil flow rates in µL/h.
5.7.5 Determination of Shear Modulus

The capillary micromechanics work of Wyss et al. (2010) and Voudouris et al. (2013) also gives a means of determining the shear modulus $G$ of the material, which is given by the gradient of a plot of $P_{wall} - P$ against $\varepsilon_r - \varepsilon_z$. While these measurements were taken with the new devices, the results presented several problems. The gradient of the graph of $P_{wall} - P$ against $\varepsilon_r - \varepsilon_z$ was typically negative rather than the positive values reported in previous capillary micromechanics work. As a negative value for the shear modulus is unknown except in certain exotic theoretical metamaterials (Wu et al., 2011), it is more likely that the equations used need to be altered to take into account the different geometry of the devices in this work compared to an axisymmetric capillary. However, as this appears to only be the case for the alginate microgels and not for the protein-based microgels measured later in this work, and the alginate experiments also demonstrate an extremely poor linear relationship between $P_{wall} - P$
and $\varepsilon_r - \varepsilon_z$, this may be a function of errors in this particular set of experiments rather than a weakness of the method as a whole.

In other measurements of alginate gels, while the relative values of the errors were smaller, the graph did not give a good linear relationship between $\frac{P_{wall} - P}{2}$ and $\varepsilon_r - \varepsilon_z$. In Figure 5.12, the $R^2$ value for a linear fit in the graph to determine $G$ for the gel in channel DD’ is only 0.67, while that for the gel in channel CC’ is even lower at 0.23 and it is clear that no sensible linear fit can be obtained for the data from the gel in channel BB’.

![Figure 5.12: Poor linear fit of the graphs of $\frac{P_{wall} - P}{2}$ against $\varepsilon_r - \varepsilon_z$ for alginate microgels](image)

However, in the measurements for the soy protein isolate microgels, the graphs were much closer to linear, and a value for the gradient could be obtained (see Figure 5.13) with $R^2$ values of at least 0.85. These values were broadly in agreement with each other and with literature values for protein hydrogels such as those seen in Ramachandran et al. (2005). However, it is noticeable that the modulus appears to be lower at lower flow rates, and hence lower values of $\frac{P_{wall} - P}{2}$ and $\varepsilon_r - \varepsilon_z$. This may represent some type of transition from one regime to another, and either be a property of the device or of the material being tested. Further experiments are required to ascertain this.

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5.8 Conclusion

In this chapter, a new method for the mechanical characterisation of soft mesoscale particles is developed. While conceptually based on existing capillary micromechanics methods, this method has several advantages. For instance, the PDMS devices it uses can be easily and identically fabricated in large numbers, and their design means that they are easier to use and can measure multiple particles simultaneously. These devices were shown to be able to measure the mechanical properties of different varieties of hydrogel, and also work with non-hydrogel particles in water. The values obtained for the compressive modulus were in good agreement with the literature, as were those for shear modulus in some cases.

Currently the measurements obtained from these new devices are not particularly precise, but improvements in image acquisition or processing could dramatically increase this precision in the future. In addition, further study
is needed to determine the reliability of the values obtained for shear modulus from the results obtained with these devices. However, they work well enough for comparative measurements of the compressive modulus, as is detailed in the next chapter where they are used to characterise a new class of materials.
Chapter 6

Microgels from Repeat Proteins and Amyloid Fibrils

6.1 Summary

In this chapter, the alkyne-functionalised amyloid fibrils produced and characterised as detailed in Chapter 3 and the azide-functionalised proteins produced and characterised as detailed in Chapter 4 are combined to form a nanomaterial. As shown in 4.4, the reaction between the azide and alkyne components of the material was successfully optimised to give a good level of cross-linking between the repeat protein and amyloid fibril components of the material. The decision was taken to produce this material in the form of microgels, both as part of a broader ongoing research programme of work on microgels based on proteins and other biomolecules (Shimanovich et al., 2015; Zhou et al., 2015), and because microgels are of interest for controlled release of drug molecules (Alvarez-Lorenzo and Concheiro, 2014; Jiang et al., 2014). The microgels were formed using droplet microfluidics as detailed in 2.7.3 and characterised by a
variety of methods. Although time limitations precluded a full test of the responsive behaviour of functional microgels, the results outlined in this chapter show that a new class of materials with a range of potential applications has been developed.

6.2 Initial Experiments

Initially, experiments were carried out using the droplets made from TTR-A1 fibrils (see Chapter 3) and the azide-functionalised CTPR4 prepared in 4.1.2 and characterised in 4.3.2 to determine whether there was a difference in the behaviour of droplets in which the ‘click’ reaction had taken place and those formed without the copper (I) catalyst, resulting in no reaction. If the reaction did take place in the droplet phase as it does in the bulk (see 4.4), then the droplets which formed microgels would behave differently from the unreacted ‘control’ droplets— the latter would simply behave as drops of water in oil.

The gel-forming and control droplets were initially tested in various microfluidic devices. When flowed through a small microfluidic nozzle similar to the mechanical testing devices in Chapter 5, the droplets made with the catalyst became stuck, while those made without it went through the nozzle or broke up.

The behaviour of droplets in a high-salt solution (5 M zinc bromide) was also tested. A droplet of aqueous solution in oil that has not formed a material will simply dissolve if the continuous oil phase is replaced by an aqueous one. On the other hand, a hydrogel droplet will maintain its integrity, shrink due to osmotic loss of water in a high-salt solution, then swell again when the high-salt solution is itself replaced with pure water. The protein microgel droplets exhibited the behaviour expected for a hydrogel. Finally, the hydrogel droplets were stable in a capillary for a period of days at 37 °C, as shown in Figure 6.1, whereas water-in-oil droplets produced under the same conditions tended to coalesce.
6.3 Confocal Microscopy

In order to show incorporation of repeat proteins into the microgels, microgels were made using azide-functionalised CTPR4 that had previously been fluorescently labelled with FITC. These microgels were then imaged on a Leica SP5 confocal microscope, using a 492nm laser for excitation, as shown in Figure 6.2. Several attempts were made to label the amyloid fibril components of these droplets with Nile Red, which exhibits augmented and blue-shifted fluorescence in contact with amyloid (Mishra et al., 2011), in order to localise them and give a superimposed image. These were not successful, probably due to ‘cross-talk’ between the two dyes as the emission of FITC at 518 nm can excite Nile Red, whose excitation peak is at 530 nm. Therefore, the FITC and Nile Red channels gave indistinguishable images.
Figure 6.2: Confocal image of a suspension of microgels, produced from azide-functionalised CTPR4 labelled with FITC before microgel formation and 100% TTR-A1 fibrils, in FC-40 fluorinated oil. Image is of 518 nm emission in response to excitation at 492 nm.
6.4 Mechanical Testing

As well as the experiments above, the protein-based microgels were tested in the mechanical testing devices developed in Chapter 5. These experiments determined the mechanical properties of the new material that had been produced.

6.4.1 Mechanical Testing of Microgels from CTPR4 and Amyloid Fibrils

Initial tests were carried out using the CTPR4 microgels which had also been characterised in 6.2 and imaged by confocal microscopy in 6.3. These experiments were in fact the first use of the mechanical testing devices—before they were tested with known materials. Therefore, an earlier version of the devices was used here, in which there was only one inlet rather than the Y-shaped double inlet of the devices used in other sections of this work. This meant that the flow rate of continuous phase could not be varied independently of the rate at which microgels were potentially introduced to the channels, as can be seen from the fact that new microgels appear in some of the channels (for instance the second channel from the bottom in Figure 6.3) during the measurement, rendering data taken from those channels useless.

Calculations of the mechanical properties of the microgels in the top and bottom channels were carried out using the methods detailed in Chapter 5. While the data are not very high quality due to the small number of points and large errors, they do show that at least some of the microgels here are behaving as gels (though relatively weak gels) rather than liquid droplets.
Figure 6.3: CTPRA/amyloid microgels in a mechanical testing device at varying oil flow rates. Numbers refer to oil flow rates in µL/h.
Figure 6.4: Determination of compressive modulus $K$ for CTPR4/amyloid microgels in Figure 6.3. Gradient of the graph corresponds to the value of $K$.

Figure 6.5: Errors comparable in size to the value of $\varepsilon_r - \varepsilon_z$ in the determination of $G$ for CTPR4/amyloid microgels in Figure 6.3.
6.4.2 Mechanical Testing of Microgels from TNKS2ARC4 and Amyloid Fibrils

After the newer design of mechanical testing devices (with double inlets) were tested on materials of known properties in Chapter 5, they were used to test microgels made from TTR-A1 fibrils and the azide-functionalised TNKS2ARC4 produced as detailed in 2.3.4 using the method detailed in 2.7.3. By this point, due to experience gained in testing the devices with other materials, it was clear that more data points needed to be acquired to obtain a good fit in the linear regression. In addition, these microgels were tested both while increasing and decreasing the flow rate (and hence the pressure) in order to check for any potential hysteresis effects, as shown in Figure 6.6. These measurements are referred to as "forward" and "back" respectively.

As shown in Figure 6.7, the compressive modulus of the TNKS2ARC4 microgels is similar to that of the soy protein isolate microgels measured in 5.7.4. It is not clear why the modulus of CTPR4 microgels was so much lower, but the CTPR4 data are much less reliable due to being derived from fewer measurements. Alternatively, the CTPR4 droplets might not have gelled as fully as the TNKS2ARC4 droplets for various reasons, including old click catalyst and lower incorporation of azide in the protein.

In all of the TNKS2ARC4 measurements in Figure 6.7, the lines for forward and backwards measurements are parallel to within error.

Similarly, the values of shear modulus $G$ obtained for the TNKS2ARC4 microgels at room temperature were within a believable range for the shear modulus of a protein-based hydrogel, as shown in Figure 6.8. In this case the modulus appeared to be lower when measured with decreasing flow rate, which may be due either to a hysteresis effect or to damage to the gel as it is forced into the tapering channel.
Figure 6.6: TNKS2ARC4/amyloid microgels in a mechanical testing device at varying oil flow rates. Numbers refer to oil flow rates in µL/h. Red numbers denote images taken while increasing the flow rate, while images with blue numbers were taken while decreasing the flow rate.
Figure 6.7: Determination of compressive modulus $K$ for TNKS2ARC4/amyloid microgels in Figure 6.6. Gradient of the graph corresponds to the value of $K$. 
Figure 6.8: Determination of shear modulus $G$ for TNKS2ARC4/amyloid microgels in Figure 6.6. Gradient of the graph corresponds to the value of $G$. 

Left channel

Forward $G = 1800 \pm 160$ Pa
Back $G = 1400 \pm 180$ Pa

Centre channel

Forward $G = 3700 \pm 400$ Pa
Back $G = 3000 \pm 400$ Pa

Right channel

Forward $G = 2300 \pm 180$ Pa
Back $G = 1550 \pm 250$ Pa
6.5 Thermal Response of TNKS2ARC4/Amyloid Microgels

Whereas full-consensus tandem repeat proteins consisting of more than two or three repeats are so stable that they cannot be denatured thermally (see the work of Wetzel et al. (2008) or Section 4.2), this is not the case for naturally evolved tandem repeat proteins. For instance, TNKS2ARC4 has a melting point in the region of 47 °C (Guettler et al., 2011). Therefore, the properties of a material consisting of amyloid fibrils linked by TNKS2ARC4 might change if the material were heated to a temperature higher than the melting point of the protein, so that the links consisted of unfolded rather than folded protein.

A new batch of TNKS2ARC4/amyloid microgels was produced and tested as in 6.4.2. Initially, these gels were tested at room temperature. With the microgels still in the test chamber, the device was heated to 55 °C using a WP-16 heated microscope stage connected to a TC-124A temperature controller (Warner Instruments, Hamden, CT, USA). A temperature significantly higher than the known melting point of the protein was chosen because the temperature controller only controls the temperature of the stage, and the actual temperature within the microfluidic channels may be lower. Images of the gels under testing are shown in Figures 6.9 and 6.11.

Mechanical testing of the gels at room temperature gave a compressive and shear modulus similar to, if slightly lower than, those found for the batch of gels measured in 6.4.2, as shown in Figure 6.10. This variation between batches could be explained by various reasons, including components of the catalyst degrading and inaccuracies in measuring reagents.

The gels behaved in an unexpected way at 55 °C. Although the reduction in their compressive modulus could potentially be explained by the denaturation of the repeat protein component, the gels also appeared to be breaking down or losing material. This is shown by the fact that their volume continued to decrease under flow of FC-40 oil continuous phase (see Figures 6.11 and 6.12).
Figure 6.9: TNKS2ARC4/amyloid microgels in a mechanical testing device at room temperature and varying oil flow rates. Numbers refer to oil flow rates in µL/h. Red numbers denote images taken while increasing the flow rate, while images with blue numbers were taken while decreasing the flow rate even as the flow rate was decreased, whereas in all previous experiments at room temperature the gels returned to close to their original volume as the flow rate decreased.

This constant decrease in volume, even under decreasing flow rate, may be partly or entirely responsible for the apparent decrease in the compressive modulus, so it is not clear that the compressive modulus actually does decrease at temperatures above the melting point of the repeat protein component.

Interestingly, the gels appeared to continue to shrink even once the heating had been switched off and the temperature allowed to return to room temperature. Therefore, whatever effect is responsible for the breakdown of the microgels at elevated temperatures—whether it is related to the protein components of the gels or their fluorosurfactant coating—appears to be irreversible, as the gels continue to lose material even once returned to a lower temperature. This apparent breakdown of the gels requires further investigation, but this was not possible due to time limitations.

As can be seen in Figure 6.13, the value of G remains constant or only
Figure 6.10: Graphs for the determination of $K$ and $G$ for TNKS2ARC4/amyloid microgel at room temperature in Figure 6.9
Figure 6.11: TNKS2ARC4/amyloid microgel in a mechanical testing device at 55 °C and varying oil flow rates. Numbers refer to oil flow rates in µL/h. Red numbers denote images taken while increasing the flow rate, while images with blue numbers were taken while decreasing the flow rate.

Figure 6.12: Graph for the determination of $K$ for TNKS2ARC4/amyloid microgels at 55 °C in Figure 6.11. Gradient of the graph corresponds to the value of $K$. 

Forward $K = 600 \pm 60$ Pa
Figure 6.13: Graph for the determination of $G$ for TNKS2ARC4/amyloid microgels at 55 °C in Figure 6.11 Gradient of the graph corresponds to the value of $G$.

decreases slightly at 55 °C compared to the room temperature values shown in Figure 6.10. While this decrease is apparently more pronounced at decreasing flow rates, this cannot be relied on due to the apparent breakdown of the gels detailed above. If it is indeed the case that the mechanical properties of these gels do not change upon thermal denaturation of the repeat protein component, then their mechanical properties probably rely more on the amyloid component than on the elastic properties of the repeat proteins. This would limit the use of the gels to provide a mechanical response to a chemical stimulus, though they would probably still be able to provide a chemical response to a mechanical stimulus. Again due to time limitations, this effect could not be investigated further.

6.6 Comparison to Other Nanomaterials

Researchers have used a wide range of methods, both macroscopic and microscopic, to measure the mechanical properties of protein hydrogels and other hydrogels and soft materials of biological interest (Markert et al., 2013), including cells (Ravetto et al., 2014). The most popular method is parallel plate
Table 6.1: Comparison of elastic or storage modulus of a range of protein and other biological hydrogels with the values obtained in this work. ELPs are Elastin-like Peptides. For fibrinogen/thrombin, salt content gel as well as method varies between the two values. Gelatin/agarose composites are a range of different ratios of gelatin to agarose. For lysozyme, modulus varies with concentration. For monocyte cells, modulus varies depending on degree of deformation.

<table>
<thead>
<tr>
<th>Material</th>
<th>Value (kPa)</th>
<th>Method</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose (1%)</td>
<td>3.6 ± 0.7</td>
<td>AFM</td>
<td>Markert et al. (2013)</td>
</tr>
<tr>
<td>Agarose (2%)</td>
<td>6 ± 0.9</td>
<td>AFM</td>
<td>Markert et al. (2013)</td>
</tr>
<tr>
<td>ELPs (various)</td>
<td>0.24-15</td>
<td>Parallel Plate Rheometer</td>
<td>Trabbic-Carlson et al. (2003)</td>
</tr>
<tr>
<td>Fibrinogen/thrombin (25 mg/mL)</td>
<td>0.5 ± 0.1</td>
<td>AFM</td>
<td>Markert et al. (2013)</td>
</tr>
<tr>
<td>Fibrinogen/thrombin (20 mg/mL)</td>
<td>9.29-17.24</td>
<td>Parallel Plate Rheometer</td>
<td>Murphy and Leach (2012)</td>
</tr>
<tr>
<td>Gelatin-Agarose Composites</td>
<td>0.2-0.85</td>
<td>Micropipette Aspiration</td>
<td>Chan et al. (2014)</td>
</tr>
<tr>
<td>Lysozyme Hydrogels</td>
<td>1-4</td>
<td>Parallel Plate Rheometer</td>
<td>Yang et al. (2008)</td>
</tr>
<tr>
<td>Monocyte Cells</td>
<td>0.8-4</td>
<td>Capillary Micromechanics</td>
<td>Ravetto et al. (2014)</td>
</tr>
<tr>
<td>TPR Gels</td>
<td>0.27</td>
<td>Microrheology</td>
<td>Grove et al. (2010)</td>
</tr>
<tr>
<td>CTPR/Amyloid Microgels</td>
<td>0.19-0.42</td>
<td>Single Inlet Device (K)</td>
<td>Figure 6.4</td>
</tr>
<tr>
<td>TNKS2ARC4/Amyloid Microgels</td>
<td>1.5-2.5</td>
<td>Double Inlet Device (K)</td>
<td>Figure 6.7</td>
</tr>
</tbody>
</table>

rheometry, a macroscopic and (usually) dynamic method, which therefore obtains a value for the storage modulus rather than the bulk elastic modulus. As can be seen in Table 6.1, particularly the values obtained by Markert et al. (2013) and Murphy and Leach (2012) for relatively similar materials, the values obtained for the modulus of a hydrogel are extremely method dependent. In addition to the consideration of whether the method used is static or dynamic and macroscopic or microscopic, we must consider whether it measures the whole material (such as capillary micromechanics) or only a small area of it (such as AFM or micropipette aspiration).

Nonetheless, over a wide range of materials and measurement techniques, it can be seen that the elastic modulus of protein-based nanomaterials tends to fall in the range from 100 Pa to 10 kPa. The hydrogels produced and measured in this chapter also have compressive and shear moduli falling within this range. From this, it can be said that the measurements of their mechanical properties obtained by the new variation of capillary micromechanics developed in Chapter 5 are believable, although further work is required to properly validate this method and establish whether, and how, measurements obtained using it can be compared to those using other mechanical testing methods.
6.7 Conclusions

In this chapter, I detail the production and characterisation of a new class of protein-based materials. These materials clearly have some properties of a protein hydrogel. More research is required to further characterise these materials, and in particular to examine and apply their predicted chemical responsiveness to a physical or mechanical stimulus. In addition, the method used to produce the materials needs to be improved so that they do not break down at high temperatures.
Chapter 7

Conclusions and Further Work

In this work, I have developed a new class of protein-based materials and a microfluidic method which was used to characterise them. While it was not possible to fully test the responsive behaviour of the materials due to time constraints, they are a promising avenue for future research into drug-delivery methods or protein-based sensors and actuators. The mechanical testing devices I developed to characterise these materials could be applied to the measurement of the mechanical properties of a wide range of mesoscale objects, including polymer microparticles, hydrogels, and living cells.

The initial part of this work involved producing repeat protein and amyloid components for the desired material that contained azide and alkyne components respectively, allowing them to be covalently linked together by a copper-catalysed ‘click’ reaction. In the case of the repeat protein component, various methods were tried, including amber codon suppression which has the advantage that a wider range of non-canonical amino acids can be added compared to the relatively small range available using methionine-replacement techniques. This would have opened the possibility of using other varieties of ‘click’ reaction that
do not involve copper catalysis, and is an interesting potential avenue for future research. However, the original methionine-replacement method, with some optimisation, proved to be the most efficient in terms of time and resources so was the method eventually used.

While ‘clickable’ repeat proteins were already known in the literature at the time that this project started, ‘clickable’ amyloid fibrils were not. The nature of the material meant that they were required, as the material was assembled from fibrils that had already self-assembled from peptide monomers. Therefore, a method was developed to form fibrils that displayed an alkyne functionality on their surface, allowing a wide range of chemical species, including functional proteins, to be attached to them via a ‘click’ reaction. As the peptides that form these fibrils are made using solid-phase peptide synthesis, which is a chemical rather than biological method, the alkyne functionality on their surface could in principle be replaced by any of an extremely wide range of chemical functionalities to allow the cargo species to be attached to the fibrils by means of other reactions such as strain-promoted azide-alkyne cycloaddition or a tetrazine-norbornene ‘click’ reaction. This is therefore a novel and extremely general method for using amyloid fibrils to organise molecules.

After the components were produced, characterised, and tested to show their reactivity with other ‘clickable’ molecules and with each other, they were used to form microgels. These microgels were characterised by various means, including a new method which improves in several respects, such as reproducibility and the ability to perform multiple measurements in parallel, on existing capillary-micromechanics methods. However, it is currently only able to measure the compressive modulus of microgels, and not their shear modulus. In principle, all the data needed to measure the shear modulus of a material are acquired using this new method, but the analysis of these data presents problems as the methods used to analyse existing capillary-micromechanics measurements give clearly incorrect values or no values at all. Further work is needed to determine how the different geometry of my devices affects the analysis of data to give a
value for the shear modulus.

Microgels were produced using both consensus-designed repeat proteins and TNKS2ARC4, a natural repeat protein that has well-characterised ligand binding abilities and a significantly lower melting point than consensus-designed proteins. The aim of using TNKS2ARC4 was to explore whether the microgels could bind ligands and release them in response to thermal denaturation of the repeat protein component. Although it was not possible to perform these experiments within the timescale of the thesis, the TNKS2ARC4 microgels were characterised at temperatures above the melting point of the repeat protein. They appeared to exhibit a change in their mechanical properties, but this was masked by a marked decrease in volume of the gels due to loss of material for an unknown reason.

7.1 Further Work

A wide range of further experiments are possible with these protein microgels. First, the method of producing them needs to be improved so that they do not break down at high temperatures—or alternatively the repeat protein component replaced with one that denatures at a lower, physiologically-relevant temperature. Once the new mechanical testing method is improved to allow measurement of shear as well as compressive modulus, the change in mechanical properties of the gel with increasing temperature could be measured.

Due to time constraints, it was not possible to perform many experiments that would have yielded useful information about the properties of the material that was produced, and further proved that it had the intended structure. Probably the most important of these would be nano-IR, as discussed in Chapters 3 and 4 which allows the localisation of infrared absorptions, and hence of particular covalent bonds, by scanning probe microscopy. In this case, nano-IR would be used to observe both the ‘clicked’ amyloid fibrils described in Chapter 3 and the final material in order to localise the distinctive absorption of
the triazole linkage produced by a ‘click’ reaction to the fibrils. Standard FTIR could also be useful to simply detect the presence of a triazole linkage in the material, though it would not be able to localise it.

In addition, the structure of the microgels could be examined by scanning electron microscopy (either standard or environmental) or transmission electron microscopy. In the latter case, while the results from Chapter 3 show that amyloid fibrils are visible under TEM without any negative staining, staining of some form might be necessary in order to visualise the repeat protein component of the material. In addition, the microgels might be too thick and contain too much for TEM to give useful information, in which case TEM samples would have to be produced as thin films instead.

More importantly, it was not possible to perform experiments to prove the ‘smart’ nature of the materials formed with TNKS2ARC4. The obvious experiment here would be to use light microscopy to monitor the release of a fluorescent TNKS2ARC4-binding peptide such as those developed by Xu et al. (2017) as the material was heated past the melting temperature of TNKS2ARC4. The gels would either be produced with these peptides already encapsulated or allowed to take the peptides up from aqueous solution, and their properties measured in water in already existing microfluidic droplet trapping devices similar to those developed by Huebner et al. (2009) as well as in the mechanical testing devices developed as part of this work. Release of a bound ligand as the repeat protein unfolded under conditions of either elevated temperature or mechanical stress would show that the new material gave a chemical response to a mechanical stimulus, showing that it is a “smart nanomaterial”.

CD spectroscopy of this material would also be desirable, as at room temperature, the CD spectrum of the material would show peaks for both beta-sheet-rich amyloid fibrils and alpha-helix-rich ankyrin repeat proteins. However, when the material was heated, the alpha signal would be replaced by a signal for unstructured protein. It might also be possible to detect unfolding in response to force by circular dichroism, but this would require a custom-built setup to apply
force to the material in the CD spectrometer which might not be practicable.

### 7.2 Importance of Findings

This work is the first example of repeat proteins and amyloid fibrils being combined with each other as components of the same material. Therefore, it is best considered in terms of its contribution to the hitherto separate fields of repeat protein-based and amyloid fibril-based nanomaterials.

Various researchers have synthesised and characterised materials from repeat proteins. However, none of these materials hitherto has been able to exhibit reversible ligand binding and release when immersed in water or buffer, as would be required for applications such as drug delivery.

One example is the films produced by Grove et al. (2013). These consist of designed CTPR repeat proteins that self-organise into regularly packed arrays upon drying. They also contain ligand-binding sequences, allowing them to bind other peptides that also form part of the material. However, as these films are only held together by packing forces in the absence of solvent, they break down in water, which limits their potential applications until some method is found to chemically cross-link them without affecting their functionality.

Gurunatha et al. (2016) used mutual recognition and affinity of repeat proteins to drive the self-assembly of gold nanoparticles. While this is an interesting example of using the properties of repeat proteins to produce a material, it does not usefully incorporate their ligand-binding or recognition functionality into one.

In the work of Nicholes et al. (2015), the functionality of repeat proteins is used as a component of a nanodevice, specifically a switch to modulate enzyme activity depending on the presence or absence of a ligand. This is a solution-based device, though, lacking the distinctive practical advantages of a solid material.

Perhaps the closest previous work is the ligand-binding repeat-protein-based
hydrogels of Grove et al. (2010). These are solid materials that will bind ligands. However, the material will lose its structure once the protein unfolds, and therefore cannot release the ligand once bound without eroding away. This limits its applications, as it is ‘single-use’ and care must be taken that the release of free repeat protein into a biological system does not itself have an effect. In contrast, adding amyloid fibrils to covalently hold the material together allows the repeat protein to unfold and release the ligand without compromising the network structure of the material—though its mechanical properties may well change, it will not break down.

Amyloid fibrils have been used both as structural components for protein-based materials (Knowles et al., 2010) with a wide range of function derived from the fibrils themselves (Li et al., 2014; Shimanovich et al., 2015) and as scaffolds for organising other proteins (Baxa et al., 2002; Forman et al., 2012, 2013) in the past. However, current methods for attaching proteins to amyloid fibrils, either by indiscriminate cross-linking (Pilkington et al., 2010) or by conjugating them to peptides that are then incorporated into the fibril (Hudalla et al., 2014), limit the range of proteins that can be organised in this way. The new method developed in Chapter 3 allows a much wider range of proteins to be conjugated to fibrils. Relying on this method, this work is to my knowledge the first example of repeat proteins being conjugated to amyloid fibrils while retaining their structure and function.

Finally, this work has contributed to the separate field of microfluidics. The method detailed in Chapter 5 for measuring the mechanical properties of microgels, though it requires some further work, particularly regarding data analysis, before it is fully usable, improves on the existing state of the art in capillary micromechanics in terms of repeatability, ease of use and the capacity to make parallel measurements.
Appendix A

Sequences of Proteins and DNA Constructs

Proteins Ank6, CTPR4 and TNKS2ARC4 were expressed both as ‘control’ proteins containing only natural amino acids (sequences as below) and with methionine replaced by azidohomoalanine. In PR65, X in the protein sequence denotes the non-canonical amino acid propargyllysine. All constructs were codon optimised for expression in *E.coli*. DNA sequences are given from the initiator methionine ATG codon to the first stop codon (excluding Amber codons which are read through in the case of PR65).

A.1 Sequences of Proteins
<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ank6</td>
<td>MRGSHHHHHHGLVPRGSDLGKKLLEAARAGQDDEVRILLANGADVNAKDKDGYTPLHLAAREGHEIVELLKAGADV NAKSKDGYTPLHLAAREGHEIVELLKAGADVNAKDKDGYTPTLH LAAREGHEIVELLKAGADVNAKDKFGKTAFDIDNGN EDLAEILQM</td>
</tr>
<tr>
<td>CTPR4</td>
<td>MRGSHHHHHHGLVPRGSAEAWYNLGAYYYKQGDYQKAI EYYQKALELDPNNAEAWYNLGAYYYKQGDYQKAI3 EDYQKALE LDPNNRSAEAWYNLGAYYYKQGDYQKAIEYYQKALELDPNNM</td>
</tr>
<tr>
<td>TNKS2ARC4</td>
<td>MRHHHHHHSDLSDLVPRGSGNSEADRQLLEAAKAGDVET VKKLCTVQSVNCRDIEGRQSTPLHFAAGYNRVSVVEYLLQ HGADVHAKDKGGGLVPLHACSYGYHEVAELLVHGAVV NVADLVKFTPLHLAACEKGYEICKLQLQHGADPTKNNRD GNTPLDLMGDTDIQDLDLGDAALM</td>
</tr>
<tr>
<td>PR65</td>
<td>MAAADXDDSLYPIAVLDELDRENEDVQLRLNSIKKLSTIALAL GVERTRSHELLPFLTDTDITYDEDEVLLALAEQLGTFTTLVGG PEYVHCPLPPLASLTVEETVVRDKAVESLRAISHEHSPSD LEAHFVPLVKRLAGGDWFTSRSTACGLFSCYVPRVSSAVK AELRQYFRNLCSDDTMPVMRRAASKLGEFAKVLELDNVK SEIPMFSNLASDEQDSVRLAVEACVNAQLLQPQEDLEAVL MPTLRLQAEKDJSWRVRYMVADKFTELQKAVGPEITKTD LVPAFQNLKMDCEAVRRAASHKVKEFCENLSADCRENVI MSQILPCKELVSANQHVKSALASVIMGLSPILGKDNTIEH LLPLFAQLKDCEPVRLNIISNLDCVNEVIGRQLSQQSLPA IVELEADAKWRVLIAIEYMPLLAGQLGVEFFDEKLNLC MAVLVDHYAIREAAATSNLKLLVEKFGEWAHATIPKV LAMSGDPNYLHRMTTLFCINVLSEVCQGDIITTKHMPLTV LRMAGDPVANVRNFLQKIGPLDNSTLQSEVKPILEKL TQDQDVVKYFAQETLVSXAHHHHH</td>
</tr>
</tbody>
</table>

Table A.1: Amino Acid Sequences of Repeat Proteins Studied in this Work
A.2 Sequences of DNA constructs

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ank6</td>
<td>ATGCAGGGGTTCATCATCATCATCATCATGATGTCTGGTGCAGGGGTTCTCATCATCATCATCATCATGGTCTGGTTCCGCGTGAGATCCTCGTGAAGGATTCGGGATGATGAAGTTCGTATTCTGCTGGAAATTGTTGAAGTTCTGCTGAAAGCCGGTGCCGATGTGAACGCCAAAGACAAAGACGGCTATACCCCTCTGCACCTGGCTGCACGCGAAGGCCACCTGGAAATCGTAGAGAGGGCCATTTAGAGATTGTCGAGGTACTGCTGAAAGCGGGTGTCAGACGTTAACGCGAAAGATAAAGACGATACACTCCGCTGCACCTGGCAGCTCGCGAGGGTCATCTAGAGATCGTGGAAGTCCTGCTGAAAGCTGGGGCGACGTGAATGCAAAAGACAAATTTGGTAAAACCGCCTTGACATTAGCATCGATAATGGCAATGAAGATCTGGCAGAAATCCTGCAGATGTAA</td>
</tr>
<tr>
<td>CTPR4</td>
<td>ATGCAGGGGTTCATCATCATCATCATCATCATGATGTCTGGTGCAGGGGTTCTCATCATCATCATCATCATGGTCTGGTTCCGCGTGAGATCCTCGTGAAGGATTCGGGATGATGAAGTTCGTATTCTGCTGGAAATTGTTGAAGTTCTGCTGAAAGCCGGTGCCGATGTGAACGCCAAAGACAAAGACGGCTATACCCCTCTGCACCTGGCTGCACGCGAAGGCCACCTGGAAATCGTAGAGAGGGCCATTTAGAGATTGTCGAGGTACTGCTGAAAGCGGGTGTCAGACGTTAACGCGAAAGATAAAGACGATACACTCCGCTGCACCTGGCAGCTCGCGAGGGTCATCTAGAGATCGTGGAAGTCCTGCTGAAAGCTGGGGCGACGTGAATGCAAAAGACAAATTTGGTAAAACCGCCTTGACATTAGCATCGATAATGGCAATGAAGATCTGGCAGAAATCCTGCAGATGTAA</td>
</tr>
</tbody>
</table>
**TNKS2ARC4**

ATGGCTCACCACCATCACCATACACTCGGATCTGCGGA<br>TCTTGTTTCCGCGTGGATCCGGATATAGCGAAGCAGATTTGA<br>AACCGTAAAACACTTGTTACGTCGCTACGACCCGCCTGCAAT<br>TTCGCTGCAACATGGTCAAGTCATGTGGAAATTTACCC<br>CTCTGCTGCAACATGGTCAAGTCATGTGGAAATTTACCC

**PR65**

ATGTCCCTTATACGTAGGTATTGGAAAAATTAAGGGCCT<br>TGTCGCAACCACCTGCTCTTTTTGGAAAATATTTGAAG<br>AAAAATGGTCAAGACTTTTTTATGAGGCGGAGTAAAGG<br>TGATAATGCGCAAAACCAAGAAAATTGGAATTGGTTTG<br>GATATTAGATACGCAATATTGAAAAATATGCTATCTACTATAG<br>AAGCTCTTTGCTGGCCCGCGCGCCGCTCCCAAAATCGGATCTGGTTCCGCGTGGATCCA

CTGGCGGCGGCCGACTAGGACGACTCGCTGTACCCCATC<br>GCGGTGCTCATAGACGAACTCCGCAATGAGGACGTTCA<br>GCTTCGCCTCAACAGCATCAAGAAGCTGTCCACCATCG<br>CCTTGGCCCTTGGGAGGCCCAGAGTACGTGCACTGCCTG<br>CTGCCACCGGTGGAGTGCGTCCTGGCCCAAGCAGTGGAAGA<br>CAGTGGTGCAGCAGAAGCGAGTGCTCTTACCGGACC

**129**
| GGAGTTTGCCAAGGTGCTGGAGCTGGACAACGTCAA |
| GAGTGAGATCATCCCCATGTTCTCCAACCTGGCCTCTG |
| ACGACGGACTCGTGCTGGAGCTGGGAGGGGACAGC |
| GTGGTGAACATCCAGCTTGCCCTCTGTGCCAGGAGATC |
| TGGAGGCCCTTGTTGATGCCAACACTTGCACTCAGTTG |
| GAAAGACACAGTGGCGCTGGCCTACATGGTGGCTG |
| ACAAGTTCACAGAGCTCCAGAAAGCAGTGGGGCCTGA |
| GATCACCAAGACAGACCTGGTCCCTGCCTTCCAGAACC |
| TGATGAAAGACTGTGAGGCCGAGGTTGAGGGCCGAGC |
| CTGCCAAGTCTTGCCCTGGCCTAGTCTCATAGGCTGCTC |
| CTCATCTTGGGCAAAGCAAACACCATCGAGACCTC |
| TGAGGTGCGCTGACATGATCTCTACTAAGCTTGGAGCTG |
| TGAACGAGGTTGATGGCACTCCGGCAGCTGGTCCCTCC |
| GTGCTCCGATGGCTGTGAGCTGGTGGAGCTTCTT |
| AGAAACTAACTTGTTGATGCTGTTTGGGGATATA |
| CAGTTGATGCTGACTCCACACTCCCAGTCAGAGAAGGGAGG |
| GAGGAGGCTGTCTGCCAGGCTGACTCAGGCTCTT |
| GCCATATGCGACGCTGCTGTGCTGCTGCTGAGGACG |
| ACCACCAAGCAGACATGCTACCCACAGGTCTGCTGCAG |
| TGTTGCCGACGCTGCTGCTTGGCAATGTGGCAGT |
| AGTCTCAGATGACAGATGAGGCCATCTGGAGAAGC |
| ACCGTGCAGATGACAGATGAGGCATCCCAGTCAGAGAAGG |
| GACACCAAGCAGACATGCTACCCACAGGTCTGCTGCAG |
| CACCACCAAGCAGACATGCTACCCACAGGTCTGCTGCAG |

Table A.2: Sequences of Constructs Coding for Proteins Used in this Work
Appendix B

Aggregation Kinetics of TTR105-115 Peptide

The aggregation kinetics of the TTR105-115 peptide (see Table 2.1) were investigated using Thioflavin T, a dye which exhibits a distinctive fluorescence in the presence of β-sheet-rich material such as amyloid fibrils (Biancalana and Koide, 2010). In particular, the aim of this study was to investigate whether this peptide exhibits secondary nucleation or seeding behaviour, in which the surfaces of existing fibrils act to nucleate the formation of new fibrils. TTR105-115 peptide synthesised as in 2.4.2 was dissolved in water with 10% v/v acetonitrile at concentrations of 5, 10 and 15 mg/mL. In addition, 10 mg/mL solutions were made up with 1%, 3% and 5% of the peptide replaced by pre-formed amyloid fibrils for seeding. Thioflavin T (20 µM) was added and the fluorescence at 480 nm in response to excitation at 440 nm was followed for 10-18 hours at 37 °C on a Fluostar or ClarioStar plate reader (BMG Labtech) using LoBind clear-bottomed black 96-well plates (Corning) with shaking. Fluorescence values were normalised so that the highest value observed in each experiment was equal to 1.

Some seeding behaviour was observed, in that reactions which contained
more pre-aggregated fibrils showed a much shorter lag phase before the fluorescence increased. However, the results showed poor reproducibility. Historically, it has often proved difficult to obtain reproducible values for the aggregation kinetics of TTR105-115 (Bongiovanni et al., 2012). This is probably not due to the stochastic nature of the aggregation process (Hellstrand et al., 2010; Gillam and MacPhee, 2013) but instead to variations in the size or quantity of the seed fibrils, or to other factors such as impurities or pre-existing aggregates in the monomer solution. Much more carefully controlled experiments—for instance, using monomers freshly purified by size exclusion chromatography—would be necessary to obtain more reproducible results. There was also an unexplained decrease in fluorescence after it reached its peak value. While such a decrease is sometimes caused by sedimentation, these experiments were carried out using plate readers that read from below so should be unaffected by sedimentation. This decrease made it impossible to fit the data using the AmyloFit software (Meisl et al., 2016).

Shaking may also have affected results by causing the fibrils to break up, as potentially broken ends of fibrils could also be acting as nucleation sites. Therefore, an experiment was carried out under the same conditions but without shaking. While no seeding effect was observed here, this may have been because the duration of the experiment was too short due to the plate reader malfunctioning—all the samples gave identical results, none of which showed the rapid increase in fluorescence typical of aggregation behaviour.

Due to lack of availability of equipment and peptide and time constraints, further experiments were not performed.
Figure B.1: Aggregation Kinetics of the TTR105-115 Peptide Under Different Seeding Conditions
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