SELF-ASSEMBLED PEPTIDE HYDROGELS

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University of Bath

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DECLARATION OF WORK DONE IN CONJUNCTION WITH OTHERS

All work is acknowledged in the appropriate section of each Chapter. I am responsible for all other work presented in this thesis.

Circular Dichroism, Transmission Electron Microscopy and Fibre X-Ray Diffraction data presented in Chapter 7 was collected and analysed at the University of Sussex, in conjunction with Kyle Morris and Dr Tom Williams.

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ABSTRACT

The use of low-molecular weight peptide-based hydrogelators (LMWGs) for the immobilisation of enzymes is presented in this thesis. Low-molecular weight hydrogelators are a class of materials which are highly suitable for increasing enzyme lifetimes as they create a suitable biomimetic environment. Immobilised enzymes can be utilised in enzyme fuel cells, providing low-energy conversion routes for chemical processes. The hydrogels also possess tunable properties which allow their structure to be manipulated to give desirable properties.

This work begins with an exploration of dipeptide hydrogelators by investigating the effect of varying salt solutions and concentrations of dipeptide on final hydrogel structures. A wide range of characterisation techniques are employed to provide information about the micro- and macro-structure of the hydrogels.

The creation of dipeptide hydrogel materials via an electrochemical method is explored, which allows the production of nanometre thick, membrane-like materials. These layers are measured using Surface Plasmon Resonance techniques. The electrochemical technique for dipeptide gelation is expanded in later chapters to produce a range of novel materials.

Finally, an exploration into the effect of additives on dipeptide hydrogels is conducted, where the effect of adding chiral molecules is investigated. This provides interesting information regarding the self-assembly processes involved with hydrogelation processes, which has important implications for studying the folding and unfolding processes of peptides.
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<tr>
<td>AFM</td>
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<td>A / Ala</td>
<td>Alanine</td>
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<td>Ag</td>
<td>Silver</td>
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<td>Silver Chloride</td>
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<td>ATR</td>
<td>Attenuated Total Reflection</td>
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<td>Au</td>
<td>Gold</td>
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<td>BrNapAV</td>
<td>Br-Nap-Ala-Val-OH</td>
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<td>Boc</td>
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<td>Counter Electrode</td>
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<td>F / Phe</td>
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<td>Fluorenylmethyloxycarbonyl</td>
</tr>
<tr>
<td>FmocFF</td>
<td>Fmoc-Phe-Phe</td>
</tr>
<tr>
<td>FmocLG</td>
<td>Fmoc-Leu-Gly</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform IR</td>
</tr>
<tr>
<td>FTO</td>
<td>Fluorine-doped Tin Oxide</td>
</tr>
<tr>
<td>G</td>
<td>Gradient</td>
</tr>
<tr>
<td>Gδ-L</td>
<td>Glucono-δ-lactone</td>
</tr>
<tr>
<td>G / Gly</td>
<td>Glycine</td>
</tr>
<tr>
<td>H</td>
<td>Proton</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrogen Chloride (aq)</td>
</tr>
<tr>
<td>H₂O</td>
<td>Water</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen Peroxide (30%)</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase, Type I</td>
</tr>
<tr>
<td>HQ</td>
<td>Hydroquinone</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>I</td>
<td>Current</td>
</tr>
<tr>
<td>IR</td>
<td>Infra-Red Spectroscopy</td>
</tr>
<tr>
<td>ITO</td>
<td>Indium Tin Oxide</td>
</tr>
<tr>
<td>K</td>
<td>Kelvin</td>
</tr>
<tr>
<td>K / Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>L</td>
<td>Leucine</td>
</tr>
<tr>
<td>Lp</td>
<td>Penetration Depth</td>
</tr>
<tr>
<td>L-Phe</td>
<td>L-Phenylalanine</td>
</tr>
<tr>
<td>LR SP</td>
<td>Long Range Surface Plasmon</td>
</tr>
<tr>
<td>LSR SP</td>
<td>Long and Short Range Surface Plasmon</td>
</tr>
<tr>
<td>Abbr.</td>
<td>Meaning</td>
</tr>
<tr>
<td>-------</td>
<td>---------</td>
</tr>
<tr>
<td>M</td>
<td>Metre</td>
</tr>
<tr>
<td>mbar</td>
<td>Millibar</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulphonic acid</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetre</td>
</tr>
<tr>
<td>N</td>
<td>Refractive Index</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide (aq)</td>
</tr>
<tr>
<td>Nap</td>
<td>1-napthyloxymethylcarbonyl</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>ns</td>
<td>Nanosecond</td>
</tr>
<tr>
<td>O</td>
<td>o-phenylenediamine dihydrochloride</td>
</tr>
<tr>
<td>OPD</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>F / Phe</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>pKa</td>
<td>Dissociation Constant</td>
</tr>
<tr>
<td>Pt</td>
<td>Platinum</td>
</tr>
<tr>
<td>R</td>
<td>Arginine</td>
</tr>
<tr>
<td>Arg / R</td>
<td></td>
</tr>
<tr>
<td>RE</td>
<td>Reference Electrode</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>S</td>
<td>Seconds</td>
</tr>
<tr>
<td>s</td>
<td></td>
</tr>
<tr>
<td>SAXS</td>
<td>Small Angle X-Ray Scattering</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>SP</td>
<td>Surface Plasmon</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface Plasmon Resonance</td>
</tr>
<tr>
<td>SR SP</td>
<td>Short Range Surface Plasmon</td>
</tr>
<tr>
<td>T</td>
<td>Time</td>
</tr>
<tr>
<td>t</td>
<td></td>
</tr>
</tbody>
</table>
TEM  
Transmission Electron Microscopy

ThT  
Thioflavin T

U  

µL  
Microlitre

V  

V  
Volt

V / Val  
Valine

W  

W  
Watt

WE  
Working Electrode

X  

X  

XRD  
X-Ray Diffraction

SYMBOLS

ε  
Dielectric Constant

θ  
Angle

θ_c  
Critical Angle

θ_{min}  
Minimum Angle

λ  
Wavelength

α  
Alpha

β  
Beta
# CHAPTER ONE:
## INTRODUCTION TO LITERATURE

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1. INTRODUCTION

Low-molecular weight peptide hydrogelators (LMW gelators) exhibit a wide range of interesting properties and show excellent biocompatibility with biological molecules. In this chapter, the self-assembly processes of LMW gelators are explored in detail, with many examples of the processes described in the literature. A wide range of applications for these materials are discussed, along with a comprehensive explanation of characterisation techniques which have been explored to afford a greater understanding of their properties, at the nano- and macro- scale. The applicability of these materials for use in enzyme immobilisation is described, particularly highlighting the large potential for their use in enzyme fuel cells.

1.1 Hydrogels

Traditional cross-linked hydrogels based on polymerised hydrocarbons produce a highly hydrated three-dimensional network.¹ Hydrogels are utilised in bio-devices due
to their operational stability and control within the system, facile product recovery, and avoiding any contamination to the catalyst from solid supports. A variety of conventional and well-characterised polymeric hydrogels have been used for enzyme immobilisation, including poly(acrylic acid), poly(ethylene glycol), PEG-acrylamide beads, and poly(hydroxyl cellulose).

Hydrogels are highly responsive materials, reacting in a variety of ways to external stimuli. These responses, outlined in Table 1.1, can be exploited by materials scientists depending on their requirements.

Table 1.1. Stimuli and Responses of hydrogels. Reproduced from Cells and Gels, Pollack.

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>Responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>Chemical (stimulates or inhibits reactions or recognition processes)</td>
</tr>
<tr>
<td>Temperature</td>
<td>Phase separation (precipitation)</td>
</tr>
<tr>
<td>Chemical/biological agents</td>
<td>Shape change (shrinks or swells)</td>
</tr>
<tr>
<td>Solvents &amp; salts</td>
<td>Surface (becomes non-wetting)</td>
</tr>
<tr>
<td>Electrical field</td>
<td>Permeability (increases or decreases sharply)</td>
</tr>
<tr>
<td>EM radiation</td>
<td>Mechanical (hardens or softens)</td>
</tr>
<tr>
<td>Mechanical Stress</td>
<td>Optical (clears up or opacifies; becomes coloured)</td>
</tr>
<tr>
<td></td>
<td>Electrical (generates signal; electrochemical reaction occurs)</td>
</tr>
</tbody>
</table>

There are several general key properties for hydrogels for use in bio-devices; they must avoid non-specific protein adsorption, contain a high functional group density for incorporation of biomolecules, be highly hydrated, and optically transparent.

There are several drawbacks of using polymeric cross-linked hydrogels in bio-devices. In the main, cross-linking (gelation) tends to be a non-reversible process. This means that materials cannot be altered once gelled, and require high temperatures to break them down. Polymers are also derived from limited petrochemical resources, and can have toxic effects on organisms. Therefore, the next section will discuss the use of a new class of bio-compatible hydrogels which come from short chain amino acids (peptides) for use in bio-applications and bio-devices.
2. LOW-MOLECULAR WEIGHT PEPTIDE HYDROGELS

2.1 Introduction to low-molecular weight peptide-based gelators

There is currently significant interest in low molecular weight peptide hydrogelators (LMW gelators) in the research community, as they can be used for many potential applications. In particular, tuneable hydrogels composed of amphiphilic peptides are receiving a good deal of attention due to the wide range of properties accessible. A range of self-assembling di- or tri-peptides, and single amino acids, form hydrogel matrices via intermolecular interactions in water.\textsuperscript{9-11} LMW gelators contain a high solvent content, typically more than 99 \% w / w. The solvent within a hydrogel is mobile on a molecular level, but does not flow on a bulk level.\textsuperscript{12} This gives rise to a solid-like, transparent material, comprised of a three-dimensional matrix of non-covalently linked peptide molecules.

The recent surge of interest lies in their ease and low cost of synthesis, rapid response to external stimuli, thermoreversibility, and low bioaccumulation.\textsuperscript{13,14} Applications in areas such as healthcare and advanced materials are wide ranging due to their ability to mimic biological systems, exhibiting extra-cellular properties. Some examples which exploit these characteristics are tissue repair,\textsuperscript{15} immobilisation of enzymes,\textsuperscript{16} and drug delivery.\textsuperscript{17}

A recent review article has highlighted the progress made in the last few years in the area; it is now possible to “design polypeptide-based nanostructures de novo, or engineer naturally derived systems bothrationally and with confidence.”\textsuperscript{9} The next section will cover much of the important research over the last few years in the area.

2.2 Which peptides can form hydrogels?

There are several requirements for peptide gelation which must be balanced to allow for the self-assembly of LMW gelators to occur. The molecule must be partially soluble and partially insoluble in the solvent,\textsuperscript{12} which allows the drive for self-assembly and avoids complete dissolution or precipitation of the molecule. Also, to have the potential to form directional “multiple non-covalent interactions with itself.”\textsuperscript{12} Optimal molecules for self-assembly are able to organise into “one-dimensional molecular arrays connected by intermolecular hydrogen bonds.” Those with the potential for forming many hydrogen bonds with water are either too insoluble or too soluble to be efficient hydrogelators.\textsuperscript{12} Typical self-assembled hydrogels contain amide, urea, and carbohydrate functional groups, and less commonly, consist of steroids and bile salts with hydrophobic surfaces, long chain alkanes, and nucleobases.
Diphenylalanine (H-FF-OH, Figure 1) is the smallest known self-assembling peptide moiety, forming vesicles and nanotubes in water (not hydrogels).\textsuperscript{18-20} A wide range of protected diamino acids at the amine terminus have been shown to form hydrogels (protecting group: Fmoc\textsuperscript{21-28}, Cbz, and Nap\textsuperscript{29-31}). Many constrained dipeptides (e.g. FF dimers with unsaturation at one of the α-carbons) have also been investigated, and shown to form hydrogels. Figure 1.1 shows the structure of two generic amino acids protected with Cbz and Fmoc groups in [1] and [2], and H-FF-OH in [3].

![Figure 1.1](image)


Adams et al have reported a comprehensive library of dipeptide gelators,\textsuperscript{33} and surmise that whilst the prediction of potential gelators is difficult, controlling the hydrophobicity of the dipeptide is a key factor in achieving a stable hydrogel. Their work has shown that when dipeptides were hydrophobic (logP (partition coefficient) of 2.8 and below), any hydrogels that formed were very weak, and several did not form at all. The same was found with more hydrophilic dipeptides (logP of 5.5 and above). LMW peptides with logP values between 3.4 and 5.5 formed strong, stable, transparent and homogenous hydrogels.

There is inherent chirality within peptide hydrogel structures,\textsuperscript{12} being comprised of β-sheets\textsuperscript{34-37} and α-helices.\textsuperscript{38} There is also inherent chirality in the molecules as either L- or D- amino acids are used. These motifs form hierarchical extended nano-structures comprised of nano-tubes, rods and fibrils, also termed ribbons. From here the peptides form long fibres by aggregating and entangling together (10 nm to several microns in length), as shown with Fmoc-diphenylalanine (FmocFF) in Figure 1.2.\textsuperscript{25,39,40}
The initial structure arises solely from non-covalent forces, hydrogen bonding and π-π stacking between the amino acids.\textsuperscript{11,25,28} These structures form a rigid scaffold, with G’ values obtained from rheological measurements of 1 to 104 Pa.\textsuperscript{25,28,42} The hydrogels can be re-dissolved in water, and destroyed by shear forces. They have been shown to be stable up to temperatures of 100 °C.\textsuperscript{43}

2.3 Nano-structures accessible through peptide gelators

A range of nano-structures have been accessed with self-assembling peptide gelators. Generally, nanofibres are observed which resemble amyloid fibrils. However, other shapes observed in the literature include nano-spheres, e.g. with Boc-protected diphenylalanine (BocFF) under certain conditions, and nanotubes with Fmoc-protected diphenylalanine (FmocFF). These structures were created using a printing and patterning technique onto ITO and aluminium surfaces, with SEM showing the presence of a “dense patterning of nanostructures.”\textsuperscript{44}

Elsewhere, H-FF-OH has been shown to form nanotubes upon exposure to water vapour at room temperature, and nano-wires under anhydrous conditions at room temperature and subsequent exposure to aniline vapour at 150 °C.\textsuperscript{45} The different hydrogel structures possessed different properties; the nano-wires were shown to be stable up to 200 °C, and from pH 1 – 14. This enhanced stability was attributed to a dominant β-sheet conformation in the nano-wires. The nano-tubes were stable up to only 100 °C, yet in a range of solvents, from non-polar to polar. These examples show
the differences in structure which can be accessed by using peptides with very similar molecular properties.

3. PEPTIDE HYDROGELS: APPLICATIONS

Peptide based hydrogels are being investigated for a wide range of applications in the fields of healthcare and advanced materials. According to Ulijn et al, peptide materials can (as of 2011) “be fully encoded at the genetic level,” which provides “exciting applications in bio-medicine and bio-inspired energy-related technologies.”

Another important area is the investigation of peptide and protein self-assembly by studying the self-assembly of small amphiphilic peptide building blocks. This has particular relevance for understanding protein unfolding processes which can lead to degenerative diseases such as Alzheimer’s. This section will explore the potential applications in detail.

3.1 Peptide hydrogels as Extra-Cellular Matrices (ECMs)

Due to their extended three-dimensional structure and inherent biomimetic properties, dipeptides have been investigated for tissue engineering, wound healing, and cell culturing. There is considerable potential for observing cell growth behaviour in environments with complex mixed soft matter systems. For example, in 2009 Jayawarna et al used a “finely tuned” Fmoc-FF and Fmoc-RGD (Fmoc-protected Arginine-Glycine-Aspartic acid) composite hydrogel successfully for cell growth studies. In another study by Haines-Butterick et al, β-hairpin peptide hydrogels have been shown to exhibit non-cytotoxic properties towards mouse peritoneal macrophage cells. These studies show the potential for peptide hydrogel structures in the field.

3.2 Conducting peptide hydrogels

Peptide gelators can also be used in electronic devices. It has been recently highlighted that the future benefit of peptide hydrogels will come from functionalising gels and creating multi-component systems, using them as components in electronic devices. These include “biosensors and lab-on-a-chip devices, bio-inspired light harvesting devices, bio-molecular motors and so on.”

Potential applications for conducting peptide hydrogels lie in bio-energy devices. It has been shown with impedance measurements that the enzyme-triggered Fmoc-protected tri-leucine (FmocL₃) hydrogel possesses “minimum sheet resistances of 100 kΩ sq⁻¹ in air,” with conductivity scaling linearly with the mass of peptide in the network. Whilst
this value is relatively low, it does provide a “platform for interfacing biological component with electronics” which provides a starting point for the technology.

Introducing metal ions into the fibrous structure of peptide hydrogels could allow for efficient electron transfer throughout hydrogel materials. At the time of writing, there have been a few studies incorporating Cu into self-assembling peptide structures, however no conductivity studies have been carried out on these systems. L-Phe and Cu(II), mixed in a ratio of 2:1 formed a hydrogel (in the pH range 6.5 – 8.0), which was stable for a short time (20 min). The authors suggested the system could be used for the stimuli-responsive release of entrapped molecules upon the hydrogels shrinking or undergoing gel-to-sol transitions. (Interestingly, when 50 % or more D-Phe was used in the mixture, no hydrogels formed with Cu(II)). In a separate study, a trinuclear Cu(II) complex of inositol and 2,2’-bipyridine was shown to form a stable hydrogel (pH 12.4). Another application has used dipeptide nanostructures as degradable moulds, where nanotubes are filled, or coated with metallic materials for use in nano-electronic devices.

The potential of dipeptide hydrogels for light harvesting materials has been shown by Adams et al, where a naphthalene-protected diphenylalanine (NapFF) dipeptide transfers energy to donor and acceptor chromophores, specifically dansyl and anthracene acceptors. Recently, the optical properties of FmocFF have been investigated and show quantum confinement effects below gelation concentrations. This was attributed to the molecules organising into structures with nano-sized crystalline regions. The absorption spectrum was similar to quantum dots, with a sharp photoluminescence in the UV. This technology could be used for creating low-cost solar cells or LEDs.

3.3 Responsive peptide materials

Applications in drug delivery arise from peptide hydrogels responsiveness to external stimuli, which can allow the release of molecules upon controlled dissolution of the material. They are particularly useful when they can “interact with, adapt to, or direct biological processes as relevant to next generation molecular biomaterials.” One example is the dehydrophenylalanine gelator, H-Phe-ΔPhe. This molecule forms a fractaline hydrogel with high mechanical strength, which was responsive to external conditions including pH, temperature, and ionic strength. Due to the lack of protecting group, the material is biologically compatible, and presents no cytotoxicity to cells. Panda et al have used H-Phe-ΔPhe to immobilise vitamin B\textsubscript{12} and ascorbic acid, with
release rates decreasing with increasing salt concentration. A change in pH induced loss of gel structure and faster release of the molecules.\textsuperscript{14}

Debnath \textit{et al}\textsuperscript{56} have explored the bacteriocidal properties of Fmoc-protected dipeptides by introducing a pyridinium moiety positioned at the C terminus. Their work has explored the effect of different positions of the pyridinium group. The most effective anti-microbial properties were found when the -Phe side group was adjacent to the Fmoc group, providing “optimal hydrophobicity.”\textsuperscript{56} This is another reminder that the structure of the peptide is very important in affecting the characteristics of the hydrogel.

A study by Zhou \textit{et al}\textsuperscript{57} presented a pH-responsive hydrogel, consisting of two small amphiphilic molecules gelled together, which exhibited shrinking and swelling properties. Hydrogel mixtures of GalNAc glutamate ester and amphiphilic carboxylic acids gave more densely entangled fibres in acidic environments than at neutral pH. The incorporation of counter-ions caused an inter-fibrous repulsion within the structure, leading to its swelling.\textsuperscript{57}

Lin \textit{et al}\textsuperscript{58} have shown responsiveness to UV light activity when an azobenzene group was attached to a C\textsubscript{4}-Azo-Gly-Gly dipeptide. The hydrogel structure was observed to change reversibly as the Azo moiety changed from \textit{trans-} to \textit{cis-} with application of UV light. This is an interesting area for energy applications, and for tuneable materials. The next section will briefly introduce the concept of electrochemically gelating polymers at surfaces, for control over thickness and surface properties.

3.4 Future Potential for Peptide Hydrogel Materials

A limiting factor for the exploitation of LMW gelators rests in the lack of control over the gelation process, the nucleation, the structure of growth,\textsuperscript{50} and hence the patterning of their assembly.\textsuperscript{44} Gelation is a process which occurs in the bulk phase, therefore routes to triggering self-assembly in specific areas of the precursor solution is an important step in developing this area. This work develops novel methods for creating pH-triggered hydrogels which allow hydrogels to be formed at specific sites and giving specific properties such as thickness and density. This will allow increased complexity in these systems, with “enhanced molecular, temporal and spatial control over the self-assembly process.”\textsuperscript{9}
4. PEPTIDE HYDROGELS: SELF-ASSEMBLY MECHANISMS

4.1 Self-assembly processes
Peptide self-assembly is a spontaneous, reversible process, involving the organisation of ‘molecular building blocks’ into ordered structures. The driving forces behind the self-assembly process are non-covalent, ordered, weak interactions, specifically hydrophobic interactions, hydrogen bonding, and electrostatic interactions. Self-assembly occurs when the carboxylic acid group of the peptide is protonated, i.e. below the pKa of the acid.

Self-assembly is induced via π-π interactions between aromatic groups creating β-sheet arrangements, or hydrophobic interactions creating α-helices. The extent of π-π stacking and hydrophobic interactions are significant for the final hydrogel strength. Hydrogen bonding then allows these structures to form β-sheet structures, such as tapes and ribbons, which are one dimensional tightly coiled molecular columns. These stack together to produce nano-fibrils. These fibrils assemble into “nano-scale bundles”, fibres of between 15 and 100 nm in diameter, and micrometres in length. When gelled, the hydrophilic part of the peptide molecule is located on the surface of the self-assembled nanofibres, forming hydrogen bonds with water molecules. The fibres then “tangle and interact with one another to form a self-supporting, sample-spanning ‘solid-like’ network.”

4.2 Triggers for peptide hydrogelation
Self-assembled peptide structures react globally to a local stimulus, inducing spontaneous gelation. Typically the triggers used are pH, temperature, and salt concentration. Another approach includes combining (heterogeneous) catalysis with molecular self-assembly, which produces the molecular building blocks locally. This involves the use of enzymes, where non-gelling molecules are cleaved by enzymes to give gelating molecules. This technique avoids the exposure of any immobilised species to extreme conditions during the gelation process. Other methods of initiating gelation reported in the literature include local X-ray irradiation, gelating cyclic dipeptides with shear forces, sonication in the presence of NaYF₄ nanoparticles, and light activated methods.
4.3 Electro-addressed hydrogels

Until the work outlined in Chapter 4 in this thesis was published, there were no examples of using electro-addressing as a trigger for forming dipeptide hydrogels in the literature, although the technique had been used for the electrochemical deposition of the polysaccharide chitosan onto microelectrode surfaces.\textsuperscript{75-78} Hydrogel film thicknesses were shown to increase with increasing applied voltage, time, and concentration of chitosan.\textsuperscript{75} A fluorescent dye was added to the film to observe the development of the layers using a fluorescence microscope.\textsuperscript{76} In recent years, the technique has been developed to create a ‘bio-device interface’, which used a chitosan layer with proteins tethered by glycine (G) and lysine (K) residues, to afford spatial selectivity of proteins at a surface under mild conditions.\textsuperscript{78}

At the time of writing it has been shown to be possible to trigger gelation with a locally applied stimulus via a “localised activation of non-assembling precursors.”\textsuperscript{74,79} This area is developed in this thesis, by using electrochemical techniques to trigger controlled gelation at electrode surfaces.\textsuperscript{80}

4.4 Importance of self-assembly processes on hydrogel structures

The self-assembly processes of peptide hydrogelators has been well researched in recent years. Understanding the gelation processes involved has implications in studying general peptide self-assembly, predicting which peptides will form structures, and predicting the nature of the structures which will be present in resulting hydrogels. LMW gelators have been shown to be affected by many factors which influence self-assembly mechanisms, and influence the final properties and physical structure of the hydrogel. Ulijn particularly notes in a recent highlight article that “the route of self-assembly dictates supramolecular order, and therefore functionality.”\textsuperscript{9}

The degree of hydration influences the packing of the dipeptide molecules, as water can interact with hydrogen bond donor or acceptor sites. The size, shape and material of the container the hydrogels are formed in has also been shown to result in a range of different structures.\textsuperscript{11} A recent study by Chen \textit{et al}.\textsuperscript{81} have shown the kinetics of self-assembly were dependent upon the final pH of the hydrogel; for example, those with lower final pHs had fewer β-sheet-type structures, and were more rigid than those with a higher final pH.\textsuperscript{81} This group has also have provided a deeper understanding of the self-assembly process by utilising low energy crystal packing possibilities together with computer modelling to predict possible molecular arrangements.\textsuperscript{67} Computer modelling based on Fibre-XRD measurements have shown hydrogel structures to be affected by differences in conformational and hydrogen bonding properties.\textsuperscript{67} Despite this
promising result, they suggest the research community is “a long way from predicting from first principles whether a molecule will form a gel or crystallise.”

4.5 **Examples of self-assembling peptides in the literature**

i) **Fmoc-diphenylalanine (FmocFF)**

Smith *et al.* have investigated the stacking of FmocFF in water. Their work found there to be four distinct stages of self-assembly, shown pictorially in Figure 1.3. First, molecules aligned into anti-parallel β-sheets, in [a]. Second, interlocking Fmoc groups from alternate β-sheets created anti-parallel π-stacked pairs in [b], with Wide-Angle X-Ray Scattering (WAXS) data measuring the separation between Fmoc groups as 4 Å. Next, these interlocked into four twisted anti-parallel β-sheets (hairpins), brought together by lateral π-π interactions, to form cylinders with a 3 nm radius, shown from the top view in [c]. Finally, the cylinders lined up side-by-side via facial interactions to form ribbons, collapsing to give a bi-layer structure. This led to the long, covalent supramolecular assemblies of fibrils shown in [d].

![Figure 1.3](image_url)

*Figure 1.3. Diagram showing an Fmoc-diphenylalanine gelator at each stage of the self-assembly process. Reproduced from Smith *et al.* “In [a], [b] and [d] fluorenyl groups are coloured orange and the phenyl groups are coloured dark blue to illustrate the paired π-stacked nature of the fluorenyl groups.”*

Figure 1.3 shows diagrammatically the predicted structures described above, which form the long fibres characteristic of a hydrogel network seen earlier in Figure 1.2.
ii) Nap-Alanine-Glycine (NapAG)

A detailed investigation by the Adams group into the potential arrangements of Nap-protected dipeptides has used computer modelling based on data acquired from Fibre-XRD measurements. The group found two plausible structures for the dipeptides, consisting of “open tapes of hydrogen bonded molecules and structures with compact columns of molecules”. In the case of NapAG, they found “pairs of amide-carbonyl hydrogen bonds between neighbouring dipeptide molecules, resulting in a β-sheet arrangement”. As in Figure 1.3[c], hydrogen bonding between end groups served in “extending the β-sheet structures into kinked molecular tapes with a typical width of 30 Å”. They found the π-π stacking of NapAG helped to stabilise the packing of the molecules.

The second structure most commonly observed for NapAG was found to have a “more compact” structure, similar but with smaller and “less extended” motifs, with less hydrogen bonding between end carboxylic groups. Overall, this led to “tightly coiled helices with smaller dimensions”. These findings were backed up by using characterisation methods such as Electron Microscopy, Circular Dichroism and fluorescence spectra of similar Nap-protected dipeptides.

4.5 Importance of the sequence of amino acids in self-assembly

As well as the amino acids themselves being important in determining which peptides self-assemble into hydrogels, their order within the molecules also affects their potential for gelation. For example, Fmoc-Lysine-Leucine-Valine (FmocKLV) (the lysine group is Boc-protected) has been shown to form hydrogels with branched fibrils, whereas Fmoc-Valine-Leucine-Lysine (FmocVLK) formed ordered aligned fibrils. Elsewhere, Nap-AG was shown to form a hydrogel, but Nap-GA did not. Currently, it is difficult to predict which order of amino acids will aid or hinder gelation. The next section discusses the end groups and how they affect gelation of peptides.

4.6 Importance of end groups in self-assembly

Several groups have performed studies with different end-groups to determine their importance in self-assembly. One result reported by Sanchez de Groot et al showed that Valine-Phenylalanine (VF) formed an extended structure but Isoleucine-Phenylalanine (IF), with an extra methyl group, did not. This suggests that the initial driving force for self-assembly is not solely due to head-to-tail interactions or the stacking between aromatic rings, and that hydrophobic interactions are also important.
A protecting group can be added to the peptide molecule in order to provide increased hydrophobicity, preventing the molecule from dissolving completely in water. Fmoc and Nap are used most frequently, as they allow $\pi-\pi$ interactions to occur between adjacent molecules. The importance of the type of aromatic group used is also in question; there are examples which show that with Nap and Fmoc groups protecting the N-terminus of the amino acid, hydrogels form (e.g. with Fmoc-F$_5$ gelator), but with Cbz and Boc protecting groups, no hydrogel structure was observed.  

Interestingly, with a Br group added to the Nap protecting group in BrNapAV, Chen et al showed hydrogels were still able to form, despite the presence of the Br group, which was thought to interrupt $\pi-\pi$ stacking between the Nap groups. However, the peptide gelled due to the increase in hydrophobicity, forming a homogenous, translucent hydrogel.

4.7 Importance of side groups in self-assembly

Generally, peptides with aromatic side chains tend to form stronger hydrogels than those with aliphatic chains. -Phe is a common group which works well, being critical in the folding of amyloid fibres. It is unclear whether $\pi-\pi$ stacking in side chains is important, as it has been shown that a cyclohexane (Cha) group gives stronger hydrogels than phenylalanine, in the case of Fmoc-dipeptides. The importance of the positions of side phenylalanine groups have been shown for pentapeptides in organic solvents.

Ryan et al have investigated the effects of adding F / Cl / Br in ortho- / meta- / para-positions on the phenylalanine moiety of FmocFF dipeptides. The position of the halogen atom affected the gelation rate and rheological structure. In terms of the speed of gelation, when F / Cl / Br were in the para- position, self-assembly was accelerated, and gelation occurred within 0.5 min. With the halogen in the meta- position, gelation time was relatively unaffected, occurring between 5 and 15 min. When the halogen was in the ortho- position the molecules took the longest to gelate (between 30 to 50 min). In the case of the meta- positioned halogens the gels were the strongest, followed by the ortho- position, then para-. Interestingly, the para- systems formed the largest fibre bundles. The influence of the halogen atom itself was found to have "subtle and unpredictable effects."

These studies and examples reiterate how small changes in the molecules can create very different hydrogels. In the next section, the importance of pKa and electrostatic interactions in self-assembly are presented.
4.8 Electrostatic interactions and pK\textsubscript{a} shifts

As peptide hydrogels are unstable at extremes of pH and temperature, electro-static interactions are thought to have a role in self-assembly, specifically a head-to-tail coupling between COO\textsuperscript{-} and NH\textsubscript{3}\textsuperscript{+} which is disrupted with increased ionic strength.\textsuperscript{88}

The pKa of dipeptides within hierarchical structures is higher than in free solution.\textsuperscript{51} For example, NapAV showed a ‘higher than expected’ value of 5.9 in the hydrogel structure.\textsuperscript{81} Similarly, the pK\textsubscript{a} of FmocFF in solution is 3.5, yet in the hydrogel two pK\textsubscript{a} shifts are seen\textsuperscript{82} at pH 10.2 – 9.5 (when molecules associate into paired fibrils) and pH 6.2 – 5.2 (when fibrils associate into flat ribbons). There are two reasons for why this occurs; the first is due to the carboxylic acid group being in a highly hydrophobic environment, and the second is due to the stabilisation of the carboxylic acid group by neighbouring molecules. These influences lead to a lower pK\textsubscript{a} than expected.

5. PEPTIDE HYDROGELS: CHARACTERISATION TECHNIQUES

There is a good deal of precedent in the literature for the characterisation of dipeptide hydrogels,\textsuperscript{11} however analysis is made challenging by their properties, being highly solvated and soft materials. There is a recognised need amongst the research community for detailed characterisation as systems become more sophisticated which “span the atomic, molecular, supramolecular and bulk regimes.”\textsuperscript{51} Most commonly, Fibre-XRD, Circular and Linear Dichroism, Rheology, and Electron Microscopy Imaging techniques are used. WAXS and Impedance spectroscopy are also employed (not discussed here). A range of the techniques used in the literature are given with detailed examples in this section.

6.1 Imaging techniques for dipeptide hydrogels

There are many imaging techniques available for the investigation of hydrogels. This section outlines the various methods for imaging dipeptide hydrogels used in the literature. There are some drawbacks in utilising these techniques, as sample preparation for TEM, SEM and AFM can influence the morphology observed, giving artefacts caused by drying procedures. Cryo- techniques are more appropriate as the structures are ‘frozen’ in place, although structures can be affected by ice crystals forming artificial structures.
Peptide hydrogels consist of 0.1 – 1 % solid material by mass and 99 – 99.9 % water. When the water phase is removed, all the interactions of the peptides with water are affected. The hydrogel is dependent upon the water phase in retaining its delicate structure and fluidity. Shear forces during drying also disrupt the structure, which causes a range of artefacts. It is not possible to observe the chirality in the fibres, as under Electron Microscopy (EM) techniques only smooth fibres and bundles can be seen.\textsuperscript{12}

6.2 Scanning Electron Microscopy (SEM)

SEM shows fibrous structures on the nanometer scale.\textsuperscript{2} Kumaraswamy et al\textsuperscript{89} have recently used SEM to report the effects on the nanostructures of diphenylalanine structures grown on various substrates, at different pHs and temperatures, and in a range of buffers and salt concentrations. In this paper a variety of materials (aluminium, glass, silicon, etc) were used as substrates on which to grow the nanotubes, which affected the observed structures significantly due to variations in surface topography, hydrophobicity, and conductivity (which is related to how the water phase evaporates).\textsuperscript{89}

One study by Scanlon et al has shown how freeze-drying samples for SEM can create artificial structures upon imaging.\textsuperscript{90} They explored the self-assembly of peptides and found that tapes, ribbons and fibrils gave flat lamellar-like structures after undergoing the freeze-drying process. This was attributed to the water crystallisation within the hydrogels.

Cryogenically freezing samples and using cryo-SEM techniques allows the fibrous structure within the hydrogel to be preserved. There are many examples of cryo-SEM images in the literature (see Figure 1.4), with images of hydrogels showing networks of fibres with some examples of diameters reported of $22 \pm 5 \text{ nm}$\textsuperscript{21,28} and $56 \pm 13 \text{ nm}$.\textsuperscript{22}
Figure 1.4. Cryo-SEM of FmocLG after 24 hours gelation with Gδ-L. Scale bar 1 µm. Reproduced from Adams et al.21

Figure 1.5 shows cryo-SEM images of three examples of three dipeptide hydrogels, FmocGG, FmocAA and FmocFF.

Figure 1.5. From left to right: Cryo-SEM images of FmocGG, FmocAA and FmocFF (concentrations 0.15 - 0.66 wt %, 0.16 - 0.66 % and 0.22 - 2.14 %, respectively). Scale bar = 1 µm. Reproduced from Jayawarna et al.22

In practice, higher concentrations of dipeptide and long gelation times tend to give more rigid hydrogel network structures, and are more suited to imaging techniques. It is not possible to encase the hydrogels in resin for SEM as with other biological structures, as the hydrogels dissolve in high ethanol solutions (>90 %).

6.3 Transmission Electron Microscopy (TEM)
Polypeptide or alpha-protein-like structures with β-sheets and α-helices were first shown by TEM.41 Reddy et al have successfully used cryo-TEM to image polymer architecture in proteins of less than 10 nm.91 It is possible to gain pictorial information into the macrostructure of dipeptide hydrogels, and nucleation points of fibres with TEM, which shows the association to give bundles of fibres.2,3 Thin films of hydrogels grown on a supported surface such as a grid coated with a carbon film are preferred.
The samples require a negative stain (uranyl acetate) and are air dried directly onto the grids used for imaging.

Figure 1.6 shows a set of TEM images taken at various times throughout the gelation of BrNapAV. The emergence of a dense fibrous network was observed over time.

![Figure 1.6. BrNapAV at various times after addition of GδL. [a] 0 min [b] 40 [c] 80 [d] 120 [e] 160 [f] 200 [g] 240 [h] 280 and [i] 400 min, showing the evolution of fibres into a dense network over time (scale bars 0.2 µm). Reproduced from Chen et al.](image)

It is important to record the methodology of sample preparation for TEM, as different preparation techniques can give different results. There can also be discrepancies between specific TEM techniques. Sample concentration and hydrogel strength also affect the structures observed.

### 6.4 Atomic Force Microscopy (AFM)

AFM is a useful technique for providing information about hydrogel samples in semi-solvated conditions. The advantage is that the hydrogels retain some of the water
phase whilst the measurement is taking place. Williams et al have used AFM to show a FmocL / L₂ system (see Figure 1.7), measured using tapping mode. The network of fibres is clear, and corresponds to similar images obtained with TEM.

An interesting study by Duan et al has used AFM to show the different morphologies of a hydrogel comprised of three dendrite L-glutamic acid units and a long alkyl chain at different pHs. The fibrils of the hydrogel changed significantly from pH 10 to 11. Figure 1.8 shows the transition from nanotubes to dendrite structures ('pine' at pH 10[a,b] and 'feathers' at pH 11[c,d]).

Figure 1.7. AFM image of an enzymatically induced hydrogel of Fmoc/L₂ system showing a network of interlinked fibres (scale bar 250 nm). Reproduced from Williams et al.
Figure 1.8. AFM showing gels from three-dendrite L-glutamic acid units combined with a long alkyl chain. Nanotubes are shown in [a] and [b] (scale bars 600 nm and 500 nm). At pH 10 and 11 dendrite structures were observed, shown in [c] and [d] (scale bars 4 µm and 3 µm. Reproduced from Duan et al.93

In both studies there was no detailed methodology given for AFM sample preparation and measurements.

6.5 Confocal Microscopy

Confocal microscopy uses a fluorescent stain, such as Nile Blue, which does not significantly affect the hydrogel structure. Work by Adams et al.21 provides confocal microscopy images of FmocLG upon addition of Gδ-L, which hydrolyses to release protons and lowered the sample pH sufficiently to produce a stable hydrogel. Confocal microscopy was used to show the evolution of fibres, and showed the sample becoming less mobile after five minutes (Figure 1.9).
Figure 1.9. Confocal microscopy images showing evolution of fibres as a $1.46 \times 10^{-5}$ mol dm$^{-3}$ FmocLG solution is mixed with $\delta$-L after [a] 1, [b] 2, [c] 3, [d] 4, [e] 10 and [f] 45 minutes, stained with Nile Blue and using a cavity well slide (scale bar 50 µm). Reproduced from Adams et al.$^{21}$

The hydrogel sample shown in (f) was solvated, with the fibres ‘waving’, which provided less well resolved images, but a more accurate overall picture of the fully solvated hydrogel structure than other techniques.

Coupling imaging techniques with spectroscopic techniques provides a deeper understanding of the fine structure of peptide hydrogels. Techniques such as Fourier Transform Infra-Red Spectroscopy (FTIR), Rheology, Fibre X-Ray Diffraction (Fibre-XRD) (although this aligns the structure in one direction to give ‘pulled’ fibres), Linear and Circular Dichroism (LD and CD) (which can align the fibres in one direction due to the narrow cell window), fluorescence measurements, and Small Angle X-Ray Scattering (SAS) have been investigated for the study of peptide hydrogels. Combining a range of techniques builds up an overall analysis of the solvated system and provides information about how the hydrogels are formed. These techniques are discussed in more detail below.
6.6 Fourier Transform Infra-Red (FTIR)

FTIR is used to positively assign the amide I peak at 1607 and 1691 cm\(^{-1}\) which are attributed to $\beta$-sheet and $\beta$-turns.\textsuperscript{25,57} Experiments are carried out in D\(_2\)O, which does not appear to significantly affect gelation properties.

6.7 Rheology

Rheology is a useful tool for measuring elasticity of materials and properties of peptide hydrogels.\textsuperscript{81} In general, a sample is considered to be a hydrogel if the value for G’ (elastic modulus) is one order of magnitude higher than the value for G” (storage modulus). Procedures for taking rheological measurements include time sweeps and frequency sweeps, which provide information about the timescale and extent of fibrillar entanglement.\textsuperscript{87} One study by Mehler et al has used a plot of viscosity versus shear rate, which gives a power law relationship, providing evidence of a structured hydrogel.\textsuperscript{25}

6.8 Circular Dichroism (CD)

CD signals arise in peptide hydrogels due to the inherent chirality of both the molecules and the fibres,\textsuperscript{94} which also provides information about rates of fibril formation.\textsuperscript{87} It has also been used to test hydrogel stability, to see if the CD bands disappear as temperature is increased.\textsuperscript{12} CD has been used by Chen et al\textsuperscript{92} to show the left-handed helices which appear as samples of BrNapAV and G$\delta$-L gelate over time. Linear Dichroism spectra were also taken, which gave the same set of peaks, so it was certain that the structure was not artificially aligned in one plane in the cell. The results are shown in Figure 1.10.
The peaks at -218 show $n \rightarrow \pi^*$ transitions, and at +230 nm, $\pi \rightarrow \pi^*$ transitions. At higher wavelengths, peaks represent near-UV peaks and give information about the tertiary structure, arising from effects of the surrounding environment. An increase in the peak CD signal was observed with increasing hydrogel strength. Smith et al have also measured a peak in the CD spectrum for FmocFF, at -218 nm. They attributed it to a $\beta$-sheet structure.\textsuperscript{28}

6.9 Fluorescence

Fluorescence measurements use the well-documented molecular rotor Thioflavin T (ThT) for observing the formation of amyloid fibres.\textsuperscript{95-97} When a hydrogel forms, the ThT can no longer rotate freely, and is removed from the aqueous environment, allowing fluorescence to occur. The excitation wavelength for ThT is at 455 nm, and the maximum emission peak is measured at 485 nm. ThT is used in micro-molar concentrations. An example of hydrogel formation followed by fluorescence measurements in real time is shown in Figure 1.11.
Fluorescence is a useful technique for the positive confirmation of the existence of peptide fibres, and an indication of gelation speed and extent of gelation. Long time (24 hour) experiments also suggest the structure does not change significantly after the hydrogel has ‘set’.

6.10 Fibre X-Ray Diffraction

Fibre diffraction samples are prepared by dropping 10 µL of precursor hydrogel solution between two capillary tubes filled with wax. The solution forms a hydrogel that is then allowed to dry. This produces a “partially aligned fibre sample.” Samples are mounted on a goniometer head to take measurements. Chen et al. have used Fibre-XRD to compare BrNapAV hydrogels with different concentrations of Gδ-L (Figure 1.12).
Figure 1.12. Fibre-XRD of BrNapAV hydrogels with Gδ-L added at different concentrations: [a] 1.82 mg mL$^{-1}$, final pH = 5.0; [b] 2.94 mg mL$^{-1}$, final pH = 4.5; [c] 5.96 mg mL$^{-1}$, final pH = 3.6; (d) 14.42 mg mL$^{-1}$, final pH = 3.1. Reproduced from Chen et al.$^{92}$

The results show a signal at 4.5 Å on the meridian, which corresponds to the spacing of fibre strands along the fibril axis. The two equatorial patterns were found at 16 and 7.3 Å, which were attributed to the packing distance of the Nap- groups. Unit cell parameters for the organisation of peptides within the hydrogel were also obtained from this data. The results showed that each sample were similar on this scale. Smith et al.$^{99}$ have also shown FmocFF to have fibre spacing along the fibril axis to have a separation of 4.3 Å, which corresponds with these findings.

6.11 Small Angle Scattering (SAS)

SAS has recently been shown to be a useful technique for the characterisation of peptide hydrogels in the wet state. Saiani and Guilbaud$^{100}$ introduce the topic in a recent highlight article, showing it is possible to observe the dissociation and association of peptide nanotubes. In conjunction with other microscopy techniques, ambiguous data can be interpreted to give a picture of the dynamic self-assembly, whilst dimensional information can be obtained using structural peaks and form factors from spectra.
Recently, SAS has been used to measure the thickness of nanotubes walls within an (Ala)₆Lys hydrogel. The nanotubes were found to have a 26 nm radius, a wall thickness of 0.65 nm, with a peptide repeat distance of 0.5 nm. The study was also able to find a novel phase formed by the peptides at higher concentrations, showing a flat bilayer structure. This shows a promising precedent for giving accurate details of average dimensions within fully hydrated hydrogels.

6. PEPTIDE HYDROGELS: USE IN BIOCELLS

7.1 Introduction to biocells
There is currently a great deal of attention given to biocells in the literature as a topic which has grown steadily over the last decade. Biocells use biological molecules to generate energy, typically using proteins, enzymes or whole organisms to convert energy from a chemical to an electrical form. They offer diverse 'green' alternatives to conventional solid state fuel cells, which require high temperatures (between 300 – 500 °C), and require p-group or transition metal catalysts which are expensive and non-abundant. Contrary to this, biocells operate at physiological temperatures and their widespread use and mass-production drives down the cost, making them attractive as long-term investments.

Biocells have many applications in the energy field, including: bioremediation, biotransformation, biomass engines, harvesting hydrogen, sensors, and power recovery from industrial waste streams. The focus of the next section is enzyme fuel cells. One of the challenges associated with this technology is enzyme lifetime, which can be enhanced by several methods, discussed in detail.

7.2 Enzyme fuel cells
Enzymes provide a reaction route for the conversion of a wide range of small molecules, at low temperatures (physiological), using environmentally acceptable solvents (aqueous buffer solutions) and providing high yields and high specificity (between 90 – 100% yields), all with a biodegradable catalyst. Enzymatic catalysis also removes the requirement for functional group protection and functional group activation, affording shorter reaction pathways and less waste.

Current enzymatic devices use isolated and purified enzymes as specific catalysts, together with an electrochemical device to recover the energy in an electrical form.
These work by either using an activated solution, where the active component is freely moving in the electrolyte, or an activated electrode, where the active component is localised at the electrode surface. It is common to have several enzymes working together in one cell to ensure complete oxidation of the substrate. Due to recombinant DNA techniques it is possible to produce a wide range of enzymes for commercially viable prices. Advances in protein engineering allow the possibility of producing enzymes with specifically designed properties, such as substrate specificity, activity, selectivity, and stability for increased performance. Figure 1.13 is a graphical representation of an enzyme fuel cell, with examples of enzymes for oxidising fuel at the anode and reducing oxygen at the cathode. Cells use molecular oxygen as the electron acceptor and catalyse the reduction to water, using neutral or slightly acidic media.

![Figure 1.13. Schematic of an enzyme fuel cell for oxidation of biofuels. Reproduced from Atanassov et al.](image)

Alcohol dehydrogenase enzymes can be used in a biocell such as this one for the oxidation of methanol to carbon dioxide, in which one half of the cell contains biological molecules and the other half using nicotine adenine diamine (NAD\(^+\)) as an electron mediator. The back reaction consuming carbon dioxide is a sustainable method for the reduction of carbon dioxide to produce fuel, with low impact on the environment.
7.3 Denaturation of enzymes

Enzymes are prone to denaturation and loss of activity in vitro. Factors causing this are comprehensively outlined by Iyer \textsuperscript{115} and involve the unfolding of the enzyme structure, leading to a disordered polypeptide chain. Enzymes are catalytic due to their conformational properties and functional group alignments, so an unfolding leads to a state where key residues are no longer closely aligned to be able to sufficiently co-operate in interactions with a substrate. The unfolding process can be reversible, but chemical changes can then lead to irreversible denaturation.

The Lumry-Eyring mechanism for protein unfolding \textsuperscript{116} defines enzyme inactivation as a two step process; the enzymes reversibly unfold, which is followed by several kinetically irreversible steps leading to disordered structure and aggregates. External factors causing denaturation include extremes of temperature, mechanical forces, pH, chemical agents, proteases, and ionic strength, all of which cause destruction of hydrophobic bonds, solvated groups, and peptide bonds. \textsuperscript{117} The next section will discuss various methods of immobilising enzymes to improve their lifetimes.

7. IMMOBILISATION OF ENZYMES

8.1 Introduction to Enzyme Immobilisation

There are many immobilisation techniques available for the prevention of enzyme denaturation. \textsuperscript{2,118,119} Immobilisation increases enzyme stability by introducing molecular rigidity, which stabilises the quaternary structure and prevents the structure from unfolding. This support provides thermal and long-term stability for the enzyme. It also provides a barrier to undesirable conditions such as extremes of temperature, pH, and exposure to protease contamination. Improvements in enzyme stability are attributed to an appropriate bio-mimetic micro-environment which offers ideal stabilisation and diffusion characteristics. \textsuperscript{120}

Applications of entrapped enzymes include industrial biotransformations, medical implants, and biosensors, all devices which must stand up to many cycles of high yield processes. Increasing enzyme and cofactor lifetime has been widely shown to increase catalytic activity, reducing denaturation and significantly improving yields. \textsuperscript{118,119} Longer lifetimes will also drive down the cost of biofuel cells in general. \textsuperscript{107}
There are many methods available for enzyme immobilisation described in the literature, including: prefabricated supports, entrapment in organic or inorganic polymeric matrices, cross-linking of enzyme molecules, and more recently, using novel supports such as hydrogels. A challenge only recently being addressed is the immobilisation of enzymes without exposing them to sub-optimal conditions which affect lifetime and stability, an example of which is the encapsulation into titania particles.

8.2 Prefabricated Enzyme Supports

‘Attaching’ enzymes to a rigid support enhances their stability due to the provision of a protected micro-environment, in which enzymes are either adsorbed, covalently bound, or entrapped within a material. These systems are convenient as the enzymes can be more easily handled and recovered from the support material. To preserve enzyme activity the material must simultaneously immobilise both sub-units of the enzyme structure, whilst also providing stabilisation of the quaternary structure.

Adsorption into mesoporous substrates is considered a simple method for immobilisation, without damaging the enzyme. Several theories exist as to why these networks stabilise enzymes. Firstly, the charge attraction between the enzymes and the mesoporous surface retains the shape of the enzyme. Secondly, the physical containment of the enzyme within appropriately sized pores protects it from denaturing, and also helps to rigidify the enzyme. Finally, covalent bonds between enzymes and the solid support add stability.

A variety of prefabricated inorganic materials have been tested for enzyme immobilisation, including silica, alumina, and titania networks. Enzymes have also been immobilised within nanoporous silica, where they are surrounded by a polymeric network (organic or inorganic) and further immobilised into nanoporous silica, with a high surface area and controllable pore sizes.

Other more complex methods in this area include the rigidification of the enzyme by attaching short molecular spacer arms to the enzyme and chemical modification (amination or succinylation of amino groups) for immobilisation into inorganic supports.

8.3 Polymer Matrices as Enzyme Supports

Widely used biopolymers for immobilisation include cellulose, starch, agarose, chitosan, gelatin and albumin. Synthetic polymers such as poly(ethylene glycol) (PEG) and poly(vinyl alcohol) (PVA) beads have also been used for the immobilisation
of enzymes. As well as providing much improved long-term kinetic and thermal stability, it is also possible to retrieve and recycle enzymes from these materials.

There are also examples within the literature of a range of polymeric supports for enzymes. One example uses tubular combinations of poly(caprolactone) and poly(ethylene oxide) (PEO), which have improved lifetime and stability of an alkaline phosphatase (AP) enzyme. Elsewhere, Bolivar et al have used highly activated glyoxyl agarose beads for immobilising alcohol dehydrogenase and formate dehydrogenase enzymes. The group have also explored using ionic adsorption to immobilise enzymes in poly(ethylene imine) (PEI) coated amino-epoxy supports, which gave a 100% activity retention of formate dehydrogenase.

Leaching of the enzyme can occur if there are a lack of electrostatic interactions or chemical bonds between the support and the enzyme. This can be overcome by including covalent attachments between the enzyme and the hydrogel, typically using the lysine group as the attachment.

Cross-linking the enzymes increases their overall size and reduce leaching from hydrogel networks. Another way of overcoming this problem is to create a polyelectrolyte with an opposite charge to the enzyme, so electrostatic interactions hold the enzymes within the network. This was shown by Bruns et al, who using a poly(2-hydroxyethylacrylate) hydrogel, loaded the gel by immersion into a solution of enzyme, and found enzymes located in hydrophilic domains.

Cross-Linked Enzyme Aggregates (CLEAs) allow precipitation of the enzyme by cross-linking them with a bi-functional reagent, such as glutaraldehyde. Precipitation is simple, purifying and immobilising the enzyme. This process can also be used for a mixture of enzymes. CLEAs retain activity and structure of the enzyme, as the enzyme is locked into a favourable conformation.

8.5 Peptide hydrogels for Immobilising Enzymes

Recently, self-assembling amphiphilc molecular hydrogels have been explored for enzyme immobilisation. These materials provide a biomimetic environment with physiological properties, minimising denaturation and inactivation of the enzyme. Jayawarna et al have used the spontaneously self-assembling diphenylalanine molecule for creating scaffolds in aqueous conditions for enzyme immobilisation.

One problem associated with using molecular hydrogels is that in order to trigger gelation, a change in pH, ionic strength, or temperature is required, which exposes the
enzyme to sub-optimal conditions. A method of avoiding this is to use enzyme catalysed hydrogelation processes. Wang et al\textsuperscript{136} have used a variety of enzymes encapsulated into hydrogels comprised of FmocFF, FmocK, and sodium carbonate. They report ‘superactivity’ of enzymes in the hydrogel in various solvents, up to eight times of that of the enzymes in water. They attributed the high enzyme activity to the localisation of the enzymes on the nanofibres of the hydrogel, coupled with the nanoporous channels in the gel facilitating the movement of substrates across the hydrogel network.

Hu et al have built on this work with peptide based systems, using the protein cross-linking enzyme transglutaminase (TGase) to form isopeptide linkages in molecular hydrogels, resulting in in-situ immobilisation of the enzymes.\textsuperscript{137} High activity of enzymes immobilised in this way was observed in organic solvents. Another example used two derivatives of amino acids to form a hydrogel with an immobilised enzyme, which showed an eightfold increase in activity compared to the native enzyme.\textsuperscript{136} The increased activity was attributed to the unique role of the self-assembled nanofibres. Enzymatic hydrogelation of Fmoc-tyrosine phosphate using a kinase / phosphatase switch has also been successfully explored.\textsuperscript{138}

8.6 Immobilisation of horseradish peroxidase

The enzyme horseradish peroxidase (HRP) is used in this study as a model, as it has a colorimetric assay and is relatively simple to study using UV-Vis spectroscopy. HRP has been immobilised in the literature in many ways to date,\textsuperscript{7} and is most commonly used as a H$_2$O$_2$ sensor. One procedure for ‘trapping’ this enzyme in a network has used heme models, joined with hydrogelators to form artificial immobilised peroxidase enzymes with high catalytic activity.\textsuperscript{139} Another method is the use of a cross-linked network of poly(hydroxyl cellulose) for immobilisation.\textsuperscript{7} Finally, a mixture of the spontaneously gelating dipeptide FmocFF has recently been used as a biosensor with quantum dots and HRP immobilised within its structure.\textsuperscript{140} The study found minimal leakage of the enzyme from the hydrogel.

9. CONCLUSIONS

Peptide-based gelators have large potential as biomimetic materials for many biologically-inspired energy harvesting/producing applications. An understanding of
their formation mechanisms has high relevance in understanding protein folding and unfolding. A new method for locally controlling self-assembly is required for expanding the potential applications of peptide hydrogels, where novel and specific designs can be created accurately. Finally, immobilisation of enzymes within dipeptide hydrogels will provide tuneable, biomimetic, and low-cost supports for enzyme fuel cells, which have shown promising results in the literature.

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CHAPTER TWO:
THEORY AND EXPERIMENTAL TECHNIQUES

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1. INTRODUCTION

This chapter presents the background theory and experimental details for all the techniques used in this thesis. The chapter covers a range of characterisation techniques used for measuring dipeptide hydrogels; their mechanical properties, gelation processes, structures, and so on. The methods used for creating hydrogels are also presented, for spontaneously gelating and pH-triggered dipeptides. Finally, enzyme immobilisation and the procedure for measuring enzyme activities are given in detail.

2. GELATION OF FMOC-PHE-PHE-OH (FMOCFF)

Fmoc-Phe-Phe-OH (FmocFF) gelates spontaneously when added to water at pH 7. A range of concentrations form hydrogels which are stable to inversion, ranging from 1 to 10 mg mL⁻¹. The dipeptide was made from the natural form of the amino acids, in the L-form.
Experimental procedure for gelating FmocFF: A stock solution of FmocFF (Bachem, 534.61 g mol⁻¹) in dimethylsulphoxide (DMSO, 99 %, anhydrous, Sigma-Aldrich) was prepared by dissolving 100 mg FmocFF in 1 mL DMSO (to give 100 mg mL⁻¹ solution). Small volumes of this solution (10 to 100 µL per 1 mL MilliQ water) were diluted into water, and quickly mixed before allowing to set. FmocFF hydrogels set over several minutes at high concentrations. The gelation could take up to three hours at the lowest concentrations.

3. GELATION OF FMOC-LEU-GLY-OH (FMOCLG)

The synthesis of the FmocLG dipeptide (Figure 2.1[a]) used in this work has been outlined in detail by Adams et al.,¹ along with gelation methods and detailed characterisation. The compound was obtained from the Adams group (University of Liverpool). The dipeptide was also made from the natural form of the amino acids, in the L-form.

FmocLG forms hydrogels when the acidity is lowered below pH 4 (Adams et al.¹⁵). Gelation is induced by addition of acid, and it has been shown that both HCl and glucono-δ-lactone (Gδ-L) gelate FmocLG at concentrations above 1.46 x 10⁻² mol dm⁻³ (5.8 mg mL⁻¹). Gδ-L is preferred as the slow hydrolysis in water yielding gluconic acid² leads to the formation of more transparent and homogeneous hydrogels. This was the first instance in which homogeneous dipeptide hydrogels could be formed reproducibly.

Electrochemical gelation of FmocLG: Using an electrochemically induced local pH drop to trigger the gelation of pH-sensitive dipeptides gives great advantages over addition of protons or addition of an acid which slowly hydrolyses, due to the added control over speed, time, intensity of current allowed to flow, or potential applied.

Experimental procedure for the electrochemical gelation of FmocLG:
The oxidation of hydroquinone to 1,4-benzoquinone and production of hydrogen ions was induced by allowing an oxidation current to flow at an electrode (Figure 2.1[b]). This created a localised pH gradient which was calculated to reach pH 3.6 after 60 s at the electrode surface (see Section 3.1). This pH drop was used to gelate FmocLG at a gold electrode surface.
To grow hydrogel films, a stock solution of $2.43 \times 10^{-3}$ mol dm$^{-3}$ (100 mg mL$^{-1}$) of FmocLG in DMSO was prepared. 10 µL of the stock was added to 1 cm$^3$ of a solution containing $6.6 \times 10^{-2}$ mol dm$^{-3}$ hydroquinone (HQ, Sigma-Aldrich, > 99 %) and $1 \times 10^{-2}$ mol dm$^{-3}$ NaCl (Sigma-Aldrich, ≥ 99.5 %). 4 µL of 1 mol dm$^{-3}$ NaOH (Sigma-Aldrich, ≥ 98 %) was added to fully dissolve the dipeptide, then 8 µL 1 mol dm$^{-3}$ HCl (Sigma-Aldrich 36.5 - 38.0 %) added to bring the pH to 7.0. The final solution was introduced to the surface of the electrode via an electrochemical flow cell.

A hydrogel was grown using an electrochemically induced pH drop. Film growth was initiated when hydroquinone (HQ) was oxidised at the electrode surface, to produce quinone and two protons. 20 µA cm$^{-2}$ was allowed to flow for times between 100 s and 3 hours, creating a surface pH of approximately 3.65, which was sufficient to initiate hydrogel formation in the presence of FmocLG.

Figure 2.1 [a] Molecular structure of FmocLG (MW: 410.47 g mol$^{-1}$) [b] Electrochemical two electron oxidation of hydroquinone to 1,4-benzoquinone releases two equivalents of H$^+$. Reproduced from Johnson et al.$^3$
3.1 Calculating the electrochemically induced pH drop

The pH drop shown in Figure 2.1[b] was calculated as follows: The application of 10 µA current for 60 s corresponds to the generation of $5.6 \times 10^{-4}$ C (with correction for the background charging current). This corresponds to $5.8 \times 10^{13}$ protons s$^{-1}$ or $9.6 \times 10^{-11}$ moles H$^+$ s$^{-1}$. The diffusion layer thickness $l_d$ after 1 s can be estimated from $l_d = \sqrt{\frac{aD}{\pi}}$ as $1.7 \times 10^{-2}$ cm (assuming the diffusion coefficient for protons in water is $9.3 \times 10^{-5}$ cm$^2$ s$^{-1}$). The surface area of the electrode was 0.5 cm$^2$ which gives a cylindrical volume of $3.34 \times 10^{-6}$ cm$^3$. An increase of $9.6 \times 10^{-11}$ moles H$^+$ in this volume corresponds to a surface localised pH change from pH 7 to pH 4.5 after 1 s. After 60 s the diffusion layer thickness was 0.13 cm and the surface pH had decreased to 3.65.

4. CIRCULAR DICHROISM (CD)

4.1 Introduction to Circular Dichroism (CD)

Circular Dichroism (CD) is a technique used to determine the degree of folding of proteins in solution, providing estimates of type and ratios of secondary structures present in a protein, from $\alpha$, $\beta$ and random coil. It is possible to follow structural changes as they occur using CD, monitoring folding or unfolding. CD is also commonly used as a technique to quantify structures present within dipeptide hydrogels. It illuminates the sample with linearly-polarised light and measures the resulting elliptically-polarised light.

CD effects occur in the UV spectrum, associated with electron transitions in molecules. CD arises in a sample when there is a difference in absorption (dichroism) between R- and L- circularly polarised light. The changes in chiral environments are measured as a function of incident light, $\lambda$. Asymmetric chromophores absorb the R- and L- polarised light to different extents, giving different extinction coefficients for the two components. The sample is irradiated with alternately R- and L- polarised light, and the difference is the measured CD signal.

Electronic spectroscopy transitions are rapid ($10^{15}$ s$^{-1}$) and much faster than vibrational transitions, therefore showing vibrational and rotational fine structure. In solution the fine structure is not resolved and electronic transitions are broad, bell-shaped bands, known as Cotton effects. There are two types of Cotton effect; those associated with chiral chromophores (intrinsic) and those with achiral chromophores in asymmetric...
environments (extrinsic). Extrinsic effects are usually much weaker than intrinsic. The electron transitions available to proteins and their peak wavelengths are given in Table 2.1.

Table 2.1. Chromophore types and their electron transitions.

<table>
<thead>
<tr>
<th>Chromophore</th>
<th>Type</th>
<th>Electron transitions</th>
<th>( \lambda_{\text{max}} )</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide bond</td>
<td>intrinsic</td>
<td>( \pi-\pi^* ), n-( \pi^* )</td>
<td>&lt;200, 200-240</td>
<td>2°</td>
</tr>
<tr>
<td>Cystine bridge</td>
<td>intrinsic</td>
<td>n-( \sigma^* )</td>
<td>240-360</td>
<td>2°</td>
</tr>
<tr>
<td>Aromatic (Tyr, Trp, bases)</td>
<td>extrinsic</td>
<td>( \pi-\pi^* )</td>
<td>250-310</td>
<td>3°</td>
</tr>
<tr>
<td>Haem</td>
<td>extrinsic</td>
<td>( \pi-\pi^* ) (and others)</td>
<td>360-460</td>
<td>3°</td>
</tr>
</tbody>
</table>

Molar ellipticity is the signal obtained from the CD spectrum. It is used to show the extent of ellipticity in the sample. It is a ratio of change in CD signal to molar concentration, see Equation 2.1.

\[
[\phi]_{\text{molar}} = \frac{100 \phi \lambda}{m d}
\]

Equation 2.1. Molar ellipticity \([\phi]_{\text{molar}} \) (deg cm\(^2\) dmol\(^{-1}\)) as a ratio of change in CD signal to molar concentration. \( \phi \lambda \) = change in CD signal; \( m \) = molar concentration (mol dm\(^{-3}\)); \( d \) = path length (cm).

Peaks in the CD spectrum are generally assigned as follows; the far UV section (180 – 250 nm) of the CD graph is shown in Table 2.2.

Table 2.2. Far UV CD absorption bands with assignments to structure.

<table>
<thead>
<tr>
<th>2°</th>
<th>Absorption bands</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha )</td>
<td>+ve 192, -ve 208, -ve 222</td>
</tr>
<tr>
<td>( \beta )</td>
<td>+ve 198, -ve 215</td>
</tr>
<tr>
<td>Random coil</td>
<td>-ve 198</td>
</tr>
</tbody>
</table>

Peaks arising from the near-UV section of the spectrum (250 – 310 nm) contain bands from aromatic side chains in asymmetric environments. The CD signal comes from an induced effect and is much weaker at these wavelengths, so peaks are not generally assigned to a specific structure. They are attributed to the tertiary structure which is affected by the absorption and dipole orientation of the amino acids.
4.2 Sample preparation for CD experiments

CD experiments were carried out in the Serpell group at the University of Sussex. Samples for CD were made as follows. FmocFF was added to and mixed with water (or a solution of D-Phe or L-Phe), in a sample tube, as in Section 2.1. The pre-gelled mixture was transferred to the cell sample holder via pipette. The path length of the cell was 0.005 cm, holding approximately 20 µL of hydrogel.

Blank samples were measured with MilliQ water, and solutions of D-Phe and L-Phe at concentrations of 9.5 mg mL\(^{-1}\) (0.05751 mol dm\(^{-3}\)). These spectra were subtracted from the hydrogel spectra.

Linear Dichroism (LD) spectra were also taken, by rotating the sample 90°. This ensures the CD spectra are not aligned in one direction.

5. DIFFUSION ORDERED NMR SPECTROSCOPY (DOSY)

5.1 Introduction to DOSY (Diffusion Ordered NMR Spectroscopy)

DOSY (Diffusion Ordered NMR Spectroscopy) can be used to measure the diffusion coefficient (D) of water in a sample, given by Equation 2:

\[ d = \sqrt{2 Dt} \]

*Equation 2.2. Diffusion coefficient; d: diffusion distance (µm), D: diffusion coefficient (m\(^2\) s\(^{-1}\)), t: time (ms).*

In the DOSY experiment, the \(^1\)H spectrum is acquired 16 times using a “pulsed gradient stimulated echo” (PGSte). This gives a “normal” \(^1\)H spectrum with reduced signal intensity. A magnetic field gradient is applied across the sample. The stronger the gradient (g), the more the signal intensity (I) is reduced in Equation 2.3:

\[ I = I(0) \exp(-D \gamma^2 g^2 \delta^2 (\Delta - \delta/3)) \]

*Equation 2.3. I(0): Signal intensity without gradient, D: Diffusion coefficient of the molecule providing the signal, g: gradient strength, \(\gamma\), \(\delta\), and \(\Delta\): constants: 4258 Hz G\(^{-1}\), 2 ms and 50 ms respectively.*

The result from acquiring the spectrum 16 times with increasing gradient strength (g) is that the signal intensity (I) becomes progressively weaker. The amount by which the signal intensity is reduced is also related to the diffusion speed. A plot of \(\ln(I/I(0))\)
versus $g^2$ gives a straight line from which the diffusion coefficient, $D$ can be obtained. The Bruker software performs a `least squares fit' of $\ln[I/I(0)]$ versus $g$ to obtain $D$.

Recently, DOSY measurements have been used to determine the diffusion coefficient of water inside low-molecular weight hydrogels, and natural polymers such as cellulose.$^9$-$^{11}$

5.2 Sample Preparation for DOSY experiments
FmocFF hydrogels were made directly into NMR tubes for analysis. FmocLG hydrogels were made outside and transferred to NMR tubes for analysis. The measurements were taken using a Bruker Avance 400 NMR spectrometer operating at 400.13 mHz and 298 K, using the parameters described above. The samples were measured for 1 hour, and the H$_2$O peak measured with time to give a value for the diffusion coefficient, $D$.

6. FOURIER TRANSFORM INFRA-RED (ATTENUATED TOTAL REFLECTION) SPECTROSCOPY (ATR FTIR)

6.1 Introduction to Fourier Transform Infra-Red Spectroscopy (FTIR)
Fourier Transform Infra-Red (FTIR) can be used to assign peaks to the internal structure of peptide hydrogels by using the amide regions to discern orientations of hierarchical structures. According to Kong et al.$^{12}$ peaks at 1684 cm$^{-1}$ and 1669 cm$^{-1}$ can be assigned to $\alpha$-helices, and the peak at 1637 cm$^{-1}$ to $\beta$-sheets.

FTIR passes infra-red (IR) radiation through a sample, giving a molecular absorption and transmission spectrum. It is a qualitative technique which measures the amount of sample present as well as specific molecular bonds. A beamsplitter is used to split the incident light, allowing the detector to collect data from a range of wavelengths at the same time. Fourier transform is applied to each data point to generate a spectrum.$^{13}$

In Attenuated Total Reflection (ATR) FTIR, the sample rests on a crystal which enhances the signal and allows the spectrum to be generated in reflectance mode. This is shown in Figure 2.2.
The hydrogel sample was measured on a gold slide. This was placed onto the FTIR sample holder, enhancing the signal via reflection from the gold layer.

6.2 Sample preparation of FmocFF hydrogels for FTIR experiments

10 µL of a 100 mg cm\(^{-3}\) FmocFF stock solution in DMSO was added to D\(_2\)O to make a 0.5 cm\(^3\) hydrogel volume with 3.47 x 10\(^{-3}\) mol dm\(^{-3}\) FmocFF. The hydrogels were sealed and left for 24 hours, before being transferred onto a gold slide. Measurements were taken using a Perkin Elmer Spectrum 100, with the same force applied to each sample.

6.3 Sample preparation of FmocLG hydrogels for FTIR experiments

FmocLG hydrogels were grown directly onto gold slides via an electrochemical deposition method. Samples were made in D\(_2\)O. Measurements were taken using a Perkin Elmer Spectrum 100, as above.

7. FIBRE X-RAY DIFFRACTION (FIBRE-XRD)

7.1 Introduction to Fibre X-Ray Diffraction (Fibre-XRD)

When combined with modelling, Fibre-XRD is a powerful tool for providing information on the crystallinity and unit cell dimensions of β-sheet samples, including peptide hydrogel materials\(^{15}\). The samples must be prepared in order to align the fibres / fibrils in one axis. Figure 2.3[a] (below) shows one preparation method for aligning fibres in the hydrogel sample. A blob of pre-gelled mixture is placed between two capillaries sealed with wax, and left to air dry.
Figure 2.3[a] Schematic showing sample preparation for Fibre-XRD to align fibres by allowing a hydrogel to form between two wax sealed capillary tubes. [b] Example Fibre-XRD pattern from a cross-β architecture in a sample. Reproduced from Morris et al.\textsuperscript{15}

Figure 2.3[b] is an example Fibre-XRD diffraction pattern for a cross-β sample with hydrogen-bonded strands on the fibre axis (arrow), and associating β-sheets perpendicular to the fibre axis.\textsuperscript{15} The directionality and repetition of structure in the sample gives rise to a signal of 4.7-8 Å on the meridian and 10 – 12 Å on the equator. The signal on the meridian arises from the crystallinity of the sample along the fibre axis and corresponds to the distance between each H-bonded β-strand.\textsuperscript{15,16} This gives information on unit cell dimensions in this plane. The signal on the equator arises from associations of β-sheets, and is more diffuse as it is the average of the order present perpendicular to the plane of the fibril axis. It is also affected by the size of the side chains present in the molecules.\textsuperscript{16}

7.2 Sample preparation of hydrogels for Fibre-XRD experiments

Fibre-XRD experiments were carried out in the Serpell group at the University of Sussex. A 4 µL blob of pre-gelled, freshly mixed FmocFF and water was placed between two capillary tubes, the ends of which were sealed with wax as shown in Figure 2.3[a]. The capillaries were slowly pulled apart, and the pre-gelled mixture was allowed to air dry. Samples with excess salt or additive required washing prior to measuring. Fibre sample is mounted on a goniometer, and data collected using a Rigaku Cu Kα rotating anode and R Axis IV++ detector. Images were analysed using a structural modelling and testing program, CLEARER.
8. FLUORESCENCE SPECTROSCOPY

8.1 Introduction to Fluorescence Spectroscopy
Fluorescence can be used to follow the hydrogelation kinetics of the spontaneously gelating dipeptide FmocFF, when coupled with a fluorescent amyloid fibre stain. Fluorescence is light emission from the singlet-excited state of a molecule. The return to the ground state is spin-allowed and occurs rapidly (typically $10^6$ s$^{-1}$) by emission of a photon, giving a typical fluorescence lifetime of approximately 10 ns. Fluorescence spectral data is acquired by the illumination of a sample with a specific wavelength of light, and recording the emission spectrum across a range of wavelengths.

8.2 Thioflavin T (ThT) as a fluorescent stain for dipeptide fibres
Though the exact mechanism of binding and subsequent fluorescence from the binding of the benzothiazole dye Thioflavin T (ThT – see Figure 2.4) to amyloid fibres is not entirely understood, the assay is widely utilised in following dipeptide hydrogel formation, as it fluoresces when in the presences of α- and β- structure.$^{17,18}$ An increase in fluorescence is observed proportional to the extent of amyloid structure present in the sample.$^{19}$ The signal is dependent on fibril morphology.$^{20}$

![Figure 2.4. Molecular structure of Thioflavin T (ThT).](image)

ThT exists in solution as micelles of 3 nm diameter at the concentrations generally used for fluorescence binding assays (10 – 20 μ mol dm$^{-3}$).$^{21}$ The micelles bind along the length of the fibrils and change conformation such that the fluorescence is no longer quenched. The positive charge on the ThT molecule has an effect on micelle formation and is considered to be the force binding them to amyloid fibres.

8.3 Sample preparation of hydrogels for fluorescence measurements
A USB4000 Ocean Optics fluorimeter with Spectra Suite software was used to measure the hydrogelation kinetics of $3.47 \times 10^{-3}$ (2 mg mL$^{-1}$) FmocFF in MilliQ water. The excitation wavelength was at 450 nm and the emission measured at 494.5 nm. The emission of the scattering from the pre-gel solution was of a much higher intensity, collected at 474 nm. Figure 2.5 shows an example emission spectrum as a solution of
FmocFF formed a hydrogel, following the two traces at 494.5 and 474 nm in real time. ThT was added to give a final concentration of $5 \times 10^{-3}$ mol dm$^{-3}$.

**Figure 2.5[a]** Emission over time due to scattering at 474 nm (blue squares; left axis) and ThT response at 494.5 nm (green triangles; right axis) with 200 ms window, showing the gelation of $3.47 \times 10^{-3}$ mol dm$^{-3}$ FmocFF over 10 minutes. The scattering of the pre-gel solution caused the ThT response to be shielded until the point where clearing (gelation) occurred, which caused a linear increase in fluorescence to be observed. The gradient of the increase in fluorescence is measured and used as an indication of the extent of hydrogel (fibril) structure. **[b]** ThT response upon gel formation at 494.5 nm. The lag phase is the time taken before the FmocFF started to form structure. The growth phase began when the hydrogel was beginning to form. The slope of this curve correlates to the time taken for a hydrogel to form.

Information regarding gelation time and final hydrogel strength can be gained from recording the ThT trace at 494.5 nm, as shown in Figure 2.5[b]. ThT is generally
measured at is $\lambda_{\text{max}}$, but here the wavelength is higher than in literature because the scattering peak largely obscures the ThT signal. The values obtained for fluorescence were for a 200 ms integration time. Real data in results sections are corrected to give counts per second.

There is an issue with this measurement in that at short times the hydrogels are too cloudy for the ThT response to be observed through the sample. This means the gradient of the increase in ThT response (494.5 nm) is directly related to the gradient due to the decrease in the scattering of the sample (474 nm). This is unavoidable, yet the ThT gradient does seem to correspond to the gelation of the hydrogels, although it is not a direct measurement. Many other ways of measuring gelation were calculated, such as the negative gradient of the scattering trace corresponding to the clearing of the hydrogel, the area under the peak, etc. However, the gradient of the ThT response gave by far the most consistent data, and does seem to correspond to an increase in $\beta$-sheet structure.

9. RHEOLOGY

9.1 Introduction to Rheology

Rheology can be used to measure the strength of hydrogels and their properties. Rheology is the science of the deformation and flow of fluids, semi-solids and solids under controlled conditions.\textsuperscript{22,23} It is used in the characterisation of hydrogels to determine their viscoelastic properties, by measuring viscosity over a range of shear rates. There are two experimental methods used in this section; time sweep (response to stress or strain at a constant frequency, amplitude and temperature), and frequency sweep (response to increasing frequency at constant amplitude and temperature). Hydrogels exhibit non-Newtonian behaviour (elastic behaviour).

Stress is defined as the force per unit area acting upon and within a material. Strain is the amount of deformation induced by the stress. The rheometer induces strain and measures stress, or viscosity (resistance to flow) over a wide range of shear rates. As a force is applied to a volume of material, deformation occurs (see Figure 2.6). Newton’s law states shear stress (force / area) is proportional to shear strain rate (volume / height, with the dynamic viscosity $\eta$ as the proportionality constant (see below):
Figure 2.6. Force applied in the x-direction results in deformation V.

Shear stress: \( \tau = \frac{f}{A} \); Shear strain: \( \gamma = \tan \theta \);

Shear strain rate: \( \dot{\gamma} = \frac{x(t)}{y(t)} \); Shear modulus: \( G = \frac{\tau}{\gamma} \); Viscosity: \( \eta = \frac{\tau}{\dot{\gamma}} \).

The viscoelastic properties of the molecular structure of materials can be quantified using these equations. Viscoelastic materials respond to applied stress in a time-dependant way, as shown in Figure 2.7 below.\(^{24}\)

Figure 2.7. The regions of viscous (Newtonian behaviour) and elastic (non-Newtonian behaviour) are shown in relation to time and shear strain. The point where the two cross, \( \tau \), is the relaxation time of the material. From this graph, the gradient \( \eta \sim G_o \tau \).

With dynamic testing of materials, an oscillatory (sinusoidal) deformation (stress or strain) is applied to a sample and the response (strain or stress) is measured. The complex shear modulus \( G^* \) is the measure of the overall resistance to deformation, defined by a ratio (Equation 2.4).

\[
G^* = \frac{\text{shear stress}^*}{\text{strain}}
\]
\[ G^* = G' + iG'' \]

*Equation 2.4. Complex shear modulus, \( G^* \).*

\( G' \) is the ‘in-phase’ storage modulus (elastic) and \( G'' \) the ‘out-of-phase’ (viscous) storage modulus. The frequency at which these parameters intersect gives the relaxation time \( \tau \) for each sample. \( G' \) is a measure of elasticity of the material and its ability to store energy (Equation 2.5).\(^{25}\)

\[ G' = \frac{\text{shear stress}^*}{\text{strain}} \cos \theta \]

*Equation 2.5. In-phase (elastic) storage modulus, \( G' \).*

\( G'' \) is a measure of the materials’ ability to dissipate energy, which is lost as heat (Equation 2.6).

\[ G'' = \frac{\text{shear stress}^*}{\text{strain}} \sin \theta \]

*Equation 2.6. Out-of-phase (viscous) storage modulus, \( G'' \).*

A time sweep is used to measure the materials response at a constant frequency, amplitude and temperature. A dynamic sweep (torque ramp) is used to measure the materials response to increasing deformation amplitude (stress or strain) at a constant frequency and temperature (Figure 2.8).
A frequency sweep is used to measure the materials response to increasing frequency (rate of deformation), which is monitored at a constant amplitude (stress or strain) and temperature.

9.2 Sample Preparation of Hydrogels for Rheological Experiments

Rheological experiments were carried out in the Adams group at the University of Liverpool. The experimental procedure for rheology experiments was similar to that described in Chen et al.\textsuperscript{27} Samples were made 24 hours prior to each experiment. The sample tubes used were 22 mm diameter and 60 mm high. Each sample had a total volume of 2 mL. The rheometer model was an Anton Paar MCR101. All samples were measured at 20 °C. Time sweep measurements for complex viscosity (storage modulus G') and shear stress (loss modulus G'"") were taken over 10 hours at 10 rad / s at a strain of 1 %. Frequency sweep measurements were carried out at 0.1 % strain, in the viscoelastic region, where G' and G'" are independent of the strain amplitude.\textsuperscript{27}
10. IMAGING TECHNIQUES: SCANNING ELECTRON MICROSCOPY (SEM)

10.1 Introduction to Scanning Electron Microscopy (SEM)
In SEM, the sample surface is imaged by scanning with a high-energy beam of electrons, under vacuum. Electrons interact with surface atoms, producing signals which contain information about the surface topography. Samples must be conducting so are coated by sputtering a thin layer of gold. The resolution achieved is 1 – 2 nm. In this section, SEM and cryo-SEM are described for FmocLG and FmocFF hydrogels at 1 wt % (2.43 x 10⁻³ and 3.74 x 10⁻³ mol dm⁻³ respectively). The samples were roughly 0.5 mm³ total volume.

10.2 Preparation of hydrogels by cryogenically-drying for low temperature SEM
The details of sample preparation for cryo-SEM are given in Figure 2.9.

10.3 Preparation of hydrogels by freeze-drying for SEM
The procedure for preparing and measuring freeze-dried samples is shown in Figure 2.10.
11. TRANSMISSION ELECTRON MICROSCOPY (TEM)

11.1 Introduction to Transmission Electron Microscopy (TEM)
Transmission Electron Microscopy (TEM) uses a beam of electrons which is transmitted through an ultra thin sample, interacting with the material as it passes through. An image is formed from the interaction of the electrons transmitted through the specimen. The resolution is approximately 1 nm.

11.2 Sample preparation of hydrogels for negatively-stained TEM
For the FmocFF dipeptide, a drop of the pre-gelled mixture was added to a gold or nickel TEM sample grid. After the hydrogel had formed it was stained with 1 % uranyl acetate solution for 2 minutes, washed, then stained (as in Lin et al\(^ \text{28} \)). Excess stain and water was blotted with filter papers, as in Figure 2.11[a].

For FmocLG hydrogels, TEM was carried out on air-dried membranes that had been grown directly onto a gold or nickel TEM sample grid, using a three-electrode setup shown in Figure 2.11[b]. These samples were not stained with uranyl acetate.
Two different types of TEM grids were used; 95, and 430 µm diameter hexagons (holes), corresponding to 200 and 50 mesh. Measurements of FmocLG hydrogels were acquired at the University of Bath using a Jeol TEM 1200ExII. Measurements of FmocFF hydrogels were taken using a Hitachi 7100 TEM operating at 100 kV, and images acquired using a Gatan Ultrascan 100 CCD camera, at the University of Sussex.

12. CONFOCAL MICROSCOPY

12.1 Introduction to Confocal Microscopy
Confocal Microscopy is a fluorescence microscope technique. An aqueous sample is flooded evenly with light from a laser source. All parts in the optical path are excited simultaneously, with resulting fluorescence detected by a photodetector. The general setup for a confocal microscope is given in Figure 2.12.
Thin optical sectioning allows for 3D imaging and surface profiling. In this work the fibres were stained with Nile Red and excited at 488 nm.

12.2 Sample Preparation for FmocLG hydrogels for Confocal Microscopy
FmocLG hydrogels were grown directly onto a cavity microscope slide previously coated with a conducting fluorine doped tin oxide layer (FTO, prepared by Solaronix SA). A drop of $2.43 \times 10^{-3}$ mol dm$^{-3}$ FmocLG solution was added to 10 µL of $5 \times 10^{-5}$ mol dm$^{-3}$ Nile Red (Sigma-Aldrich, ~75 % dye content) in the cavity of the slide. Using the FTO slide as the working electrode, a Pt counter electrode, and an Ag$^+$/AgCl reference electrode, 5.6 µA cm$^{-2}$ was applied to the drop for 2 hours and a hydrogel formed on the surface.

Imaging was carried out on a Dual laser Nikon Eclipse 90i confocal scanning laser microscope using a 60x oil immersion microscope objective. An excitation wavelength of 488 nm, from a HeNe laser, was used to image the hydrogel.

12.3 Sample Preparation for FmocFF hydrogels for Confocal Microscopy
10 µL of 100 mg cm$^{-3}$ (3.74 $\times 10^{-3}$ mol dm$^{-3}$) FmocFF stock solution in DMSO was added to 1 cm$^3$ MilliQ water with 10 µL of $5 \times 10^{-5}$ mol dm$^{-3}$ Nile Red. A drop of the pre-gel solution was added to a cavity well slide and allowed to gelate.
13. ATOMIC FORCE MICROSCOPY (AFM)

13.1 Introduction to Atomic Force Microscopy (AFM)
Atomic Force Microscopy (AFM) utilises a cantilever and tip which oscillates close to the sample surface. The deflection of the tip is measured with a laser spot which is reflected into photodiodes. The microscope used in this work was a Nanosurf EasyScan 2 mounted onto an isostage controller. The resolution is approximately 10 to 15 nm.

13.2 Preparation of hydrogels for AFM measurements
FmocLG samples were electrochemically grown directly onto gold slides and transferred to the stage. Pre-formed FmocFF hydrogels were transferred onto a microscope slide. Measurements were carried out using a Tap190Al-G cantilever and tip with a free vibration amplitude of 200 mV, using Phase Contrast and Dynamic Force modes.

14. NMR TECHNIQUES

14.1 Use of NMR to evaluate additives in FmocFF hydrogels
In order to determine whether additives to FmocFF hydrogels became incorporated into the fibres of the hydrogels or remained in the pores, NMR experiments were carried out. Ethanol was added to the hydrogel as a marker, as it would remain free to move and produce a peak in a hydrogel. The integration of the peak relative to the additive peak was used to work out the ratio between ethanol and the additive in question. Specifically this technique was used for the addition of D- or L-Phe to FmocFF hydrogels.

14.2 Experimental procedure for NMR measurements
A 1:1 molar mixture of additive (D / L-Phe) : EtOH was dissolved in D$_2$O, to which 1 wt % FmocFF was added, and quickly transferred to a sealed NMR tube to allow the hydrogel to set. In a $^1$H (proton) NMR of an Fmoc-FF gel, the EtOH peaks are visible as it is free to move in solution. If the position of the additive molecules (-Phe) were within
the hydrogel fibres, its peak would not be seen in the spectrum; if the molecules remained in the pores after gelation, the peak would be visible.

The control solutions and hydrogels measured are listed in Table 2.3.

Table 2.3. Solutions and samples for an NMR experiment to determine the ratio of D/L-Phe in pores or fibres of an FmocFF hydrogel.

<table>
<thead>
<tr>
<th>Sample</th>
<th>D/L-Phe mol dm(^{-3})</th>
<th>EtOH mol dm(^{-3})</th>
<th>FmocFF x 10(^3) mol dm(^{-3})</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.05</td>
<td>0.05</td>
<td>0</td>
<td>Standard</td>
</tr>
<tr>
<td>2</td>
<td>0.005</td>
<td>0.005</td>
<td>0</td>
<td>Standard</td>
</tr>
<tr>
<td>3</td>
<td>0.05</td>
<td>0.05</td>
<td>3.47</td>
<td>Hydrogel</td>
</tr>
<tr>
<td>4</td>
<td>0.005</td>
<td>0.005</td>
<td>3.47</td>
<td>Hydrogel</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0.05</td>
<td>3.47</td>
<td>Control</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0.005</td>
<td>3.47</td>
<td>Control</td>
</tr>
</tbody>
</table>

The ratio of the integral of the EtOH peak to the -Phe peak may be calculated to find the probable positions of the additives within a hydrogel.

15. SURFACE PLASMON RESONANCE (SPR)

15.1 Theory of Surface Plasmon Resonance (SPR)

Surface Plasmon Resonance (SPR) is an optical spectroscopic method used for the quantification of attachment of molecules to surfaces, including the self-assembly of monolayers on metals. A surface plasmon (SP) propagates along a metal/dielectric interface, as shown in Figure 2.13. The dielectric constant of the metal is denoted by \(n_m\), and that of the sample by \(n_d\).
Figure 2.13. Surface Plasmon propagating along a metal surface, penetrating into the dielectric with distance $L_p$.

The SP originates from collective oscillations of light coupled into a metallic film, as the light interacts with the free electron ‘gas’ present in the metal. Resonance is achieved when the light wave vector matches the electron ‘gas’ (surface plasmon wave vector). This is shown below in Figure 2.14[a], with the cell setup in the Kretschmann configuration used in this work to achieve this resonance in [b]. A gold film is typically used for SPR of around 50 nm thick.

Figure 2.14[a] Incident light coupling into a gold film via a prism to create a surface plasmon probing into the dielectric; [b] Cell setup used in experiments. A fluid of high refractive index is used to match the prism to the LaSFN$_9$ glass.

Figure 2.14[a] shows the electromagnetic field decaying exponentially into the medium above the surface, with a maximum intensity at the surface. The field is sensitive to the analyte above the metal surface to nanometre accuracy, probing up to several hundreds of nanometres by measuring changes in the refractive index at the surface.\textsuperscript{32} The limit of detection, or penetration depth $L_p$ for SPR is approximately 200 nm away from the metal surface.\textsuperscript{33,34}
Figure 2.15[a] shows the spatial distribution of the magnetic intensity along the x-coordinate, and the penetration of an SP propagating along a metal/dielectric interface.

![Figure 2.15. Distribution of magnetic intensity $H_y$ of the SP propagating along the interface of the metal surface (gold) and the dielectric (water) at a wavelength of 633 nm. Reproduced from Dostalek et al. 35](image)

Figure 2.14[b] below is a graph of the incident light $K_{in}$ versus the angular frequency of light, $\omega$. It shows the effect of different metals on the coupling of the incident light with the surface plasmon in the metal.

![Figure 2.14[b] Dispersion relation of the surface plasmon at the interface between air and gold, silver and aluminium. Reproduced from Dostalek et al. 35](image)
The wave vector of the incident light is $K_{in} = \frac{\omega}{c}$, where $\omega$ is the angular frequency, and $c$ is the speed of light in a vacuum. SPR uses $p$-polarised incident light to excite a plasmon in the metallic film. SPs are excited when the momentum of the incident light couples with the momentum of the emerging plasmon. The component of the momentum, parallel to the plane of incidence, is transferred to the SP.\textsuperscript{32} The incident light wave vector $K_{in}$ (Equation 2.7) arises from the incident light, with a component in the plane of the metal, interacting with the surface plasmon in the metal.\textsuperscript{32}

$$K_{in}(x) = \frac{\omega}{c} \varepsilon_p \sin \theta$$

\textit{Equation 2.7. Incident wave vector $K_{in}$. $\omega =$ angular frequency, $c =$ speed of light in a vacuum, $\varepsilon_p =$ dielectric permittivity (square of the refractive index) of the prism, $\theta =$ incident angle.}

The wave vector of the surface plasmons depends upon the dielectric permittivity of the metal ($\varepsilon_m$) and the dielectric ($\varepsilon_d$). This leads to Equation 2.8, giving the complex propagation constant, or Surface Plasmon Wave Vector:

$$K_{SP} = \frac{\omega}{c} \left( \frac{\varepsilon_m \varepsilon_d}{\varepsilon_m + \varepsilon_d} \right)^{\frac{1}{2}}$$

\textit{Equation 2.8. Surface Plasmon wave vector $K_{SP}$.}

An SP mode occurs when $K_{in} = K_{SP}$. Here, the incident light is dispersed in an evanescently decaying field within the dielectric, rather than being totally internally reflected. SPs are sensitive to the angle, wavelength, and degree of polarisation of incident light.

SPR data is fitted using Fresnel equations, which model the behaviour of light moving between multi-layered systems of differing refractive indices. In a two-layered system, the reflection of light from a single interface is described, shown in Figure 2.16. When light moves from a medium with refractive index $n_1$ into another with refractive index $n_2$, reflection and refraction can occur, and is dependent upon the incident angle.
The reflectance ($R$) and transmittance ($T$) for $s$- and $p$-polarised light are given by Equations 2.9 below:

$$R_s = \frac{n_2 \cos \theta_i - n_1 \cos \theta_t}{n_2 \cos \theta_i + n_1 \cos \theta_t} \quad T_s = \frac{2n_1 \cos \theta_i}{n_2 \cos \theta_i + n_1 \cos \theta_t}$$

$$R_p = \frac{n_2 \cos \theta_t - n_1 \cos \theta_i}{n_2 \cos \theta_t + n_1 \cos \theta_i} \quad T_p = \frac{2n_1 \cos \theta_i}{n_2 \cos \theta_t + n_1 \cos \theta_i}$$


In a multi-layered system consisting of multiple interfaces, the reflections are also partially reflected. These reflections can interfere constructively or destructively with one another. A schematic is shown in Figure 2.17.
The behaviour of light can be modelled by using matrices which incorporate Maxwell’s equations, which use the fact that there are continuity conditions across boundary layers. The electric field at the beginning of a layer can be used to work out the electric field at the end of a layer. This is incorporated into the Winspall fitting software used to fit data in this work (Res-Tec, Resonant Technologies GmbH, Bahnhofstraße 70 a55234 Framersheim, Germany).

15.2 Using SPR to measure adsorption / absorption of molecules at a surface
The Kretschmann configuration shown in Figure 2.14 allows light to be coupled into the plasmon wave in the thin metal film, via a prism. The cell is mounted onto a goniometer and a 632.8 nm laser is used as the incident light in Figure 2.18.

\[ \text{Figure 2.18. Laser setup for SPR showing a 632.8 nm beam passing through polarisers and a chopper, into the sample via a prism. The cell is mounted onto a goniometer. Reflected light is measured using a photodiode.} \]

SPR can be used to measure kinetics of binding molecules to surfaces, and to measure properties of the bound material. This is possible if the molecule has a higher dielectric constant than that of the solvent used. Binding of molecules to the metal surface increases \( \varepsilon_d \), as in the example of a thiol onto a gold surface. This leads to a shift in the minimum angle. A characteristic reflection-incident angle curve is shown in Figure 2.19[a]; before and after attachment of a self-assembled thiol monolayer at the surface of the metal.
The critical angle \( \theta_c \) occurs at the point of total internal reflection, where all the light is reflected into the detector. This angle is dependent upon the average dielectric constant of the material at the gold surface. The minimum angle \( \theta_{\text{min}} \) occurs when all the light is coupled into the gold film. This is sensitive to materials adsorbed at the gold surface. Binding can be measured in real time at a fixed angle, measuring the increase in reflectivity with time, as in Figure 2.19[b]. The angle must be chosen such that any increase in reflectivity is linear, as shown by the arrow in [a]. It is possible to use the information to measure the thickness or mass density of the adsorbed layer, and to predict surface coverage from the results.

### 15.3 SPR setup used for experiments

Surface plasmon enhanced fluorescence spectroscopy (SPR) was measured in the Kretschmann attenuated total internal reflection configuration on a home-built set-up. Laser (Uniphase, HeNe, \( \lambda = 632.8 \) nm) light was passed through a chopper and two polarisers before being incident on one face of a LaSFN\(_9\) prism (Schott Glass). The first polariser adjusted the intensity of the light and the second polariser allowed only \( p \)-polarised light to reach the sample. The chopper modulated the light at 431 Hz and provided a reference signal for the lock-in amplifier.

The procedure for cleaning LaSFN\(_9\) glass for gold evaporation is outlined in Table 2.4.
Hydrogel films were formed on top of a ~ 50 nm gold (99.99 %, Advent) film, evaporated (Emitech Ltd. K975) onto a LaSFN₉ glass slide (Schott Glass). To improve its optical properties the gold film was annealed at 500 °C for 90 s. The gold coated LaSFN₉ slide was separated from the back of the LaSFN₉ prism by a thin layer of index matching fluid (Cargille Laboratories Inc., n = 1.700 ± 0.0002). The sample and the prism were mounted on a computer-operated goniometer, which was used to control the precise angle of incidence of the light. The reflected light beam was focused through a collecting lens onto a silicon photodiode.

Computer software was used to measure the magnitude of reflected light reaching the photodiode as a function of the incident angle controlled by the goniometer. Gels were formed in a Teflon electrochemical flow cell where the gold coated LaSFN₉ and a microscope slide were used as the two walls of the flow cell. Data was fitted using WINSPALL software (REStek GmbH) which uses the Fresnel equations to model the behaviour of light in a layered system.

Table 2.4. LaSFN₉ glass cleaning procedure.

<table>
<thead>
<tr>
<th>Step</th>
<th>Solution</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2% Hellmanex</td>
<td>Sonicate 15 min</td>
</tr>
<tr>
<td>2</td>
<td>MilliQ H₂O</td>
<td>Rinse 20 x</td>
</tr>
<tr>
<td>3</td>
<td>2% Hellmanex</td>
<td>Sonicate 15 min</td>
</tr>
<tr>
<td>4</td>
<td>MilliQ H₂O</td>
<td>Rinse 20 x</td>
</tr>
<tr>
<td>5</td>
<td>MilliQ H₂O</td>
<td>Sonicate 15 min</td>
</tr>
<tr>
<td>6</td>
<td>MilliQ H₂O</td>
<td>Rinse 20 x</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>Repeat steps 1 - 6</td>
</tr>
<tr>
<td>8</td>
<td>EtOH</td>
<td>Sonicate 15 min</td>
</tr>
<tr>
<td>9</td>
<td>MilliQ H₂O</td>
<td>Rinse 20 x</td>
</tr>
<tr>
<td>10</td>
<td>MilliQ H₂O</td>
<td>Sonicate 15 min</td>
</tr>
<tr>
<td>11</td>
<td>MilliQ H₂O</td>
<td>Rinse 20 x</td>
</tr>
<tr>
<td>12</td>
<td>EtOH</td>
<td>Sonicate 15 min</td>
</tr>
<tr>
<td>13</td>
<td>Dry N₂</td>
<td>Ready</td>
</tr>
</tbody>
</table>
16. LONG AND SHORT RANGE SPR

16.1 Introduction to Long and Short Range SPR
In order to gain a greater understanding of the properties of electrochemically grown dipeptide hydrogels, various Surface Plasmon Resonance techniques are used in Chapter 5. The theory behind Long Range SPR (LR SP) is similar to that of normal SPR. The penetration depths of LR SPs are much larger than normal SPR, which is typically around 200 nm. Short Range SPR (SR SP) is much more sensitive to changes closer to the Au surface. Therefore they are useful tools for analysing the electrochemically induced gelation of FmocLG.

16.2 Theory of Long and Short Range SPR
By changing the substrate on which the gold layer is evaporated, different plasmons can be observed with incident light. Long Range Surface Plasmons (LR SPs) originate from the coupling between two surface plasmons (SPs) coming from two opposite interfaces of a thin metal film. The substrates are designed to give SPs with an order of magnitude less damping along the metallic surface (Au) to probe further into the films. Figure 2.20 is a schematic showing the general setup and resultant plasmon modes in standard Long and Short Range (LSR) SPR.36

![Figure 2.20. Long and short range surface plasmon modes (LR SP and SR SP respectively) supported by a thin metal film. Reproduced from Wang et al.35](image-url)
In Figure 2.20[a] the surface plasmon modes arising from the coupling of light along a thin metallic film and into the dielectric are shown as resultant evanescent electromagnetic waves, or surface plasmons (SPs). The refractive index of the dielectric $n_b$ matches that of the sample $n_d$. The LR SP has a longer penetration depth $L_p$ (distance at which the field amplitude decreases by a factor of $1/e$) than the standard SP, and the SR SP has a higher electromagnetic density closer to the metal surface, and a shorter $L_p$.

Different SP modes can be excited depending upon the thicknesses and number of layers in the substrate. Coupled-Long Range SPs arise from a four-layered system. Figure 2.21 shows the detailed cell setup and the distributions of magnetic intensity for Long Range (LR) SPs and coupled (c-LR) SPs.\textsuperscript{37-39}
Figure 2.21[a] Long Range Surface Plasmon (LR SP) substrate. Reproduced from Dostalek et al.\textsuperscript{39} [b] Coupled-Long Range Surface Plasmon (c-LR SP) substrate. Reproduced from Dostalek et al.\textsuperscript{36}

Figure 2.21[a] shows the cell setup for LR SP substrate, which is the same for all substrates. In [b] the magnetic field distributions of symmetrical and anti-symmetrical SPs for the c-LR SP substrate is pictured with the corresponding field intensity, arising from its four-layered structure.

16.3 Substrates for Long and Short Range SPR

Substrates for Long and Short Range SPR were made in the Dostalek group at AIT, Vienna. Three Long-Range SPR substrates are used in this section, which produce different plasmons when coupled with the incident light. Those are Long and Short Range Surface Plasmons (LSR SP), Long Range Surface Plasmons (LR SP), and Coupled-Long Range Surface Plasmons (c-LR SP). These are shown in Figure 2.22, with their penetration depths $L_P$ into the dielectric.
Figure 2.22. Cell setup and thicknesses of layers used for [a] Long Range Surface Plasmons (LR SP), [b] Long and Short Range Surface Plasmons (LSR SP) and [c] Coupled-Long Range Surface Plasmon substrates (c-LR SP).

The three substrates were used separately to build up data for the FmocLG hydrogels. The aim was to use substrates with different penetration depths ($L_p$) into the hydrogel in order to observe the properties more closely throughout gelation, and at different distances from the Au surface.

In order to observe LR SPs, the metallic layer (Au) was sandwiched between two dielectrics with matching refractive indices (Figure 2.20). An amorphous fluoropolymer with a refractive index close to water was used, Cytop (Asahi Glass Company, Japan, refractive index 1.3395). As the refractive indices of these layers are very similar, the two plasmon modes couple together and give rise to symmetrical and anti-symmetrical distributions of electromagnetic intensity, observed with surface plasmon techniques as Long- and Short-Range SPs, respectively. As there is a minima for each SP observed, it is possible to fit experimental data more closely, to give values for thickness and refractive index of the films simultaneously.

By changing the thicknesses of the Au and Cytop layers, it was possible to observe various LR SPs. The substrates used in this work are shown in Figure 2.22. c-LR SPs are observed when there are two layers of Cytop and Au.
Table 2.5 below gives details for each sample produced, and their measured refractive indices and penetration depths ($L_p$).

Table 2.5. Details of layers, thicknesses, refractive indices and penetration depths into dielectric ($L_p$) for LR SP, LSR SP and c-LR SP.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Layers</th>
<th>Thickness (nm)</th>
<th>Refractive index (measured)</th>
<th>Depth of penetration into dielectric (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>desired measured</td>
<td>$\varepsilon$ (real)</td>
<td>$\varepsilon$ (imaginary)</td>
</tr>
<tr>
<td>LRSP</td>
<td>Prism</td>
<td>$\infty$ 0</td>
<td>3.413</td>
<td>1.846</td>
</tr>
<tr>
<td></td>
<td>Glass (BK9)</td>
<td>$\infty$ 0</td>
<td>1.339</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CYTOP</td>
<td>1100 1160</td>
<td>0.230</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>Gold</td>
<td>11 11</td>
<td>1.331</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Water (MilliQ)</td>
<td>$\infty$ 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LSRSP</td>
<td>Prism</td>
<td>$\infty$ 0</td>
<td>3.413</td>
<td>1.845</td>
</tr>
<tr>
<td></td>
<td>Glass (LaSFN9)</td>
<td>$\infty$ 0</td>
<td>1.339</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CYTOP</td>
<td>250 0</td>
<td>0.300</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>Gold</td>
<td>50 50</td>
<td>1.333</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Water (MilliQ)</td>
<td>$\infty$ 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-LRSP</td>
<td>Prism</td>
<td>$\infty$ 0</td>
<td>3.413</td>
<td>1.845</td>
</tr>
<tr>
<td></td>
<td>Glass (BK9)</td>
<td>$\infty$ 0</td>
<td>1.337</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CYTOP</td>
<td>660 660</td>
<td>0.300</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>Gold</td>
<td>25 22.8</td>
<td>1.337</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Water (MilliQ)</td>
<td>$\infty$ 0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The penetration depths of the LR SPs and c-LR SPs are much larger than that of standard SPR, which is typically around 200 nm. The SR SP is also much more sensitive to changes in refractive indices and thicknesses at distances closer to the metal surface.

16.4 Experimental details for making Long- and Short-Range SPR substrates
Cytop polymer (CTL-809M, refractive index 1.3395, Asahi Glass Company Japan) was mixed with various ratios of CT 180 solvent (Asahi Glass Company) and spin-coated onto glass (either LaSFN9 or BK9) substrates using a Ramgraber spin-coater. The cleaning procedure for glass slides was the same as for standard SPR (see Table 2.4). The samples were heated at 170 °C for 1 hour. The gold layer was deposited via gold sputtering (Leybold Vacuum Systems Univex 450C, 20 W, Ar atmosphere), giving a surface roughness of approximately 1 nm. It was not possible to anneal the Au layer as
in making standard SPR substrates, as the glass transition temperature \( T_g \) for Cytop is 300 °C. The gold surface was plasma cleaned with oxygen (100 W, 20 mbar, 30 s).

The conditions for the three samples are outlined in Table 2.6. Different spinning frequencies were used to coat glass with Cytop, and a calibration was performed to accurately control the thicknesses of the Cytop and Au layers (shown in Figure 2.23). Thicknesses were measured with Atomic Force Microscopy (AFM) (Molecular Imaging, Pico Plus, Tapping mode).

Table 2.6. Conditions used to making substrates for LR SP, LSR SP and c-LR SP.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Thickness (nm)</th>
<th>Conditions for Cytop Layer</th>
<th>Conditions for Au layer sputtering (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LRSP</td>
<td>Prism Glass (BK9)</td>
<td>0 0</td>
<td>1:0 1300 120</td>
</tr>
<tr>
<td></td>
<td>CYTOP</td>
<td>1000 11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gold</td>
<td>0 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water (MilliQ)</td>
<td>0 0</td>
<td></td>
</tr>
<tr>
<td>SRS LP</td>
<td>Prism Glass (LaSF9)</td>
<td>0 0</td>
<td>1:1 1990 120</td>
</tr>
<tr>
<td></td>
<td>CYTOP</td>
<td>250 50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gold</td>
<td>0 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water (MilliQ)</td>
<td>0 0</td>
<td></td>
</tr>
<tr>
<td>c-LRSP</td>
<td>Prism Glass (BK9)</td>
<td>0 0</td>
<td>1:0 1800 120</td>
</tr>
<tr>
<td></td>
<td>CYTOP</td>
<td>650 25</td>
<td></td>
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<tr>
<td></td>
<td>Gold</td>
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<td></td>
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<tr>
<td></td>
<td>CYTOP</td>
<td>650 25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gold</td>
<td>0 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water (MilliQ)</td>
<td>0 0</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.6 summarises the conditions used to make each substrate. An example of a calibration curve for the thickness of the Cytop layer is shown below in Figure 2.23, from which it was chosen to use 1990 rpm to spin-coat for a thickness of 250 nm for the LSR SP substrates.
Figure 2.23. Calibration curve to determine the spin-coating speed required for various thicknesses of CYTOP. Using 1:1 CYTOP : solvent mixture.

For each type of substrate (LSR SP, LR SP and c-LR SP) a simulation (using the parameters in Table 2.5) and experimental data (with fits) are shown for the angle scans in water (see Figure 2.24).
From Figure 2.24, the SP minima can be observed for each substrate, which occur at the following angles in water: [a] LR SP $\theta_{\text{min}} = 47.9^\circ$; [b] LR SP $\theta_{\text{min}} = 52.5^\circ$ and SR SP $= 66.5^\circ$; [c] anti-c-LR SP $\theta_{\text{min}} = 47.4^\circ$ and sym-c-LR SP $= 49.5^\circ$.

The penetration depth $L_p$ describes how fast the evanescent field amplitude decreases from the metallic surface, and were determined from ATR experiments by the Knoll group, as described by Wang et al.\textsuperscript{38}
16.5 Substrates for Long Range Surface Plasmons (LR SP)
LR SPs excitation is closer to the critical angle than a standard SP, thus giving a longer penetration depth $L_p$. Previous work by the Knoll group provided conditions to achieve optimal depth penetration into the dielectric, which was used in this work, with a 1000 nm Cytop layer and 11 nm Au layer. An $L_p$ of 550 nm for the LR SP was achieved and used in this work.

16.6 Substrates for Long and Short Range Surface Plasmons (LSR SP)
With LSR SPs there was a compromise between the sensitivity of each SP (depth of the minima, affected by thickness of the Cytop layer), and the separation of the LR SP and SR SP (affected by the thickness of the Au layer). The thickness of gold was set to 50 nm and the effect of the thickness of the Cytop layer ranged from 235 to 300 nm, as illustrated in the simulation and experimental data in Figure 2.25.
With increasing thickness of the Cytop layer the LR SP deepened, whilst the SR SP almost disappeared. Thinner Cytop layers improved the depth and resolution of the SR SP, whilst compromising the resolution of the LR SP. The optimum thicknesses were found to be 250 nm Cytop and 50 nm Au, providing an $L_p$ value of 260 nm into the hydrogel film with the LR SP, and 136 nm with the SR SP.
16.7 Substrates for Coupled-Long Range substrates (c-LSPR)
By creating a four-layered substrate (650 nm Cytop, 25 nm Au, 650 nm Cytop, 25 nm Au) a deeper depth profiling than LR SP was achieved. Again a compromise existed between the sharpness of the c-LR SPs and their separation; with increasing Au thickness the peaks were deeper, but the two minima were closer together and less well resolved. For these conditions $Lp = 950$ nm and $364$ nm were achieved for the anti-symmetrical and symmetrical SPs respectively.

17. ELECTROCHEMISTRY

17.1 Introduction to Electrochemistry
Electrochemistry is the study of electron transfer reactions in solution at conducting surfaces. The electrochemistry in this work is primarily concerned with the oxidation reaction of hydroquinone at a gold electrode (see Figure 2.1[b]). This reaction was used to study the electrochemically-induced pH drop at a gold surface and subsequent gelation of the FmocLG dipeptide, using SPR.

17.2 Electrochemical Theory
Electrochemical processes involve the transfer of charge across a conducting interface (usually a metal, m) to a species in solution (s). As this process moves towards equilibrium, a charge separation is created between m and s. This creates a potential difference, $\Phi_{m/s}$ at the interface between the metal and the solution. This is defined by Equation 2.10:

\[ \Delta \phi_{m/s} = \phi_m - \phi_s \]

Equation 2.10. Potential difference at the electrode / solution interface $\Phi_{m/s}$.

Figure 2.26 shows this potential drop with respect to the distance of the species from the electrode.
In order to study potential difference a conducting circuit must be constructed, with a working electrode (WE) and reference electrode (RE). This is discussed in further detail in Section 17.3. The potential difference between the WE and RE is given in Equation 2.11:

\[ E = (\phi^{WE} - \phi^{RE}) + iR_{\text{soln}} \]

*Equation 2.11. Potential difference \( E \) between the WE and RE.*

By considering the general charge transfer reaction of the reversible reduction of a species \( O \) to \( R \) with the transfer of \( n \) number of electrons:

\[ O(aq) + ne^{-}(m) \xrightarrow[k_{\text{red}}]{k_{\text{ox}}} R(aq) \]

*Equation 2.12. Reversible reduction of \( O \) to \( R \) with transfer of \( n \) number of electrons.*

The Nernst equation is used to define the potential established at the electrode under equilibrium conditions (Equation 2.13).

\[ E_v = E^0 + \frac{RT}{nF} \ln \frac{[O]}{[R]} \]

*Equation 2.13. The Nernst Equation. \( E^0 \): standard electrode potential of the reaction, \( E_v \): equilibrium potential of the electrode, \( n \): number of electrons transferred, \( F \): 96485 Cmol\(^{-1}\).*

Electrochemical equilibrium measurements can provide a large amount of thermodynamic information. The Nernst equation shown above can be used to give reaction free energies, entropies and enthalpies, and equilibrium constants.
The Fermi level within a conductive electrode material is where the probability of finding an electron is a half.\textsuperscript{42} When an electrode is placed into an electrolyte, the energy level of the most energetic filled state equilibrates to create an electronic equilibrium according to the Fermi level. Figure 2.27 shows two cases [a] and [b], where electron transfer from a metal to a species \( O \) in solution is made possible by altering the electrode potential.

![Figure 2.27. Energy levels and electron transfer from metal to solution. In [a], the electrode potential is too low to allow for the reduction of a species \( O \), and [b] shows a more reductive potential allowing electron transfer to the LUMO of the species \( O \).](image)

This diagram shows that if a species capable of undergoing charge transfer (\( O \)) has a LUMO which is of higher energy than the Fermi level of the metal, no reaction will occur. However, by applying a potential to the metal the Fermi level can increase in energy to that of above the LUMO of the species \( O \), and electron transfer can occur.

In ‘Electrode Dynamics’, Fisher outlines the parameters which act to influence the dynamics of an electrochemical reaction.\textsuperscript{42} These include: the electrode potential, and the nature of the electrode surface; the reactivity of the species under study, and its movement from the bulk to the electrode; and finally, the nature and structure of the interfacial region where electron transfer occurs. These steps can all affect the rate of electron transfer.

Mass transport effects influence the rate of electrochemical reactions. During the reduction of \( O \) to \( R \), there are several steps (shown in Figure 2.28) which can be rate limiting: travelling from the bulk to the surface of the electrode, adsorption at the surface of the electrode, and subsequent electron transfer, desorption, and mass transfer to the bulk. The reaction rate is influenced by the slowest step.
Figure 2.28. Steps occurring during electrochemical processes.

Diffusion, convection and migration are transport processes which influence the electrochemical experiment. The rate of diffusion of species is defined by Fick’s Laws. The first defines the diffusional flux $j$ of a species $B$ as (Equation 2.14):

$$ j = -D_B \frac{\delta [B]}{\delta x} $$


There are two types of convection, natural (from thermal gradients in solution) and forced (from stirring the solution). The effects of natural convection can be eliminated by using rotating disc electrodes or bubbling gas through the cell.

Migration arises from the electric field which exists between the electrode and the solution, due to the difference in electrical potential $\Delta \Phi_{m/s}$, described earlier. This field affects charged species at the interface and causes them to move to, or away from, the electrode. The migratory flux of a species $B$ is described in Equation 2.15:

$$ j_m \propto -u[B] \frac{\delta \Phi}{\delta x} $$

*Equation 2.15. Migratory flux $j_m$, $u$: ionic mobility, $[B]$: concentration of the ion $B$, $\Phi$: external electric potential.*

A background electrolyte is added to remove the effect of migration on an electrochemical experiment. This also improves the flow of current throughout the system.

The transport of a species $B$ to an electrode over time is described in Figure 2.29. Electron transfer occurs in the region of between 10 to 20 Å away from the electrode.
surface. Beyond this distance, there is no appreciable electrical potential gradient to draw the species B to the surface via migration, so the transport of B to the electrode is governed by convection and diffusion.

![Figure 2.29. Growth of diffusion layer thickness with time.](image)

As the reaction proceeds, B is used up at distances close to the electrode surface, and the concentration of B at the interface differs from that in solution. The thickness of the diffusion layer increases with time. It is linear at distances close to the electrode, which can be used to estimate the diffusion layer thickness. It approaches a steady state thickness, where transport within the layer occurs via diffusion alone. Transport from the bulk to the diffusion layer then occurs via diffusion and convection.

The maximum current under transport limited conditions $i_L$, is shown in Equation 2.16.

$$i_L = \frac{D_B F A[B]_{bulk}}{\delta_d}$$

*Equation 2.16. Transport limited current $i_L$, $\delta_d$: diffusion layer thickness.*

The limiting current is reduced by both adding a background electrolyte to the system and positioning the reference and working electrodes as close together as possible, which improves the conductivity of the system by removing the effects of mass transport.

An Electrical Double Layer of charged particles occurs when an electrode is immersed into a solution. This layer restricts the movement of species to the electrode by increasing the distance of the closest approach of ions to the electrode surface. The simple model devised by Helmholtz is shown in Figure 2.30.
The Double Layer can be reduced or ‘collapsed’ by adding a background electrolyte.

17.3 The Three-Electrode Cell setup

A diagram of the three electrode cell used in this work is shown in Figure 2.31[a].

Movement of charge, measured as current, is allowed to flow at the working electrode (WE). Electrons are exchanged at this electrode. As charge builds up, electrons or ions move to this surface. Gold, platinum, or fluorine-doped tin oxide (FTO) were used as WE materials. The reaction that occurs at the WE with hydroquinone (HQ) is shown in Equation 2.17.

\[
\text{Equation 2.17. Reversible oxidation of hydroquinone (HQ). For every mole of HQ oxidised two protons are released.}
\]

Current flows between the counter electrode (CE) and the WE. To reduce rate limiting effects, the surface area of the CE is large, compared with that of the WE.

The reference electrode (RE) is comprised of a metal and an insoluble salt. In this work a silver / silver chloride (Ag⁺ / AgCl) electrode was used. It holds a fixed interfacial potential and allows the potential difference between itself and the working electrode to be measured and controlled by the potentiostat, with no flow of current. Potentials are
quoted with reference to the type of reference electrode. The RE has a fixed potential of +0.971 V in KCl.

17.4 Cyclic Voltammetry (CV)

In cyclic voltammetry (CV), a voltage is applied between the WE and RE, and is ramped linearly versus time in a triangular waveform. The potential difference between WE and CE is used to control the current flowing through the system. The voltage can be started at any point and is cycled in either direction. The CV is then plotted with the current at the WE versus the applied voltage, as in Figure 2.31[b]. The oxidation and reduction of the species is observed as current peaks, and for a fully reversible system the area under the oxidation and reduction peaks is unity. The peak separation is 59 mV/n at room temperature (n = number of electrons transferred in electrode reaction).

CVs are measured in order to determine the voltage at which a species can be oxidised and reduced. CVs can be performed at a range of scan rates to provide information about the oxidised and reduced species and diffusion coefficients.

The currents observed during a CV vary with time. The Cottrell equation (Equation 2.18) describes the current response versus time, as shown in Figure 2.32.

---

**Figure 2.31[a]** Diagram showing the three-electrode setup with Working, Counter and Reference Electrodes (WE, CE and RE respectively). Electrodes are connected to a voltmeter and potentiostat. [b] CV showing current vs. voltage. From a voltage V1 to V2, showing the fully reversible oxidation and reduction of a species (oxidised species O and reduced species R). Peak potentials, currents and potential difference between reduction and oxidation peaks are shown.
Equation 2.18. The Cottrell equation.

Immediately after the potential step is initially applied, large currents flow as there is a large amount of a species A to be oxidised / reduced. This current decreases with time, as it is affected by the rate of the diffusion A to the electrode. With time the diffusion layer increases and causes the current to decrease.

17.5 Chronopotentiometry

Chronopotentiometry is a galvanostatic measurement where the current flowing through the WE is controlled during the experiment. Current is allowed to flow between the CE and WE, with the potential of the WE being monitored by measuring with respect to the RE. HQ was oxidised or reduced at the WE surface, depending on the magnitude of current flowing. A reducing current led to the reduction of quinine, with a balancing oxidation occurring at the CE. A constant rate of electrolysis was achieved. The ohmic drop was due to a constant solution resistance, so was corrected by a constant potential offset.

17.6 Experimental details and Cell Setup

The cell setups used for this work are shown in Table 2.7.
Table 2.7. Details of cell setups including working electrode material and dimensions, counter and reference electrodes, cell size, and details of gelation parameters.

<table>
<thead>
<tr>
<th>No.</th>
<th>Working Electrode (WE)</th>
<th>Area cm²</th>
<th>Counter (CE)</th>
<th>Reference (RE)</th>
<th>Cell volume</th>
<th>Current density μA cm⁻²</th>
<th>Time minutes</th>
<th>Thickness of gel mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gold slide</td>
<td>0.5</td>
<td>Platinum Wire</td>
<td>Silver/Silver Chloride</td>
<td>0.5 mL SPR cell</td>
<td>20</td>
<td>30</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Platinum Wire</td>
<td>0.01</td>
<td>Pt Mesh</td>
<td>Ag⁺/AgCl</td>
<td>5 mL beaker</td>
<td>20</td>
<td>30</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>FTO</td>
<td>1</td>
<td>Pt Wire</td>
<td>Ag⁺/AgCl</td>
<td>5 mL beaker</td>
<td>20</td>
<td>30</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>ITO</td>
<td>1</td>
<td>Pt Wire</td>
<td>Ag⁺/AgCl</td>
<td>5 mL beaker</td>
<td>20</td>
<td>30</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>Gold TEM grid</td>
<td>0.2</td>
<td>Pt Wire</td>
<td>Ag⁺/AgCl</td>
<td>5 mL beaker</td>
<td>20</td>
<td>30</td>
<td>0.5</td>
</tr>
</tbody>
</table>

18. IMMOBILISATION OF HORSERADISH PEROXIDASE (HRP) ENZYME INTO ELECTROCHEMICALLY FORMED DIPEPTIDE HYDROGELS

18.1 Introduction to Enzyme Immobilisation
In order to open up the possibilities of utilising dipeptide hydrogels as enzyme supports for use in enzyme fuel cells, the Horseradish Peroxidase (HRP) enzyme is used as a model system. This enzyme is relatively stable in solution at room temperature and its activity is measured using a colorimetric assay, and is therefore used as a starting point for this study.

The assay used for studying the activity of the immobilised HRP enzyme within the FmocLG hydrogel utilises the conversion of o-phenylenediamine (OPD) to 2,3-diaminophenazine (DAP) with hydrogen peroxide.\(^{46}\)

18.2 Enzyme kinetics
Enzyme assays study enzyme reaction kinetics. Figure 2.33 shows how a substrate (S) and enzyme (E) come together to form an activated complex (ES*), which then reacts to form a product (P). The reaction is highly specific. The enzyme acts as a catalyst.
Figure 2.33. Scheme showing an enzyme $E$ complexing with a substrate $S$ to form an activated complex $ES^*$. This complexing allows reaction with another species to form a product $P$, with regeneration of the enzyme.

The steady-state kinetics for enzyme reactions are outlined in Equation 2.19.

$$E + S \xrightarrow{k_1} ES^* \xrightarrow{k_2} E + P$$

Equation 2.19. Enzyme kinetics corresponding to the processes occurring in enzyme reactions.

18.3 The colorimetric HRP assay with Hydrogen Peroxide

The enzyme-catalysed reaction used in this section is the reaction of o-phenylenediamine dihydrochloride (OPD, >99 %, Fluka) with hydrogen peroxide ($H_2O_2$, 30% in $H_2O$, Sigma-Aldrich), which complexes with the enzyme horseradish peroxidase (HRP, Type 1, from horseradish, 50 – 150 U mg$^{-1}$, solid, Sigma-Aldrich) to form the coloured product 2,3-diaminophenazine (DAP). The activity of HRP before and after immobilisation was monitored by UV-Vis spectroscopy. Figure 2.34 shows the reaction scheme.
Figure 2.34. Scheme showing the enzymatic conversion of OPD to DAP with horseradish peroxidase. The product is measured at 450 nm.

The reaction was carried out in phosphate buffered saline (PBS) at pH 7.0.

18.4 UV-Vis setup for measuring Horseradish Peroxidase activity

The formation of DAP was monitored using the cell setup depicted below in Figure 2.35, where a UV-Vis probe (USB4000, Ocean Optics) was positioned at the back of an SPR cell behind a quartz window. The absorbance was measured in reflectance mode, using the gold surface to enhance the signal. The data was collected and analysed using the Ocean Optics program.

Figure 2.35. Diagram of the electrochemical SPR cell with a UV-Vis probe for measuring the production of DAP. The reactants are pumped into the cell after the hydrogel with HRP is formed.

The path length of the UV beam was 1 cm.
18.5 Calibration curve for Horseradish Peroxidase Assay

In order to determine the molar extinction coefficient of DAP (published value $\varepsilon = 13,000 \text{ cm}^{-1}$) a calibration was carried out using a range of concentrations of OPD. The electrochemical SPR cell was used as the reaction vessel. All the reactants (except $\text{H}_2\text{O}_2(aq)$) were mixed and introduced into the cell, which was sealed and under $\text{N}_2(g)$. The $\text{H}_2\text{O}_2$ was injected when a stable baseline at 450 nm was reached. The reaction occurred after a few minutes and the maximum absorbance of the peak at 450 nm was measured over time.

After 10 minutes the reaction had completed, producing the colour change. At longer times the product began to change to a side product which had a maximum absorbance peak at 420 nm. Interestingly, in the hydrogel layer, the maximum peak of the product remained at 450 nm. This may have been due to the product being immobilised within the hydrogel layer, restricting oxidation or back reactions.

18.6 Determining activity of immobilised HRP

$7.4 \times 10^{-5} \text{ mol dm}^{-3}$ OPD in $1 \times 10^{-2} \text{ mol dm}^{-3}$ PBS buffer was added to the cell containing $2.44 \times 10^{-3} \text{ mol dm}^{-3}$ FmocLG and HRP hydrogel. (OPD solutions oxidise quickly so were made fresh before each experiment.) $1 \mu\text{L} \text{H}_2\text{O}_2$ was injected into the cell to start the reaction.

18.7 Electrochemical gelation of FmocLG with HRP in PBS buffer

$5.35 \times 10^{-8} \text{ mol dm}^{-3}$ HRP was dissolved in $1 \times 10^{-2} \text{ mol dm}^{-3}$ PBS buffer solution at pH 7. $20 \mu\text{A cm}^{-2}$ was applied to the solution as in the method outlined in Section 15.6, except the current was allowed to flow for 3 hours. A hydrogel formed which was approximately 0.5 mm thick by eye.

19. REFERENCES

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(14) Techniologies, P. *Pike Technologies* 2010.


CHAPTER THREE:
INTRODUCING THE GELATION OF FMOC-DIPEPTIDES

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1. GELATION OF FMOC- PROTECTED DIPEPTIDES

1.1 Introduction to Dipeptide Hydrogelation

This section introduces several experimental techniques for monitoring the gelation of Fmoc-diphenylalanine (FmocFF) and Fmoc-Leucine-Glycine (FmocLG) (see Figure 3.1). The amino acids used were all in the natural L-form. Hydrogels were formed at a range of dipeptide concentrations, at different pHs, and in different salt solutions. The effects of changing conditions on gelation were investigated using titrations, rheology, and fluorescence spectroscopy measurements. Electron Microscopy imaging techniques for the characterisation of dried hydrogels are also explored in detail in this section.

Figure 3.1 shows the molecular structures for the general form of Fmoc-protected dipeptides [a], with FmocFF in [b], and FmocLG in [c].

FmocFF has a high pKa in water (8.5) which allows it to spontaneously gelate when added to water at pH 7.0. FmocLG has a lower pKa (5.8) which requires the addition of acid to initiate gelation.
1.2 Spontaneous gelation of FmocFF: Effect of concentration on hydrogel properties

The Fmoc-protected diphenylalanine dipeptide (FmocFF) self-assembles to form a rigid hydrogel when a concentrated solution in an organic solvent (DMSO) is diluted into water. Samples were made according to the procedure outlined in Chapter 2, Section 2. Figure 3.2 shows images of a series of FmocFF hydrogels at 1, 2 and 5 mg mL\(^{-1}\) (1.87, 3.74 and 7.48 x 10\(^{-3}\) mol dm\(^{-3}\) respectively) and their gelation over time. Using DMSO and water resulted in bulk phase separation, followed by self-assembly. The resulting hydrogels were translucent, homogeneous and remained intact upon inversion after 3 hours. The gels were stable over several months, as long as the tubes were sealed and the hydrogels remained solvated.

![Figure 3.2](image.png)

*Figure 3.2[a-d]. Series of FmocFF hydrogels formed in tubes at 1, 2 and 5 mg mL\(^{-1}\) (1.87, 3.74 and 7.48 x 10\(^{-3}\) mol dm\(^{-3}\) respectively). Taken at [a] 0 min [b] 2 min [c] 10 min [d] 3 hours. No flow was observed upon inversion after 24 hours.*

The samples in Figure 3.2[a] show the mixtures at 0 min, just after the concentrated dipeptide solution in DMSO was diluted into water. The solution was mixed and left to stand. The solution became cloudy upon addition of FmocFF due to its partial insolubility in water. The hydrogels were fully formed when the solution had cleared to
a translucent, self-supporting structure. The most concentrated hydrogel (5 mg mL\(^{-1}\)) was the quickest to form, taking approximately 2 to 3 min to fully gelate. The lowest concentration of FmocFF (1 mg mL\(^{-1}\)) took longer to form and was fully set after 3 hours.

Rheological experiments were carried out using different concentrations of FmocFF diluted into water, using the same procedure as above. A frequency sweep was taken at each concentration of FmocFF, and the in-phase storage modulus \(G'\) obtained at 1 % strain (see Chapter 2, Section 9.2). When \(G'\) is at least one order of magnitude higher than the out-of-phase storage modulus \(G''\), the material is strong enough to be considered a hydrogel. Each point on the graph in Figure 3.3 represents the average of three data sets.

![Figure 3.3](image)

Figure 3.3. Concentration versus \(G'\) showing effect of FmocFF concentration on \(G'\) in rheological measurements (log scale) with error bars shown. As concentration increased, hydrogel strength increased.

Figure 3.3 shows an increase in \(G'\), which represents an increase in hydrogel strength with increasing concentration of FmocFF, however, hydrogel strength did not increase linearly with concentration. As the concentration of FmocFF increased above 5 mg mL\(^{-1}\), hydrogels formed within less than a minute, and were cloudy and unhomogeneous. At lower concentrations (0.5 to 1 mg mL\(^{-1}\)) the gels formed over 20 minutes and were very clear and homogeneous. This data shows how the properties of FmocFF hydrogels can be tuned with the concentration of dipeptide. It also showed that the longer the hydrogels take to form, the more transparent and homogeneous they became.
1.3 Spontaneous gelation of FmocFF: Effect of salt solution on hydrogelation

The gelation of low-molecular weight peptide gelators is affected by different solvents and salts. The solvents and salts are specific to each dipeptide gelator. A range of salt solutions and buffers at pH 7 were used to test the stability of FmocFF at 2 mg mL\(^{-1}\) (3.74 x 10\(^{-3}\) mol dm\(^{-3}\)), in an attempt to determine the effect of changing the ionic radius, ionic charge, and counter ion on the final hydrogel properties. The results are shown in Table 3.1.

*Table 3.1. Table showing effect of salt solutions on FmocFF hydrogels at 3.74 x 10\(^{-3}\) mol dm\(^{-3}\). Arrows indicate a trend from low to high.*

<table>
<thead>
<tr>
<th>Salt added to gel at 1x10^{-3} moldm^{-3}</th>
<th>Time required for gelation</th>
<th>Viscosity of gel</th>
<th>Stability to shear stress (shaking)</th>
<th>Stability over 4 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>10 min</td>
<td>↑</td>
<td>Gel</td>
<td>Gel</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 min</td>
<td>↑</td>
<td>Gel</td>
<td>Gel</td>
</tr>
<tr>
<td>Na(_2)SO(_4)</td>
<td>20 hours</td>
<td></td>
<td>Gel wobbles</td>
<td>Gel</td>
</tr>
<tr>
<td>NaH(_2)PO(_4)</td>
<td>48 hours</td>
<td></td>
<td>Gel is destroyed</td>
<td>Gel</td>
</tr>
<tr>
<td>HEPES</td>
<td>48 hours</td>
<td></td>
<td>Portions of liquid gelated</td>
<td>No gel after 4 days</td>
</tr>
<tr>
<td>CaCl(_2)</td>
<td>10 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaSO(_4)</td>
<td>10 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>10 min (unhomogenous)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MES</td>
<td>No gel</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1 gives the results of FmocFF hydrogels made in different salts as a rank of decreasing strength, from the strongest at the top (KCl and NaCl) to the weakest at the bottom (CaCl\(_2\) and CaSO\(_4\)). Different salts affected the hydrogel formation to different extents. Buffer solutions (PBS and MES) disrupted the gelation process and the final properties of the hydrogels more markedly.

The ionic radius of counter ions and charge on the metal ion did not appear to have a definable effect on gelation. The strongest and most homogenous gels were obtained with KCl and NaCl. Hydrogels with Ca\(^{2+}\) ions formed quickly, but were not stable over time. This suggests that larger ions disrupted the structure more significantly than singly charged, small ions. This was also seen with the buffer solutions.

1.4 Titrations of FmocLG and FmocFF

Titrations of two Fmoc-dipeptides were performed as follows (as described by Adams *et al*):
3.654 \times 10^{-5} \text{ moles of each Fmoc-dipeptide was added to 2.5 cm}^3 \text{ MilliQ water with 36.5 } 
\text{µL of 1 mol dm}^{-3} \text{ NaOH. The solution was sonicated until the Fmoc-dipeptide had }
dissolved completely. 50 \text{ µL of 0.1 mol dm}^{-3} \text{ HCl was added, the mixtures were }
vortexed, and left to equilibrate for 5 minutes before each reading was taken. The }
titrations were repeated twice and errors calculated for each point. The concentration of 
dipeptide was very low to avoid hydrogelation which would have complicated the 
experiments. The results are shown in Figure 3.4.

Figure 3.4. Titrations of [a] FmocFF and [b] FmocLG where the pH of a dipeptide solution is 
measured vs. molar equivalents of HCl added. Each point represents the average of three data 
points.
The titration curve shown in Figure 3.4[a] has two equivalence points at approximately pH 5 and pH 9 for FmocFF, and one equivalence point for FmocLG. The results for FmocFF is consistent with the findings of Tang et al.\(^2\) who observed two apparent pK\(_a\)s in the titration of FmocFF. The first pK\(_a\) (at pH 10.2 to 9.5) correlated to initiating self-assembly of molecules, creating paired fibrils associating into the typical fibre structure, as pH continued to decrease. The second pK\(_a\) (pH 6.2 to 5.2) related to the precipitation of the ribbons from solution. The predicted pK\(_a\) of FmocFF in solution is approximately 8.5, and FmocLG 5.8. In reality they are much higher than expected due to the π-stacking which constrains the molecules within a self-assembled structure, with the acid groups in a hydrophobic environment. The increased pK\(_a\) allows further hydrogen bonding between structures to form fibrils.

1.5 Spontaneous gelation of FmocFF: Effect of pH on FmocFF hydrogelation kinetics using fluorescence measurements

Samples for fluorescence spectroscopy measurements were prepared as described in Chapter 2, Section 8.3, here in sodium acetate buffer at \(3.74 \times 10^{-3}\) mol dm\(^{-3}\) with a pH range 3.38 – 5.62. \(5 \times 10^{-3}\) mol dm\(^{-3}\) Thioflavin T (ThT) was added to the precursor buffer solution. 40 µL FmocFF stock solution (100 mg cm\(^{-3}\) in DMSO) was pipetted into the solution which was vortexed for a few seconds before placing in the fluorimeter. Measurements were taken at 474 (scattering – not shown) and 494.5 nm (fluorescence). Results of kinetic scans and gradient plots are shown in Figure 3.5. Each trace in [a] and each point in [b] correspond to the average of three data sets.
Figure 3.5[a] Thioflavin T (ThT) fluorescence response at 494.5 nm during hydrogelation of 3.74 x 10^{-3} mol dm^{-3} FmocFF in sodium acetate buffer, at a range of pHs (3.38: blue to 5.62: orange). Gradients are marked m. Data is from three sets. Full traces are not shown for clarity.

[b] Compiled gradients of ThT traces, showing pH versus gradient of dye trace (counts s^{-2}).

Figure 3.5[a] shows kinetic ThT fluorescence responses to the hydrogelation of FmocFF at the range of pHs in real time. The gelation process was significantly affected by changing pH. Gels formed very slowly (over 30 minutes) at low pH, with a lower final fluorescence intensity. They formed quickly (less than 1 min) at higher pH.
The final fluorescence intensity varied across the pH range. At pH 4.8, hydrogels gave much higher final fluorescence intensity, corresponding to a hydrogel with more extended 3D structure. As the pH increased to 5.2, the gels were formed more quickly again, with a lower measured final fluorescence intensity, suggesting a weaker material.

Figure 3.5[b] is a plot of pH versus the average rate of increase in fluorescence (gradient), obtained from three experiments at each pH. This is described in Chapter 2, Section 8.3. It shows the gradient increased as the pH increased. This suggests structures started to form more quickly with increase in pH. The results were very reproducible until pH 5.6, where there was a large variation in the gradient obtained. Figure 3.5[a] also showed that at this pH the hydrogels had a low final fluorescence intensity compared with the rest of the results.

It must be noted that as dipeptide hydrogels form differently in different buffers, the scans are not comparable to those obtained from those in water. Interestingly, at the highest pH tested (5.6) the gels formed very quickly (60 s) and then shrunk to half their size after 10 minutes, expelling some of the water and leaving them suspended in the buffer solution (see Figure 3.6).

This phenomenon is called syneresis. This does not occur in the same pH water, without buffer present. This may have accounted for the low final fluorescence intensity observed, as the structures were not as rigid. Adams et al have recently discussed this phenomenon of syneresis, which is affected by the hydrophobicity of the dipeptide. The less hydrophobic the dipeptide, (logP < 2.8) the more likely syneresis was to occur.
1.6  **Spontaneous gelation of FmocFF: Effect of ThT concentration on fluorescence response**

Various concentrations of ThT were added to MilliQ water prior to gelating with FmocFF to determine its effect on fluorescence response. The range of ThT was 1 to 2.5 \( \times 10^{-2} \) mol dm\(^{-3}\). Each data point in Figure 3.7 represents three experiments, with the concentration of ThT plotted against the gradient (counts s\(^{-1}\)) at 494.5 nm.

![Graph showing ThT concentration versus gradient at 494.5 nm](image)

\( m = 2.27 \times 10^{-2} \) [counts s\(^{-1}\) mol\(^{-1}\) dm\(^{3}\)]

**Figure 3.7. Effect of changing concentration of ThT on kinetic response to hydrogelation of 3.47 \( \times 10^{-3} \) mol dm\(^{-3}\) FmocFF in MilliQ water, showing ThT concentration (mol dm\(^{-3}\)) versus gradient at 494.5 nm (counts s\(^{-1}\)). Data is compiled from three sets.**

Figure 3.7 shows a linear relationship between concentration of ThT and gradient of the kinetic trace at 494.5 nm. This means the ThT response can be predicted at any concentration and does not affect the gelation of FmocFF. It suggests the ThT does not have a role in gelation, although it attaches to the \( \beta \)-sheet strands.

---

2. **ELECTRON MICROSCOPY IMAGING OF DIPEPTIDE HYDROGELS**

Several techniques were employed for imaging FmocFF and FmocLG hydrogels, outlined in Chapter 2, Sections 10-12. The results are shown in this section.
2.1 Scanning Electron Microscopy (SEM)

Samples for SEM were made according to the procedures laid out in Chapter 2, Section 10.2 and 10.3. Figure 3.8 shows five images of $2.43 \times 10^{-3}$ mol dm$^{-3}$ FmocLG hydrogels formed electrochemically at gold electrodes (described in Chapter 2, Section 3). They show the range of structures observed with different drying techniques, from air drying [a], to freeze-drying [b], and cryo-SEM [c-e].

![SEM images of FmocLG hydrogels at 2.43 x 10^-3 mol dm^-3, [a] air dried [b] freeze-dried, [c-e] cryo-SEM, with [e] showing cut side of sample. Scale bars are [a] 100 µm, [b] 10 µm, [c] 1 µm, [d] 10 µm and [e] 10 µm (insets 1 µm scale bar).]

In Figure 3.8[a], large crystals were observed, which correspond to the slow drying process of the hydrogel, allowing large crystalline structures to slowly form. The freeze-dried sample in [b] shows a three-dimensional structure which had been affected by the removal of water to some extent. It appears to show sheets of interconnected hydrogel structure. Figure 3.8[c-e] are images of three samples prepared for cryo-SEM, [c] and [d] clearly showing areas on the hydrogel surface where the sublimation of water and
growing of ice crystals has left round holes behind. In [e] the fibres were visible but had
aligned in one plane by the drying process.

Figure 3.9 shows images of freeze-dried samples where fibres were visible below the
surface.

![Figure 3.9(a-c). SEM images of freeze-dried samples showing the cut side of FmocLG
hydrogels. Scale bars are 10 µm. Fibres are visible beneath artefacts on the surface caused by
removal of water.]

The fibres visible beneath the surface in Figure 3.9[c] showed intertwined fibres
nanometres in diameter and many microns in length, concurrent with literature values.\textsuperscript{4}
It does not necessarily follow that the quicker the hydrogel freezes or dries, the more
accurate the images, because here we have shown the peptide fibres aligned in the
direction in which they were dried, and ice crystals created artificial surface structures.
As SEM was shown to give a range of different structures depending on the drying
process, it was not used for characterisation of hydrogels in this work.

2.2 Transmission Electron Microscopy (TEM)
The preparation of samples for TEM is outlined in Chapter 2, Section 11.2. Figure
3.10[a-c] shows images of an FmocFF hydrogel, with the extended structure clearly
visible in [a], fibres and interconnections in [b], and individual fibres weaved together in
[c]. Weaker hydrogels showed aligned structures arising from the washing and staining
process, breaking the structure which gave artificial features.
The length, width and scale of the fibres were in good agreement with literature values.\textsuperscript{5,6}

Although the hydrogel films for TEM required drying, staining and washing as part of the sample preparation process, the structures were not significantly affected by these processes. As the films were fairly thin, they dried quickly, and retained their fibrous structure. When films were not supported by a carbon film, they had the potential to break under the intensity of the electron beam, which is why using grids with smaller holes (200 mesh) gave better results.

2.3 Confocal Microscopy

Confocal microscopy allowed for the hydrogels to be imaged in their ‘wet’ state. It was necessary to make the hydrogel inside a well slide, and cover and image without allowing the hydrogel to dry out or have any shear forces applied to it. This protected the delicate structure and avoided breaking apart features with shear forces. FmocFF hydrogels for confocal microscopy were prepared as outlined in Chapter 2, Section 12.3.

Figure 3.11[a] shows the fibres in a $3.74 \times 10^{-3} \text{ mol dm}^{-3}$ FmocFF gel at pH 7. The image agreed with TEM and SEM, showing a network of interconnected fibres of microns in length, and nanometres in diameter. It also showed how the fibres were intertwined and appeared to come from a nucleation point, which other fibres grew out from. Figure 3.11[b] is a cross-section (Z-stack) of several fibres, the image being made up of several two-dimensional images to build up a profile of a fibre in water. It
was difficult to obtain clear resolution of the fibres due to the solvated structures ‘waving’ during the profiling.

This is a useful technique for imaging hydrogels, however it was observed that there were few sections where fibres were visible, even when the whole sample was gelled. The staining agent may not have spread across the sample to its limited solubility in water compared with Nile Blue, which may have restricted mixing and staining of fibres (Nile Red was used due to the excitation wavelength of the laser available).

![Confocal Microscopy images of FmocFF hydrogel](image)

Figure 3.11. Confocal Microscopy images of [a] 3.74 x 10^{-3} mol dm^{-3} FmocFF hydrogel stained with Nile Red [b] Cross-section showing single fibres. Scale bars are 400 and 100 µm.

A network of fibres is shown in Figure 3.11[a]. In [b], a ‘Z-stack’ was taken of an area with fibres present, showing a 3D image of the fibres in the FmocFF hydrogel.

### 3. CONCLUSIONS

#### 3.1 Gelation of FmocFF

This chapter introduced several experimental techniques used in the characterisation of FmocFF hydrogels, including titrations, rheology, fluorescence and imaging techniques. The effect of concentration of FmocFF on hydrogel strength was investigated, showing a relationship between concentration and hydrogel strength. The
The effect of salt and buffer solutions on self-assembly processes and final structures was shown. Buffers disrupted the hydrogel structure most significantly, whilst small ions with low charges gave the strongest hydrogels. The effect of pH on FmocFF in sodium acetate buffer showed an optimum pH range for hydrogels to form. Results from characterisation methods, including EM, were shown to be in good agreement with those found in the literature.

3.2 Electron Microscopy Imaging Techniques

Through various imaging techniques (SEM, TEM, and confocal microscopy), it was possible to observe FmocFF hydrogel structures on the micro- and nano-metre scales. A range of different artefacts arising from sample preparation for each technique have been presented, and ways to overcome them suggested. Where fibres were observed, it is possible to give accurate data on their dimensions which correspond to published literature values.

Overall, there are several aspects to consider when imaging dipeptide hydrogels. Minimising the distortions in structures brought about through each process is key for providing realistic representations of the three-dimensional solvated structures. Drying and washing the hydrogels introduced shear forces which significantly affected intermolecular interactions. This section has shown how the removal of water for SEM (via freeze-drying and cryo-drying) caused a range of artefacts on the surface and within the hydrogels, from artificial alignment of fibres to false cellular-type forms. These forces have less of an effect on samples prepared for TEM, as they were thinner and dried more quickly, retaining the structure more accurately. However, this method gave 2D, rigid images of the hydrogels not representative of the fluid, water-like, 3D solvated structure. In aqueous techniques, the fluidity makes imaging in high resolution challenging, and some degree of drying is necessary in order to image the structures accurately.

The addition of the fluorescent dye prior to gelation for confocal microscopy measurements may have affected the speed of gelation, and the final properties of the material. Also, in the results presented here the dye may not have stained the whole sample homogeneously. However, no significant effects have been reported in the literature.
4. FUTURE WORK

To move this work forward and to allow for more direct and quantitative comparisons between hydrogel samples, gelation methods must be standardised to limit the possibilities of widely varying structures. It would be beneficial to study experimental data coupled with computer modelling techniques to gain a better overview of gelation processes. It may be possible to study gelation in different salt solutions using this method, to observe how the salts interact and affect the self-assembly process. This understanding is important for using dipeptide hydrogels in many applications. The phenomenon of the shrinking hydrogels can also be investigated for use as a responsive material.

To continue exploring dipeptide hydrogels with imaging techniques, Atomic Force Microscopy (AFM) can be used. AFM uses a liquid cell where the sample is flooded with water. This will continue to be explored, but as the gels are very soft, the AFM tip damages and depletes the hydrogel as it measures, and strong hydrogels must be used. Temperature controlled measurements may be carried out to observe gels cooling and heating using AFM once the procedure has been refined and shown to give reproducible results. Due to their high water content it is difficult for the tip to make contact and find the surface of the hydrogel, and due to its fluidity it is difficult to gain clear images of gel structures.

5. REFERENCES

CHAPTER FOUR:
DIRECTED ELECTROCHEMICAL SELF-ASSEMBLY OF 
pH-TRIGGERED DIPEPTIDE HYDROGELATORS

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1. INTRODUCTION

The potential applications of dipeptide hydrogels may be expanded by gaining control over their gel properties. When the work presented in this Chapter began in 2009, there was very little control over size, shape and properties such as refractive index, density, and thickness. The size and shape could only be controlled by the size and volume of the container the hydrogels were formed in, and the concentration of dipeptide. In this section an electrochemical method is introduced for gelating pH-triggered dipeptides such as FmocLG. By utilising the oxidation of hydroquinone (HQ), an electrochemically induced pH drop at a gold (Au) surface could produce sufficient proton concentration to gelate pH-sensitive dipeptides. Thin films and membranes were grown using this technique. Surface initiated growth also opened the possibility of preparing multi-layered structures, where hydrogel layers with different properties are grown sequentially (see Chapter 6).

An outline of experiments carried out in this section is as follows. First, the electrochemical properties of HQ are explored. Conditions for growing Fmoc-Leucine-Glycine-OH (FmocLG) hydrogels at electrode surfaces were optimised. Surface Plasmon Resonance (SPR) was used to follow electrochemical gelation kinetics, and to obtain parameters for refractive index and thickness of electrochemically grown dipeptide hydrogels. SPR is a technique which is highly sensitive to changes at a metal surface. Imaging techniques were used to confirm the presence of fibres in hydrogel films. FTIR and DOSY were used to further characterise hydrogel films.

2. RESULTS AND DISCUSSION

2.1 Cyclic Voltammetry (CV), background charge and Chronopotentiometry

The electrochemistry of hydroquinone (HQ) was examined using cyclic voltammetry (CV) to assess at which voltages it was oxidised at different scan rates. A three-
electrode setup with a gold slide electrode was used, as described in Chapter 2, Section 17.6. The results are shown in Figure 4.1.

![Cyclic Voltammetry using a gold slide electrode](image)

Figure 4.1. Cyclic Voltammetry using a gold slide electrode of area 0.5 mm$^2$ for 0.66 mol dm$^{-3}$ HQ in with 1 x 10$^{-2}$ moldm$^{-3}$ NaCl as a background electrolyte. Measurements were performed at a range of scan rates 20 to 200 mV s$^{-1}$. Oxidation peaks lie in the range -0.025 and 0.1 V. The redox couple is reversible. Measurements w.r.t. Ag$^+$ / AgCl.

As the oxidation peak of HQ was at approximately 0.1 V at 200 mV s$^{-1}$, this potential was initially applied to the 2.44 x 10$^{-3}$ mol dm$^{-3}$ FmocLG solution at pH 7. However, it was found that a voltage of at least +0.35 V was required to generate enough protons to successfully form a hydrogel. Under potentiostatic control the current (and hence the proton flux) dropped as a hydrogel was formed on the surface. As a result, it was decided to generate films galvanostatically. The equivalent current of 20 µA cm$^{-2}$ was allowed to flow at the electrode surface to ensure a constant flow of H$^+$ ions. The background current is shown in Figure 4.2[a], with results of the electrochemically-induced oxidation of HQ and gelation experiment with FmocLG in [b].
Figure 4.2[a] Background charge measured in a solution of HQ and NaCl at a gold slide electrode of 0.5 cm$^2$ area. [b] Chronopotentiometry following gelation of $2.44 \times 10^{-3}$ mol dm$^{-3}$ FmocLG. 10 µA (20 µA cm$^{-2}$) was allowed to flow for 2000 s.

The total current was corrected for the background charge current obtained from Figure 4.2[a]. Figure 4.2[b] shows voltage over time as gelation occurred. The voltage increased from +0.4 to 0.45 V as the hydrogel film grew to maintain a constant current.

Gelation was attempted in the presence of an L-cysteine monolayer which was found to prevent the gelation of FmocLG, possibly because the production or dispersion of hydrogen ions at the electrode surface was reduced by the presence of the monolayer.
2.2 Electrochemical gelation of FmocLG without HQ

Hydrogels were shown to form without addition of hydroquinone, using an FmocLG and NaCl solution at a starting pH of 5. Under these conditions hydrogels formed at +1.3 V, as protons were released from the formation of oxides on the Au surface. The use of HQ allowed the voltage to be lowered considerably, without damaging the Au surface. It also allowed for more reproducible pH drops, as the concentration of HQ reduced and number of protons formed could be closely controlled.

2.3 Electrochemistry of HQ: different working electrodes

In Figure 4.3, two cyclic voltammagrams (CVs) of HQ with NaCl are shown for a gold slide and a gold TEM stub used as the working electrode, with 10 µA current allowed to flow.

![Figure 4.3. CVs of HQ on a gold slide compared with a gold TEM grid with 200 mesh holes at 100 mV/s scan rate. 10 µA was allowed to flow at each electrode.](image)

This result shows the oxidation peak of HQ differed in terms of current, depending on the substrate, which meant the current allowed to flow was altered to correct for this, to allow a constant current density of 20 µA cm⁻² for each material. This ensured every sample was produced under the same conditions.

2.4 Electrochemical gelation of FmocLG: Various electrode surfaces

Gelation was conducted using three materials as the working electrode: a platinum sputtered glass slide, platinum wire, and a gold coated LaSFN₉ glass slide (used for
SPR). Conducting copper tape was used to connect each working electrode to a three electrode electrochemical cell. The cell was completed by a platinum counter electrode and an Ag⁺/AgCl reference electrode (Dri-Ref). Films were grown galvanostatically by allowing 20 µA cm⁻² to flow for a set time (Ecochemie, Autolab PGSTAT 12). For a solution of 2.43 x 10⁻³ mol dm⁻³ FmocLG at pH 7.0, a voltage of +0.34 to 0.36 V was observed over 30 min. A homogeneous hydrogel of approximately 2 mm thick was present after each experiment when the cell was opened. Figure 4.4[a-c] shows images of the hydrogels formed at each electrode.

Figure 4.4[a] shows a thick (approx 2 mm) FmocLG hydrogel grown on a platinum sputter coated glass slide and [b] onto a platinum wire electrode. Interestingly, the hydrogel was formed across the gaps in the wire, a phenomenon which is exploited in later sections for producing ‘gap-spanning’ hydrogel membranes directly onto TEM grids. Figure 4.4[c] shows another thick (approx 2 mm) FmocLG hydrogel grown on a gold film using an SPR slide. For [a] and [c] the size of the hydrogel was defined by the 0.5 cm² O-ring of the electrochemical cell. The films were stable for at least one month when stored in humid conditions, and retained their shape upon inversion.

2.5 Fourier Transform (FTIR): characterisation of FmocLG hydrogels

FTIR was carried out on hydrogel layers grown directly onto gold slides, with the gold acting as the working electrode, as described in Chapter 2, Section 6.3. 20 µA cm⁻² was allowed to flow, with a 2.44 x 10⁻³ mol dm⁻³ FmocLG in a solution of NaCl and HQ for 2 hours. The spectrum was collected using a Perkin-Elmer Spectrum 100
Spectrometer. D$_2$O was used as a solvent in replacement of H$_2$O to enhance the resolution of the signals. The result is shown in Figure 4.5.

This spectrum was in agreement with that of the Gδ-L triggered FmocLG hydrogels as measured by Adams et al.$^1$ with peaks at 1684 and 1637 cm$^{-1}$ representing an anti-β sheet structure present within the hydrogel. This suggests that the gels grown using an electrochemically induced pH drop are similar in structure to those formed via addition of acid.

2.6 Diffusion Ordered NMR Spectroscopy (DOSY): characterisation of FmocLG hydrogels

DOSY was explored to obtain parameters for the diffusion of water through the hydrogel. For exact procedures see Chapter 2, Section 5.2. Experiments were carried out in H$_2$O, although it is possible to use D$_2$O. A $2.44 \times 10^{-3}$ mol dm$^{-3}$ FmocLG hydrogel was grown on a large gold slide electrode in a beaker, under normal growth conditions for 2 hours, after which the hydrogel was transferred into an NMR tube. The resulting DOSY spectra are shown in Figure 4.6.
The $^1$H peak of water was monitored in the hydrogel. The effect of changing the field gradient is measured. $D = 2.209 \times 10^{-9} \text{ m}^2\text{s}^{-1}$.

The diffusion coefficient measured for a $2.44 \times 10^{-3}$ mol dm$^{-3}$ FmocLG hydrogel was $2.209 \times 10^{-9}$ m$^2$ s$^{-1}$. This compares with the value for FmocFF hydrogel with $2.159 \times 10^{-9}$ m$^2$ s$^{-1}$ at 20 °C (see Chapter 7, Section 2.1.viii). This shows the hydrogels had an aqueous internal environment and an open structure. Parameters are included in Appendix 1.

2.7 Imaging for characterisation of FmocLG hydrogels

2.7.i Confocal Microscopy

Samples for confocal microscopy were prepared as outlined in Chapter 2, Section 12.2. Hydrogels were formed directly onto FTO-coated microscope well slides, with 20 µA cm$^{-2}$ for 2000 s. The presence of fibres was confirmed in Figure 4.7[a].
Figure 4.7. Confocal microscopy images of \(2.43 \times 10^{-3} \text{ mol dm}^{-3}\) FmocLG hydrogels. Scale bars [a] 30, [b] 100 and [c] 50 µm.

Figure 4.7[b and c] show areas more prevalent in the FmocLG hydrogel measured, which did not show long fibres, rather areas of compressed, unstructured peptide material. This may have been due to the method of the formation of the hydrogel, and again showed that there are other possible structures accessible to the dipeptide which occur other than the typical fibres usually published in the literature.

2.7.ii Scanning Electron Microscopy (SEM)

See Chapter 3, Section 2.1 for SEM of electrochemically grown FmocLG hydrogels. SEM showed the presence of an interlinked three-dimensional structure.

2.7.iii Atomic Force Microscopy (AFM)

20 µA cm\(^{-2}\) was allowed to flow at a gold slide electrode for 2000 s. Figure 4.8 shows an AFM micrograph scan of the bare gold surface, and Figure 4.9 of a blotted, partially dried FmocLG hydrogel; [a] in the centre and [b] at the edge showing the gold surface beneath.
Figure 4.8. AFM micrograph of a bare gold surface, evaporated onto LaSFNa glass. Image is a 5 µm cross-section.

Figure 4.9. AFM micrograph of a $2.43 \times 10^{-3}$ mol dm$^{-3}$ FmocLG hydrogel on a gold slide (blotted with filter paper.) [a] Amplitude and topography of the hydrogel layer. [b] Topography and phase
of the edge of the hydrogel showing the gold layer beneath. Images are 12 µm and 15 µm cross-sections.

The AFM micrograph images are in good correlation with confocal, SEM and TEM images, showing a three-dimensional extended structure of fibres in the hydrogels. Thin gels had a limited time window for imaging, as the samples dried out quickly. The thickness of the layer in Figure 4.9[b] was approximately 8 µm (difference between the top of the hydrogel layer and the gold surface).

2.7.iv Transmission Electron Microscopy (TEM)

To further the investigation into how gelation conditions affected the final hydrogel structure, TEM images were taken of films grown for a range of times of current density allowed to flow, in an FmocLG solution. The samples were prepared as outlined in Chapter 2, Section 11.2. The results are presented in Figure 4.10, showing images of hydrogels grown with 200, 400 and 1000 s of 20 µA cm$^{-2}$ current density.

Figure 4.10. TEM images of electrochemically grown FmocLG hydrogels showing [a] 20 µA cm$^{-2}$ for 200 s, [b] 400 s, and [c] 1000 s. Scale bars are 0.2, 0.5 and 0.5 µm, respectively.

Figure 4.10[a] shows a self-supporting hydrogel membrane with one single strand of fibrous structure visible, with 200 s application of current. The film is continuous, the structure of which is too small to observe using TEM. As the current was applied for longer times (400 s), the image in [b] showed the fibres were much more prevalent, and branching of fibres was observed. When 1000 s was used, the image of the hydrogel in [c] shows a dense network of fibres. This shows pictorially that the longer the current was applied, the more dense, fibrous, and strong the hydrogels became.
2.8 Measuring electrochemical gelation of FmocLG: Surface Plasmon Resonance (SPR)

SPR was used to measure the formation of FmocLG hydrogel films at the surface of a gold electrode, as described in Chapter 2, Section 3 and 15.3. The dielectric constants and thickness of hydrogels were obtained from fitting the data collected to a three-layer Fresnel model given in Table 4.1. The limit of detection of SPR for thickness of film is around 200 nm, beyond which an average of the film is given.

A $2.43 \times 10^{-3}$ mol dm$^{-3}$ FmocLG precursor solution was introduced to a gold slide via a flow cell. A three-electrode setup was used, with the gold layer acting as the working electrode (attached with conducting copper tape), a platinum wire counter electrode and Ag$^+$/AgCl reference electrode.

2.8.i Following gelation kinetically with SPR

The increase in reflectivity was measured at a fixed angle of 56.0° whilst a current of 20 µA cm$^{-2}$ was allowed to flow for 2000 s. Results are shown in Figure 4.11.

![Figure 4.11. SPR kinetic scan at a fixed angle of 56.0° showing change in reflectivity as a current is allowed to flow for a total of 2000 s.](image)

Figure 4.11 shows the reflectivity increased quickly from 30 to 56 % at short times when the current was allowed to flow (marked by the ‘on’ arrow), until the limit of detection for the plasmon was reached after around 200 s of turning the current on. The increase was due to the minimum angle position of the surface plasmon shifting to more positive angles during the formation of the hydrogel. After around 500 s no further
changes in reflectivity were observed, despite the hydrogel continuing to grow thicker. This suggested the measurable hydrogel layer (first 200 nm) retained its structure with continued oxidation of HQ and production of protons. The kinetics show that a hydrogel started to form as soon as the current was allowed to flow.

2.8.ii  Following gelation with angle scans

Angle scans were taken before and after gelation. Figure 4.12[a] gives SPR scans showing intensity of reflected light as a function of incident angle for the precursor FmocLG solution (squares) and the hydrogel layer (triangles) produced (20 µA cm$^{-2}$, 2000 s). The film was dried under vacuum overnight and is shown in Figure 4.12[b]. Experimental data is denoted with open symbols, and fits with solid lines.
When the surface plasmon is excited, a sharp dip in the reflectivity is seen. The position of both the reflectivity minimum and the critical angle is indicative of the dielectric properties of any layers above the gold substrate. The results of Figure 4.12 gave the critical angle of the Au layer in air as 22.0°, and the minimum at 25.5°. In the precursor solution, the critical angle of Au occurred at 47.4° and the minimum at 57.2°. When a 2 mm gel layer was grown on the surface and an angle scan taken of the film in air, the critical angle had shifted to 47.8° and the minimum to 58.1°.
In Figure 4.12[b] the SPR scans in air show the gold surface (squares) and the hydrogel layer (triangles) with fits (straight lines). Upon removal of excess un-gelled solution the plasmon remained the same. This was due to the hydrogel layer being thicker than the detection limit for SPR, at approximately 2 mm. It also showed that the hydrogel layer was self-supporting, remaining attached to the gold surface on washing. There was an observed shift in critical angle in the presence of the hydrogel layer which corresponded to a higher refractive index than the precursor solution.

The film was dried in a vacuum (circles in Figure 4.12[b]). The collapsed hydrogel led to a more compact film with a higher refractive index (the collapsed film was several hundred microns thick). The collapsed gel scattered the light to a much greater degree due to the presence of structure on the length scale of the wavelength of the incident light. Visually the collapsed film was translucent, and the swollen hydrogel film transparent. The results from fitting this data is given in Table 4.1.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Thickness /nm</th>
<th>ε(real)</th>
<th>ε(imaginary)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prism</td>
<td>∞</td>
<td>3.4117</td>
<td>0</td>
</tr>
<tr>
<td>Gold</td>
<td>51.21</td>
<td>-11.977</td>
<td>1.5016</td>
</tr>
<tr>
<td>Air</td>
<td>∞</td>
<td>1.0016</td>
<td>0</td>
</tr>
<tr>
<td>Precursor solution</td>
<td>∞</td>
<td>1.779</td>
<td>0</td>
</tr>
<tr>
<td>Gel</td>
<td>500</td>
<td>1.7997</td>
<td>0.002</td>
</tr>
</tbody>
</table>

The dielectric constant $\varepsilon$(real) of the FmocLG precursor solution was 1.7790, and 1.7997 for the hydrogel layer, with scattering $\varepsilon$(imaginary) at 0.002. This shows the hydrogel layer was similar in refractive index to its precursor solution. The small imaginary component of the dielectric constant was caused by either scattering or absorption. As the FmocLG hydrogel does not absorb at 632.8 nm it is likely that the curve broadened due to scattering when structures were formed on the length scale of the light. The values of the film dielectric constant confirmed the formation of an open aqueous gel network with a refractive index close to that of water (1.77); for comparison, a dense layer of protein on a metal surface typically has a much higher dielectric constant of $\varepsilon$(real) = 2.1 to 2.25.
2.8.iii Re-swelling a dried hydrogel film

In order to investigate whether a dried film would re-swell to its original size and water content, a $1 \times 10^{-2}$ mol dm$^{-3}$ NaCl solution at pH 7 was introduced to a dried hydrogel layer via the electrochemical SPR flow cell. Angle scans were taken at every hour for 7 hours, after which no further change was observed. The original hydrogel layer (squares) and the dried layer (triangles) are shown in Figure 4.13[a], with scans taken during the re-swelling process (circles). Figure 4.13[b] is a plot showing the change in the position of the minimum angle over time, following change in minimum angle (left axis) and reflectivity (right axis) over time.

![Figure 4.13(a)](image)

![Figure 4.13(b)](image)

*Figure 4.13[a] SPR angle scans in water over 7 hours showing the re-swelling of the hydrogel layer [b] plot of change in angle and reflectivity of the minimum angle from each angle scan in [a].*
In Figure 4.13[a] the minimum angle of the dried layer had shifted to more than 70°, with a broadening and an increase of the reflectivity at the minimum. The collapsed layer re-swelled slowly over 7 hours in the NaCl solution, shown by the minimum angle shifting to the right and the narrowing of the peaks. After 7 hours (grey circles) the plasmon for the re-swollen hydrogel was just to the right of the ‘as-formed’ gel, suggesting almost complete rehydration of the film. The swelling appeared to be linear on the scale of the plasmon.

Figure 4.13[b] shows a linear movement of the minimum angles with time, from the addition of the NaCl solution into the cell. This suggests the re-swelling process occurred evenly across and through the sample. This process is not possible to measure in the bulk phase and so SPR for measuring a thin film is useful for finding rehydration kinetics.

2.8.iv Dissolving hydrogel layers electrochemically

FmocLG hydrogels dissolve when exposed to high pH (9 – 10), as the dipeptide becomes soluble.\(^7\) A high pH was created electrochemically by allowing a negative current to flow which consumed protons with the reduction of quinine to HQ. It was possible to electrochemically dissolve the hydrogel layer with a -10 µA cm\(^{-2}\) current for 500 s. This was sufficient to completely remove the 2 mm thick layer. The results are shown in Figure 4.14.
The application of negative potential altered the Au layer slightly, seen by the plasmon in air in Figure 4.14[a] not being as strong, however the minimum positions were at the same angle showing no layer was present after removal of the hydrogel.

Figure 4.14[b] gives SPR angle scans in water, showing the hydrogel layer (triangles) and the gold layer before (circles) and after (squares) its removal. The kinetic scan in [c] shows the removal of the hydrogel layer at a fixed angle (56.0°), with arrows denoting where the current was turned on and off.

This result confirms it is possible to etch or dissolve the hydrogel layer away in stages or completely using an electrochemical pH reduction method. It also suggested that quinine was available at the electrode surface, or moved towards the surface in sufficient concentration to consume protons.

2.8.v Stepping current for control over thickness of hydrogel layers

In order to determine the relationship between the time the current density is allowed to flow and the thicknesses of FmocLG hydrogels, the current was allowed to flow in timed steps. After a hydrogel layer was grown to the desired thickness, the solution
remaining in the cell was removed to prevent further self-assembly. A current of 20 µA cm\(^{-2}\) was allowed to flow at a gold slide electrode for 60 s, with a 200 s rest period at open circuit to observe changes in the gel thickness when the current was switched off. The maximum potential at each step was recorded. The results are shown in Figure 4.15; kinetic data in [a], and fitted data in [b].

![Image](image-url)
In Figure 4.15[a], the reflectivity at a fixed angle was seen to increase with each current step. The maximum potential (right hand y-axis) to allow 20 µA cm\(^{-2}\) to flow increased with each FmocLG hydrogel layer, which maintained the constant electrochemical oxidation of HQ with each step. After around 600 s the evanescent tail of the surface plasmon was no longer sensitive to thickness changes, but monitored overall change in refractive index of the first 200 nm of the gel layer. Hence each additional layer resulted in a decrease in change in reflectivity, though they were of similar thicknesses.

Figure 4.15[b] shows the stepwise increase in reflectivity (taken from each rest period) plotted versus film thickness (left axis) and increase in refractive index (right axis), using the layer model outlined in Table 4.2. The thickness and refractive index are directly proportional to current time. An initial linear increase in thickness was observed, corresponding to an increase in thickness of approximately 80 nm for every 60 s of current, after which the increase in refractive index was observed.

Table 4.2. Parameters and values used to fit the SPR scans shown in Figure 4.15. [a] Infinite thickness implies a layer that is much thicker than the evanescent tail of the surface plasmon wave. Thickness was considered infinite for layers > 200 nm.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Thickness /nm</th>
<th>ε(real)</th>
<th>ε(imaginary)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prism</td>
<td>∞</td>
<td>3.4127</td>
<td>0</td>
</tr>
<tr>
<td>Gold</td>
<td>51.07</td>
<td>-12.0075</td>
<td>1.5016</td>
</tr>
<tr>
<td>Air</td>
<td>∞</td>
<td>1.0016</td>
<td>0</td>
</tr>
<tr>
<td>Precursor solution</td>
<td>∞</td>
<td>1.7782</td>
<td>0</td>
</tr>
<tr>
<td>Gel</td>
<td>∞ [a]</td>
<td>1.7972</td>
<td>0.002</td>
</tr>
</tbody>
</table>

(for layers > 500nm)

The dielectric constants of the films were obtained by carrying out Fresnel fitting of the SPR curves using commercially available software. A four layer model of glass|gold|hydrogel|water (or air) was used. In all cases, the superstrate (air, water or hydrogel film >500 nm thick) extended much further than the exponentially decaying surface plasmon wave and was therefore assumed to be infinite.

The dielectric constant of the hydrogel film was found to be just slightly higher than that of the precursor solution. The dielectric constant for the precursor solution was
measured to be $\varepsilon(\text{real}) = 1.7782$ and $\varepsilon(\text{imaginary}) = 0$ ($\lambda = 632.8$ nm). The fully formed (1 mm thick) hydrogel was found to have a dielectric constant of $\varepsilon(\text{real}) = 1.7972$ and $\varepsilon(\text{imaginary}) = 0.002$. This technique introduces the plausibility for accurate control over the thickness of layers on a nanometre scale.

2.8.vi Minimum-following SPR method: stepping current

The SPR setup was used to track the minimum peak position in terms of reflectivity and angle in real time during the gelation process. The experimental setup was similar to the step current previously: current on 60 s, open circuit (0 µA) for 200 s, for a total of 1000 s. The film was dried overnight in a vacuum and re-swelled with $1 \times 10^{-2}$ mol dm$^{-3}$ NaCl at pH 7. The results are shown in Figure 4.16.
Figure 4.16[a] Kinetic SPR data following gelation whilst stepping current minimum angle (left-hand axis) and reflectivity (right-hand axis). [b] Kinetic SPR minimum following the re-swelling of the dried hydrogel film.

Figure 4.16[a] shows the change in angle (blue) and reflectivity (black) of the minimum peak with each application of current. The two followed a similar movement. The position of the minimum shifted after the first four steps, suggesting either a re-structuring or reaching of the detection limit of the plasmon. The re-swelling of the hydrogel in Figure 4.16[b] was much faster than the 2 mm thick layer, taking only 30 minutes to reach an equilibrium which was similar to that of the original layer, as shown by the open points on the graph. This suggests the film was almost fully re-hydrated, but the hydrogel may have been disrupted by the drying process.
Table 4.3. Parameters and values for thicknesses and dielectric components of each layer, from fitting experimental data in Figure 4.16.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Thickness / nm</th>
<th>( \varepsilon ) (real)</th>
<th>( \varepsilon ) (imaginary)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prism</td>
<td>∞</td>
<td>3.4117</td>
<td>0</td>
</tr>
<tr>
<td>Gold</td>
<td>51.51</td>
<td>-12.047</td>
<td>1.4696</td>
</tr>
<tr>
<td>Air</td>
<td>∞</td>
<td>1.0016</td>
<td>0</td>
</tr>
<tr>
<td>Precursor solution</td>
<td>∞</td>
<td>1.779</td>
<td>0</td>
</tr>
<tr>
<td>Gel after 60 seconds</td>
<td>20</td>
<td>1.7997</td>
<td>0.002</td>
</tr>
<tr>
<td>120 seconds</td>
<td>70</td>
<td>1.7997</td>
<td>0.002</td>
</tr>
<tr>
<td>180 seconds</td>
<td>200</td>
<td>1.7997</td>
<td>0.002</td>
</tr>
<tr>
<td>&gt;240 seconds</td>
<td>200</td>
<td>1.7997</td>
<td>0.002</td>
</tr>
</tbody>
</table>

In Table 4.3, the minimum position for each step current is shown with fitting, measurable for the first three layers. Beyond this the thickness of the hydrogel layer moved beyond the limit of detection for SPR (200 nm). The minimum following technique provides real time kinetic data and allowed more accurate fitting of the layers as two parameters were acquired throughout the gelation process. The thickness of the first layers were 20, 70, and 200 nm, with an \( \varepsilon \) (real) = 1.7997, which was equal to the result from 2.8.ii, although the thicknesses varied.

2.8.vii Changing conditions for different hydrogel properties

To determine the effect of changing current density on hydrogel properties, all other parameters remained constant with a range of currents allowed to flow at a gold surface. The data in Figure 4.17 represents the FmocLG hydrogelation kinetics for five separate hydrogel layers, grown for 2000 s. The lowest current density required to form a hydrogel was 12 \( \mu A \) \( cm^{-2} \). As the current was increased, the gels formed more quickly, resulting in more dense, less homogeneous layers. The highest current densities, 24 and 32 \( \mu A \) \( cm^{-2} \) gave similar results, and below 12 \( \mu A \) \( cm^{-2} \) hydrogels did not form (not shown). The data was worked to show the kinetic plots in terms of the dielectric constant and the percentage of water in the films, the procedure for which is described in Section 2.9 of this Chapter.
Figure 4.17 shows the kinetic data, obtained as a function of the dielectric constant, as 12 – 32 µA cm\(^{-2}\) current densities were passed at a gold electrode. With the exception of short times (the first 200 s) the curve was due to the overall increase in refractive index of the hydrogel film. At higher current densities the films had higher final dielectric constants, and less water present in the hydrogels. The exception to this was at the highest current density (32 µA cm\(^{-2}\)), where the values were similar to that of the lowest current density (12 µA cm\(^{-2}\)). This may have been due to the speed of the gelation affecting the hydrogel properties. The final dielectric constants ranged from \(\varepsilon_{\text{real}} = 1.783\) to 1.790, with the percentage of water 95.5 to 93.5 %. This proved that the properties can be fine tuned and controlled using the electrochemical technique for hydrogelation of dipeptides.

2.9 Calculating the percentage of water in the hydrogels

Effective medium theory (EMT) was used to calculate the percentage of water inside the films when grown under different conditions. EMT describes the effective dielectric constant of a heterogeneous mixture (on the macro scale) of two different dielectric media (A and B).\(^8\) It assumes that inclusions of one dielectric material, A, are embedded in an averaged dielectric medium (A + B). It is particularly applicable when the volume fractions of A and B are similar such as in a nanoporous material. In this case the percentage of water was calculated at the limit (volume averaged approach)
where \( n_{eff} = f_{gel}n_{gel} + f_{water}n_{water} \) (\( n \) = refractive index of the gel / water, \( f \) = fraction of gel / water in the film).

2.10 Monitoring hydrogelation of FmocLG with addition of Gδ-L

In order to compare the electrochemical hydrogelation of FmocLG to the conventional method as described by Adams et al\(^7\), Gδ-L was added to an FmocLG solution and monitored with SPR. Gδ-L slowly hydrolyses to produce protons and lowers the pH, forming a hydrogel over 3 – 4 hours.

The experiment was carried out as follows. The pH of 1 cm\(^3\) MilliQ water was raised by adding 4 µL of 1 mol dm\(^{-3}\) NaOH, and 10 µL FmocLG stock solution (0.243 mol dm\(^{-3}\) in DMSO) was added to give a final concentration of 2.43 x 10\(^{-3}\) mol dm\(^{-3}\). 10.9 µL 1 mol dm\(^{-3}\) HCl was added to bring the starting pH of the solution to 7.0. A stable baseline was measured, after which 3.5 mg Gδ-L was dissolved in the sample and injected into the cell. The measurement was taken over 12 hours. The SPR kinetics and angle scans are shown in Figure 4.18.
The angle scans in Figure 4.18[a] did not show a shift in critical or minimum angles in the presence of the hydrogel. The kinetics in Figure 4.18[b] shows the change in reflectivity over the first 3 hours of the gelation process, after which no further changes were observed. The lower final reflectivity could have been due to the change in pH of the solution affecting the signal. The hydrogel produced was very clear and homogeneous, which did not show up as any change in reflectivity in the plasmon. This suggests the refractive index was identical or very close to the precursor solution.
Alternatively, the structure may not have been close enough to the surface to observe using SPR. This result is very interesting as it suggests the hydrogels made with the electrochemical technique are more dense than when formed very slowly, via this method.

3. CONCLUSIONS AND OUTLOOK

In this section the electrochemical gelation of FmocLG has been carried out at different electrode surfaces. The presence of an electrochemically formed hydrogel was proven by confocal microscopy, TEM, SEM, AFM, FTIR, and DOSY. Electrochemistry is a useful technique for the surface initiated formation of hydrogel films. It allows the rapid lowering of the pH in a known volume close to the electrode surface. By controlling the current and voltage applied it is possible to change both the final pH and the speed at which the pH falls. The pH drop can also be reversed and the gel dissolved from the surface.

Hydrogel films were controllably grown with thicknesses from tens of nanometers to millimeters. Film growth could be stopped and started at will, and the films were stable once formed. Film growth was also reversible with total disassembly occurring when the electrochemical conditions were reversed. The growth of films with thicknesses below 200 nm was followed in-situ by SPR, using kinetic scans, angle scans, and minimum following measurements. The conditions were optimised for growing hydrogels on SPR Au slides, for monitoring hydrogelation in situ. Drying and swelling experiments were also carried out, showing the reversible collapse and re-hydration of the films to almost the original structure. Hydrogels of different properties were formed using a range of electrochemical current densities.

SPR has been shown to be a useful method for studying the real-time growth of dipeptide films. Information was obtained of thicknesses and dielectric properties. SPR also allows changes in dielectric properties to be monitored during gelation and may contribute to the understanding of the evolution of peptide structures (β-sheets, fibres, ropes) in the hydrogels. The technique could be extended to give more data allowing for more accurate fitting by adding D₂O into the cell and measuring the thicknesses and dielectric constants with respect to this solvent also.
4. ACKNOWLEDGEMENTS

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Ursula Potter and John Mitchels at the University of Bath for assistance with Electron Microscopy.

5. REFERENCES

1. INTRODUCTION

In order to analyse electrochemically formed gels at an Au surface in more depth, more complex Surface Plasmon Resonance (SPR) techniques are used, which build on those in Chapter 4. Long-Range Plasmons are used to probe further into the hydrogel films. Long and Short Range and coupled-Long Range Plasmons have two minima upon interaction with p-polarised light, which allows for more accurate fitting of data for hydrogels. This section employs the use of three different substrates (Chapter 2, Section 16.3 to 16.7) to provide values for refractive indices and thicknesses of electrochemically grown FmocLG dipeptide hydrogels, at various distances from the Au surface. This is used to build up a profile of gelation at different depths into the hydrogel.
2. RESULTS AND DISCUSSION

2.1 Long and Short Range SPR (LSR SP) substrates
Substrates for Long and Short Range SPR (LSR SP) were made at AIT, Vienna and are explained fully in Chapter 2, Section 16.6. They consist of a layer of 250 nm CYTOP with 50 nm Au evaporated. The penetration depths into the dielectric $L_p$ was 136 nm (SR) and 246 nm (LR). FmocLG was dissolved at a concentration of $2.43 \times 10^{-3}$ mol dm$^{-3}$ into a solution of $6.6 \times 10^{-3}$ mol dm$^{-3}$ HQ and $1 \times 10^{-2}$ mol dm$^{-3}$ NaCl, at pH 7.0 (Chapter 2, Section 2). An electrochemical cell was used and a current of 20 $\mu$A cm$^{-2}$ was allowed to flow at the gold electrode for 6000 s.

Angle scans were taken before and after gelation, and kinetic data was collected by performing two separate minimum following experiments, measuring the SR SP and the LR SP in terms of reflectivity (%) and angle ($\theta$). Figure 5.1 shows the angle scans in [a] and kinetic data for hydrogel growth using LR and SR SPs in [b] and [c] (from two separate experiments which monitored the SR and LR SP minima individually). Data is represented by open points, and fits by solid lines.
Figure 5.1. LSR SP substrates [a] Experimental data and fits in water (black), precursor solution (blue) and gel (green). Minimum following kinetics for LR SP (red) and SR SP (black) showing [b] change in minimum reflectivity and [c] change in minimum angle.

The graph in Figure 5.1[a] shows the angle scans taken with a LSR SP substrate in water (black points), the precursor FmocLG solution (blue), and the hydrogel after 6000 s (green). The short range SP (marked SR on the graph) appeared to shift much more than the long range SP (marked LR). In [b] and [c], the change in reflectivity (%) and change in angle (θ) for the minimum angle θ_{min} is shown for the LR SP and the SR SP in a kinetic graph. The arrows denote the time at which the current was switched on.
and off. The changes in reflectivity and corresponding change in dielectric constant occurred shortly after the initial application of current, and were not affected after around 600 s (LR) and 1500 s (SR).

From this data it was possible to measure a change in the critical angle (θ\(_c\)), minimum angle (θ\(_{\text{min}}\)), and reflectivity (% intensity) for SR and LR SPs throughout gelation. The total change in \(\varepsilon\) (real) from the precursor solution to the hydrogel layer was \(n = 0.012\) for SR SP and \(n = 0.001\) for LR SP. The SR SP continued to be affected for longer times than the LR SP, indicating an increasing density or restructuring of the hydrogel (measured from the changing dielectric constant) which was observed close to the Au surface, but is not visible with the LR SP. There appeared to be small waveguides present in the final hydrogel angle scan, indicative of a layer thicker than 1000 nm. However, as θ\(_c\) had shifted throughout the gelation process, there were three unknown values to fit, which meant it was not completely accurate despite introducing two minima. The dielectric constant of the hydrogel was determined to be LR = 1.348 and SR = 1.345 (see Table 5.1 in Section 2.4). The water content was found to be LR = 89.5 % and SR = 92.9 %. This suggests that the part of the hydrogel film measured with the LR SP was slightly denser, with a higher dielectric constant and less water present, as the area measured with the SR SP had a lower dielectric constant and higher water content.

### 2.1.i Stepping current: Measuring LR SP and SR SP simultaneously

In order to minimise the effects of the changing critical angle θ\(_c\) throughout gelation, and to improve accuracy by using the same substrate to follow the LR and SR SPs simultaneously, a step current was applied to the FmocLG solution and angle scans taken after each application of current (20 µA cm\(^{-2}\) for 60 s). Fresh precursor solution was pumped into the cell after each application of current before taking angle scans to ensure a constant background dielectric constant.

Figure 5.2 shows the changes in the positions of the LR and SR SPs, and the calculated thicknesses and refractive indices of the hydrogel at each step. Also included is the change in minimum angle position and dielectric constant.
Figure 5.2. Current was applied for 60 s at intervals. Angle scans were taken in precursor solution. [a] Change in minimum angle, [b] Change in reflectivity for LR SP (green) and SR SP (blue). [c] Change in critical angle (red, left axis) and dielectric constant (blue, right axis) of precursor solution and [d] change in thickness (red, left axis) and dielectric constant (blue, right axis) of hydrogel layer.

Figure 5.2[a] and [b] show the movement of the minimum angles of the LR and SRSPs in terms of angle and reflectivity. As also observed in results from Figure 5.1[a], \( \theta_c \) changed at early stages, and throughout gelation, despite the fact that all scans were carried out in a solution with a fixed dielectric constant (the precursor was replaced with fresh solution before each scan). In Figure 5.2[c] the change in \( \theta_c \) with time is plotted.
As $\theta_c$ increased throughout the experiment it was only possible to obtain data for the density of the hydrogels, and not their thickness.

Figure 5.2[d] follows the increase in thickness and change in dielectric constant of the hydrogel layer with time, calculated from fitting each curve. From Figure 5.2[a] and [b] it is possible to know where the limit of detection of the LR SP is reached, which occurred between 480 and 540 s. The SR SP continued to be affected throughout gelation. There were also two distinct changes in gradient in the trends. $\theta_{\text{min}}$ started to increase more quickly after 180 s, flattening off at 420 s. As the penetration depth for the LR SP was 246 nm, this suggests a layer of 250 nm was present at around 480 s.

As $\theta_c$ continued to be affected until 300 s, $\varepsilon(\text{real})$ of the water layer was changed in order to fit the data. There was a difference in the magnitude of the changes between each minima, suggesting again that there were areas of different densities as the film formed. That the SR SP continued to be affected beyond 600 s shows the hydrogel layer was becoming denser closer to the Au surface, where the pH was lower, due to the increased concentration of [H+] ions being produced.

The results were as follows; $\varepsilon(\text{real}) = 1.341$ at 600 s, thickness of hydrogel = 250 nm. At longer times the SP could no longer be fitted accurately to provide a value for the thickness of the hydrogel.

2.2 Long Range SPR (LR SP) substrates
Substrates for Long Range SPR (LR SP) consisted of a 1000 nm layer of CYTOP with 11 nm Au (Chapter 2, Section 16.6), with $L_p = 550$ nm. FmocLG was dissolved into a solution of HQ and NaCl at pH 7.0 (Chapter 2, Section 2). A current density of 20 $\mu$A cm$^{-2}$ was allowed to flow for 6000 s. Angle scans were taken before and after gelation, and kinetic data collected using the minimum following technique, where the position of $\theta_{\text{min}}$ for the SP minimum was measured in terms of reflectivity (%) and angle ($\theta$). Figure 5.3 shows the angle scans and kinetic data for hydrogel growth using LR SPs.
Figure 5.3. LR SP substrate showing data with open points and fits with solid lines. [a] Experimental data and fits in water (black), precursor solution (blue) and hydrogel layer (green); [b] Minimum following kinetics for LR SP showing change in reflectivity and dielectric constant ($\Delta R_{\text{eff}}$) with time.

The angle scans for LR SPs are shown in Figure 5.3. In [a], $\theta_c$ had shifted more dramatically than with the LSR SP substrates. In the presence of the hydrogel layer,
the reflectivity of the minimum of the LR SP was significantly increased, with a large shift and a more positive minimum angle. In [b], the change in $\varepsilon(\text{real}) = 0.012$, with the sensitivity unaffected after only 500 s. This suggests the hydrogel layer was not changing significantly with additional current at this penetration depth. $\varepsilon(\text{real})$ of the hydrogel layer = 1.348, water content = 89.3%.

2.2.i Stepping current: Minimum following LR SP kinetics
Using the standard FmocLG solution, 20 µA cm$^{-2}$ was allowed to flow to the LR SP substrate for 60 s at intervals. The change in the minimum angle and change in reflectivity is shown in Figure 5.4.
The change in $\varepsilon$(real) could not be measured accurately after 240 s, as the LR SP had no clear $\theta_c$ after 240 s. Until this point, with each 60 s step of current there was a $0.1 \times 10^{-3}$ change in $\varepsilon$(real). The reflectivity and observed change in angle was affected until $12 \times 60$ s, total 720 s (similar to the LR SP in the LSR SP substrate).

2.3. **Coupled-Long Range SPR (c-LR SP) substrates**

Coupled-Long Range SPR (c-LR SP) substrates are described in Chapter 2, Section 16.7. They consisted of a four-layered system with alternate CYTOP (660 nm) and Au layers (25 nm), and the Lp = 950 (anti-symmetrical SP) and 364 (symmetrical SP).
standard FmocLG solution was used at pH 7.0, with a current of 20 µA cm\(^{-2}\) allowed to flow for 6000 s. Angle scans were taken before and after gelation, and data was collected using minimum following kinetics. Figure 5.5 shows the angle scans (symmetric and anti-symmetric SPs visible) and kinetic data (anti-symmetric SP) for hydrogel growth using coupled-LR SPs.
The parameters obtained for the c-LR SPs were not as accurate, as a $\theta_c$ could not be clearly observed in the precursor and hydrogel layers. However, the dielectric constant of the hydrogel increased by almost $0.8 \times 10^{-3}$ as the hydrogel formed over 6000 s, which is in the region of that found with the other substrates. Due to the limited number of these substrates available, a step experiment or kinetic experiment with the symmetrical SP was not carried out for this substrate. The anti-c-LR SP was unaffected after around 500 s.

2.4 Results of FmocLG gelation: Comparison of substrates

The combined results for the changes in reflectivity and minimum angle of each SP are shown in Figure 5.6.
Figure 5.6. Minimum-following experiments using each SP substrate. [a] Change in reflectivity and [b] change in angle of the minimum angle (showing initial changes).

The graphs in Figure 5.6 show the gelation kinetics of FmocLG on all three substrates, with a difference in the first 500 s observed between each one. In [a], which measured the increase in scattering of the hydrogel film, the largest change in reflectivity was observed with the LR SP, which penetrated the furthest into the dielectric, so was able to monitor the increase in thickness of the hydrogel for the longest time. The c-LR SP and the SR SP were affected less in conjunction with their penetration depths. The SP which was least affected was with the LR (LSR SP), which was an unexpected result.

In Figure 5.6[b], which measured the increase in dielectric constant, the largest change in angle observed was seen with SR SP, with the others reaching a similar, lower value, which shows the hydrogel layer was similar at these penetration depths. The LR (LR SP) and SR SPs continued to be affected for longer times than the c-LR SP and the LR (LSR SP). This showed that these plasmons were more sensitive to the change in dielectric constant over time.

Overall the combined calculated values for final water content and ε(real) of the gels were 91.0 ± 2.0% and 1.345 ± 0.003, after 6000 s. The parameters for hydrogels produced by applying 20 µA cm\(^{-2}\) for 6000 s are shown in Table 5.1.
Table 5.1. Results from each substrate giving dielectric constant $\varepsilon$(real) and water content of hydrogels.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Time of gelation (seconds)</th>
<th>Refractive Index of precursor solution</th>
<th>Refractive Index of gel (measured) $\varepsilon$ (real)</th>
<th>$\varepsilon$ (imaginary)</th>
<th>Water content in gel % (measured)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LRSP</td>
<td>6000</td>
<td>1.336</td>
<td>1.348</td>
<td>0.009</td>
<td>89.3</td>
</tr>
<tr>
<td>LR-LSRSP</td>
<td>6000</td>
<td>1.335</td>
<td>1.348</td>
<td>0.001</td>
<td>89.5</td>
</tr>
<tr>
<td>SR-LSRSP</td>
<td>6000</td>
<td>1.336</td>
<td>1.345</td>
<td>0.001</td>
<td>92.9</td>
</tr>
<tr>
<td>c-LRSP</td>
<td>6000</td>
<td>1.335</td>
<td>1.342</td>
<td>0.005</td>
<td>93.3</td>
</tr>
</tbody>
</table>

The results were fairly consistent between substrates, in terms of $\varepsilon$(real), $\varepsilon$(imaginary) and water content of the films.

3. CONCLUSIONS AND OUTLOOK

SPR is a useful method for measuring thin hydrogel films. The technique was extended in this section to use plasmons of long and short ranges by using CYTOP as a substrate for the Au layer. Hydrogels were measured using kinetic and angle scans with three substrates, and parameters obtained for FmocLG gels with 6000 s growth time. Parameters were obtained for changes in critical angle, reflectivity, dielectric constants, and water content throughout gelation. Each substrate was used to penetrate to different depths into the hydrogel films during gelation, and showed the hydrogel layer closest to the Au surface continued to become denser upon additional production of $[H^+]$ ions at the surface.

The technique was interesting to study the gelation of FmocLG, and parameters were obtained for the hydrogels at different penetration depths. However, the studies could not provide more accurate data for thick hydrogel films as the critical angle shifted or disappeared at short times. This meant there were three unknown values in the case of LSR SP and c-LR SP. To fit the data the final hydrogel layers were set as 2000 nm thickness, which was not necessarily precise. At short times or where the critical angles remained unchanged, useful data could be obtained from repeat experiments.
Future work could be continued by monitoring the growth of much thinner hydrogel films, and using lower current densities, so the films are slower growing. This way, more accurate data could be obtained for the hydrogels where the critical angle is unchanged.

4. ACKNOWLEDGEMENTS

Jakub Dostalek, Yi Wang, Chun Jen Huang and Professor Wolfgang Knoll at the Austrian Institute of Nanotechnology, Vienna for support with producing and analysing substrates.
CHAPTER SIX:
ELECTROCHEMICAL TECHNIQUES FOR THE GENERATION OF NOVEL DIPEPTIDE HYDROGEL MATERIALS

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1. INTRODUCTION

After the successful development of a technique for the controlled gelation of FmocLG using the oxidation of hydroquinone at a range of electrode surfaces, more complex and novel hydrogel systems are explored in this section. The chapter is comprised of three sections of results. The first investigates layered systems consisting of alternate layers of FmocLG and a Nap-protected dipeptide, Br-Nap-Ala-Gly-OH (BrNapAV), providing SPR data and photographic images of the layered hydrogel materials. Secondly, the spontaneous gelation of FmocLG to form thick (mm) hydrogels in the presence of a thin (nm) seeding layer is shown, with SPR and TEM used to characterise these systems.

In the final section, two methods for the immobilisation of the horseradish peroxidase (HRP) enzyme within FmocLG hydrogels are explored, and the enzymes’ activity is measured using the colorimetric hydrogen peroxidase assay.

2. LAYERING DIPEPTIDES FOR CUSTOM MATERIALS

2.1 Aim and Introduction

The aim of this section was to use the electrochemical gelation technique introduced in Chapter 4 to sequentially build up novel dipeptide hydrogel materials, consisting of layers of different dipeptide hydrogels. FmocLG and BrNapAV (see Figure 6.1, obtained from Adams group, University of Liverpool; for synthesis see Chen et al1) were used for alternate layers, and growth was measured with SPR.
This technique has important implications for creating materials for three-dimensional cell growth.\textsuperscript{2-4}

2.2 Experimental details

As described in Chapter 2, Section 2, 2.44 \times 10^{-3} \text{ mol dm}^{-3} \text{ FmocLG}, or 2.22 \times 10^{-3} \text{ mol dm}^{-3} \text{ BrNapAV} were dissolved in a solution of 6.6 \times 10^{-3} \text{ mol dm}^{-3} \text{ HQ} and 1 \times 10^{-2} \text{ mol dm}^{-3} \text{ NaCl}, and the pH adjusted with 1 \text{ mol dm}^{-3} \text{ HCl} to 7.0. The pKa of \text{BrNapAV} is 5.9 when titrated against 0.1 \text{ mol dm}^{-3} \text{ HCl}.\textsuperscript{1} Its molecular structure is show below in Figure 6.1.

![Figure 6.1. Structure of \text{BrNapAV}, MW = 451.32 g mol\textsuperscript{-1}.](image)

The electrochemical SPR cell was used, with the three electrode setup described in Chapter 2, Section 17.6.

SPR angle scans were taken in fresh buffer solution after each layer had been washed. 20 µA cm\textsuperscript{-2} was allowed to flow in the FmocLG solution for 100 s, and in the BrNapAV solution for 200 s. This was due to the BrNapAV hydrogels having a lower dielectric constant than FmocLG, and therefore required longer gelation times to register as a significant shift in the surface plasmon. The layers and corresponding times of applying current are shown in Table 6.1.

\textit{Table 6.1. Showing the dipeptides used for the layers and corresponding time of current applied to create a multi-layered hydrogel.}

<table>
<thead>
<tr>
<th>Layer no.</th>
<th>Solution/Gel</th>
<th>Time [seconds]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Precursor FmocLG</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>FmocLG</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>BrNapAV</td>
<td>200</td>
</tr>
<tr>
<td>3</td>
<td>FmocLG</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>BrNapAV</td>
<td>200</td>
</tr>
</tbody>
</table>
2.2 Controls

Two control experiments were performed. The first, shown in Figure 6.2, involved growing the first FmocLG layer with 100 s of current allowed to flow at the electrode. The excess solution was replaced with a ‘buffer’ solution of NaCl at pH 7.0. Further current was allowed to flow at this layer for 200 s, then 100 s, then another 200 s (shown by blue sections on the graph).

![Figure 6.2. Control experiment showing reflectivity in real time. Current was switched on for 100 s to create the first FmocLG layer. A blank buffer was inserted into the cell and current switched on for 200 s, followed by 100 s and 200 s. Blue portions show where the current was switched on.

Figure 6.2 shows no change in reflectivity with each application of current, suggesting the layer remained the same and did not become more dense or increase in thickness. From the angle scans (not shown) the critical and minimum angles were also unaffected with each further application of current.

In the second control experiment, the buffer solution was changed before each subsequent application of current, as in the real experiment.
Figure 6.3. Control experiment showing reflectivity in real time. Current was switched on for 100 s to create the first FmocLG layer. Buffer was used to wash the layer after each application of current. Current was switched on for a further 200 s, followed by 100 s, then 200 s.

Figure 6.3 shows the decreasing reflectivity with each washing step. This process slowly removed most of the original layer away from the surface. This gave a decrease in minimum angle with each angle scan (not shown).

2.3 Results and Discussion

Angle scans were taken before gelation and after each addition of gel layer, in buffer at pH 7. The kinetics were followed at 55.8°. The kinetic results are shown in Figure 6.4[d] with the SPR angle scans for the four-layered FmocLG / BrNapAV hydrogel in Figure 6.4[a-c]. Each layer was approximately 50 nm thick (see fitting in Figure 6.5[b]).
Figure 6.4[a] Critical angle for FmocLG solution and each layer added. [b] Minimum angle (data only); [c] SPR angle scans for FmocLG precursor solution and each layer of hydrogel after application of current, with data (open points) and fits (solid lines) shown.
Figure 6.4[d] Kinetics at 55.8°, showing the increase in reflectivity with each application of current.

It was possible to observe the change in critical and minimum angle with each layer of hydrogel formed at the surface. When the buffer solution was removed from the cell at the end of the experiment, the surface plasmon was unchanged from that of the 4th layer. This indicates the layered hydrogel was thicker than the detection limit of the surface plasmon, i.e. thicker than 200 nm. The critical and minimum angles are focused on in Figure 5.4[a-b]. An increase in the minimum and critical angles were observed with each additional layer of dipeptide. From the data in the control experiments, it was possible to correlate the increase in reflectivity with an increase in layer thickness. The exact changes in angle are plotted in Figure 6.5[a] below.
Figure 6.5[a] Change in critical angle and minimum angle from individual SPR scans for each layer. [b] Table of results from fitting experimental data.

Figure 6.5[a] shows the changes in both critical and minimum angles for each FmocLG and BrNapAV layer. As the minimum and critical angles shifted upon adding each layer, fits for the thicknesses and refractive indices of the layers given in Figure 6.5[b] are approximate. The value of 50 nm is based on the results from Chapter 4 which showed a current of 100 s gave a layer of FmocLG of 50 nm thick, in the linear region where it was possible to measure thickness of the film. The dielectric constant of the fourth layer was very high, due to the incompatibility of the fitting software with multi-layered materials. In reality the dielectric constants of each layer are likely to be very similar.

This technique has potential for creating a range of custom materials. In Chapter 4 it was shown that different electrochemical conditions lead to different hydrogel structures, and using this in the layered structures it would be possible to create materials with a range of properties throughout. Also, materials with more layers could be made, or with different molecules inserted into specific layers, and so on.

2.3.i Layers for photography

A two-layered hydrogel with dye was made for photography: On ITO a two-layered system was made for the purposes of photographing the gel. The first FmocLG layer contained a pink fluorescent dye (Rhodamine B). The second BrNapAV layer contained no dye. The current was applied for 30 minutes for each layer. The electrode was dipped in the FmocLG to 1 cm, and the BrNapAV layer to 2 cm depth.

By applying the current for longer times, it was possible to view individual layers by eye, as in Figure 6.6 below.
Figure 6.6[a] An FmocLG layer with Rhodamine B dye (pink) incorporated into hydrogel, front and side view. [b] BrNapAV gel layer grown on top of FmocLG layer, with original layer intact beneath.

Figure 6.6[a] shows the first FmocLG layer on an ITO electrode (working electrode, connected with conducting copper tape) and [b] the second BrNapAV layer. This shows pictorially the original layer (2 mm) remaining more or less intact after another layer was grown on top of it (4 mm total thickness). This could suggest the nano-layers grown for SPR remained intact as more layers were grown on top.

2.4 Conclusions

Multi-layered nano-gels have been successfully created by sequentially electrochemically growing thin hydrogel layers on top of one another. Alternate ~ 50 nm layers of FmocLG and BrNapAV were grown to build up a thick hydrogel. Control experiments showed no increase in density of an FmocLG layer with further application of current, so increases in reflectivity and minimum angles can be attributed to increases in thickness of each layer. The dielectric constants were measured with SPR to range from 1.79 (first layer) to 2.29 (fourth layer). An image of a two-layered gel approximately 4 mm thick was also provided, showing the first layer intact beneath the second.
3. SPONTANEOUS GROWTH OF THICK HYDROGEL FILMS ON SURFACES PRE-SEEDED WITH NANOMETRE THICK HYDROGEL LAYERS

3.1 Aim and Introduction

The spontaneous growth of hydrogel films in the presence of an electrochemically formed seeding layer was investigated. It was found that a very thin FmocLG layer (around 50 nm) would seed the formation of a thick hydrogel. The films were allowed to form over 48 hours in the absence of any significant pH drop, suggesting a templating mechanism of film growth.

A seeding layer (< 100 nm thick) was first grown at an electrode surface (gold SPR slide) electrochemically. The electrolyte was then rinsed from the cell and replaced by a $2.43 \times 10^{-3}$ mol dm$^{-3}$ FmocLG in $1 \times 10^{-2}$ mol dm$^{-3}$ NaCl solution at pH 7. Despite the fact that there were not enough protons remaining to bring the pH below the $pK_a$ to form the hydrogel by the usual mechanism, the hydrogel continued to grow and a thick fibrous film developed over 48 hours (see Figure 6.7). The spontaneous assembly of peptides on the surface of a seeding layer has important implications for understanding the self-assembly of bio-mimetic hydrogel films.

Figure 6.7. Schematic of the FmocLG hydrogel layer spontaneously gelating over 48 hours in the presence of a seeding layer at a gold electrode surface.

This has potential applications in the immobilisation of enzymes, as a thin layer of gel could be made in the absence of enzyme, which could be added in a solution of the dipeptide later, allowing the hydrogel to form slowly. Thus the enzyme is immobilised in the hydrogel whilst not being exposed to any extremes of pH.
3.2 Experimental

A current of 20 μA cm$^{-2}$ was applied to a pH 7 solution of FmocLG for 100 s. Gelation was measured kinetically with SPR. It was shown that introducing a thin layer (< 100 nm) of gel at the surface of the electrode and then leaving the hydrogel in FmocLG solution overnight led to the spontaneous growth of a 1 mm thick layer.

The seeding layer of gel was grown using an electrochemically induced pH drop shown in Chapter 4. 20 μA cm$^{-2}$ was allowed to flow for 100 s, creating a surface pH of ~ 3.65, which was sufficient to initiate gel formation in the presence of Fmoc-LG.

Following the formation of the seeding layer, which was monitored by SPR, the electrolyte containing hydroquinone and residual FmocLG was removed from the cell. The surface was rinsed with 98 mmol dm$^{-3}$ NaCl solution at pH 7 to remove any excess protons and then the cell was re-filled with a 2.4 x 10$^{-3}$ mol dm$^{-3}$ solution of FmocLG, also at pH 7. No further electrochemical experiments were carried out, rather the thin FmocLG layer was left in the growth solution for up to 48 hours while the film thickness was monitored by SPR.

3.3 Results and Discussion

3.3.i Control experiments

Two control experiments were performed. In the case where no seeding layer was grown, and the FmocLG solution was introduced to the gold surface, a gel layer did not form. In a second control experiment, all of the experimental steps (electrochemistry, rinsing and 48 hour monitoring) were performed in the absence of hydroquinone. This prevented the growth of the initial seeding layer and again no additional layer had formed after 48 hours.

3.3.ii Application of current to create a seeding layer for spontaneous gelation of FmocLG

The SPR angle and kinetic scans are shown for the spontaneous gelation of FmocLG in Figure 6.8 and Figure 6.9, respectively. The parameters obtained from fitting the data from each layer is shown in Table 6.2.
Growth of the first hydrogel layer can be seen by the shift in the minimum angle in Figure 6.8. The hydrogel layer grown over 48 hours can be seen by a further increase in minimum angle, suggesting a thicker layer was formed. The kinetic data is shown below in Figure 6.9, with the formation of both layers shown as increases in reflectivity over time.
Figure 6.9[a] Kinetic SPR data at 55.9° showing the formation of the initial FmocLG layer by applying 20 µA cm⁻² for 100 s. [b] Spontaneous formation of a thick FmocLG hydrogel over 48 hours.

The electrochemically grown film was not visible to the naked eye, but after 48 hours in the FmocLG solution a millimetre thick gel layer spontaneously formed on the electrode surface. Interestingly it can be seen that the film changed throughout the 48 hour period, clearly structure was still developing after two days in solution. Longer experiments have not yet been carried out to determine if all the FmocLG in the cell would gel given sufficient time. The pH of the hydrogel after 24 hours was 6.5, with the remaining NaCl solution at pH 7.0.

Table 6.2 gives parameters from fitting SPR curves for thickness and dielectric constants of both hydrogel layers, at 100 s and 48 hours.

Table 6.2. Parameters used for fitting SPR data showing refractive indices and thicknesses of the first electrochemically grown FmocLG layer and spontaneously formed hydrogel.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Thickness /nm</th>
<th>ε(real)</th>
<th>ε(imaginary)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prism</td>
<td>∞</td>
<td>3.4117</td>
<td>0</td>
</tr>
<tr>
<td>Gold</td>
<td>55.6</td>
<td>-12.3326</td>
<td>1.2257</td>
</tr>
<tr>
<td>Air</td>
<td>∞</td>
<td>1.0016</td>
<td>0</td>
</tr>
<tr>
<td>Precursor solution</td>
<td>∞</td>
<td>1.7816</td>
<td>0</td>
</tr>
<tr>
<td>Gel layer (100s 20µAcm⁻²)</td>
<td>80</td>
<td>1.789</td>
<td>0.002</td>
</tr>
<tr>
<td>Gel layer (48 h)</td>
<td>∞</td>
<td>1.817</td>
<td>0.004</td>
</tr>
</tbody>
</table>
The initial hydrogel layer was approximately 80 nm, with an $\varepsilon$(real) of 1.789, and $\varepsilon$(imaginary) of 0.002, compared with an $\varepsilon$(real) of 1.7816 for the precursor solution (change of 0.0074). The final layer was more than 200 nm (fitted as infinite thickness) with an $\varepsilon$(imaginary) of 0.004. This suggests the final layer was more scattering than the precursor layer, probably due to the formation if fibres and/or features on the length scale of the light.

3.3.iii Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy (TEM) was used to image both the initial seeding layer and the thick spontaneously grown layer, following the procedure in Chapter 2, Section 11.2. The seeding layer was grown directly onto a carbon coated nickel TEM grid. The thicker layer was growth by hanging the grid with the seeding layer in a beaker containing FmocLG at pH 7 for 48 hours.
Figure 6.10. TEM images of FmocLG hydrogels showing [a, b] 10 µA cm\(^2\) applied for 100 s (scale bars 50 and 20 nm) and [c, d] subsequent structure after undergoing spontaneous growth over 48 hours in an FmocLG solution (scale bars 50 and 20 nm). The difference in structure between the two gels is apparent; nucleation points present in [a] and [b] contrast to the extended fibrous network in [c] and [d].

Figure 6.10[a-b] shows a TEM image of the initial 80 nm thick layer. No fibres were present; rather the film seemed to consist of spherical aggregates only a few nanometres in diameter. In [c-d] the film after 48 hours in FmocLG solution showed a fibrous network. The fibres were microns long with diameters of tens of nanometres.

These TEM images concur with SPR data to show that the film developed considerably over 48 hours. Some spherical aggregates were still seen, but in general the film was dominated by fibres. This suggests that the structures formed by surface initiated gelation are similar to those formed by conventional methods, such as the addition of acid and electrochemical hydrogelation.
3.3.iv Introducing the horseradish peroxidise (HRP) enzyme into spontaneously grown hydrogels

The experiment was extended by adding the HRP enzyme to the hydrogel during the second growth phase. The experiment was performed in $1 \times 10^{-2}$ mol dm$^{-3}$ PBS buffer at pH 7, with $5.35 \times 10^{-8}$ mol dm$^{-3}$ HRP. The experiment was performed using the same procedure as in 3.2, with 20 µA cm$^{-2}$ allowed to flow for 100 s, to create a seeding layer (without HRP). This layer was then allowed to gel further in a solution of FmocLG (with HRP) over 48 hours. The SPR angle scan results and parameters obtained are shown below in Figure 6.11 and Table 6.3.

![Figure 6.11. SPR angle scans showing experimental data (open points) and fits (solid lines) for the precursor solution, the FmocLG layer created by application of 20 µA cm$^{-2}$ for 100 s, and the spontaneously grown hydrogel layer after 48 hours.](image)

Both hydrogel layers can be seen by an increase in minimum angle of the SPR curve in Figure 6.11. The first layer showed a small shift in minimum angle, with the spontaneously grown layer giving a more significant increase after 48 hours. There was also a shift in critical angle between the first layer and the spontaneously formed layer, suggesting an increase in dielectric constant on the bulk level. The first hydrogel layer formed in PBS was not as thick as in NaCl, as the buffer inhibited the pH drop and the layer of diffusion of protons which cause hydrogelation. The final layer was observed by eye to be approximately 0.5 mm thick.
Table 6.3. Results of parameters obtained from fitting SPR data showing refractive indices and thicknesses of the first electrochemically grown FmocLG layer and spontaneously formed hydrogel with HRP after 48 hours.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Thickness/nm</th>
<th>ε(real)</th>
<th>ε( imaginary)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prism</td>
<td>∞</td>
<td>3.4117</td>
<td>0</td>
</tr>
<tr>
<td>Gold</td>
<td>52.46</td>
<td>-11.3187</td>
<td>1.5279</td>
</tr>
<tr>
<td>Air</td>
<td>∞</td>
<td>1.0016</td>
<td>0</td>
</tr>
<tr>
<td>Precursor solution</td>
<td>∞</td>
<td>1.7844</td>
<td>0</td>
</tr>
<tr>
<td>Gel layer (100s 20μAcm²)</td>
<td>100</td>
<td>1.803</td>
<td>0.002</td>
</tr>
<tr>
<td>Gel layer (48 h)</td>
<td>∞</td>
<td>1.808</td>
<td>0.001</td>
</tr>
</tbody>
</table>

The results in Table 6.3 shows \( \varepsilon \) (real) = 1.803 for the first seeding layer and \( \varepsilon \) (imaginary) = 1.808 for the HRP layer. \( \varepsilon \) (real) for the final hydrogel layer was lower than that of the layer formed without HRP. This may be due to the large HRP molecules creating a denser layer onto the gold surface. There was a small amount of scattering in the layers, shown by \( \varepsilon \) (imaginary) = 0.002 and 0.001 for the first and second layers, respectively.

SPR curve fitting suggests that the initial layer was 70 – 80 nm thick. As the cell and the surface were then rinsed with NaCl solution at pH 7 the majority of the excess protons were likely to have been removed, thus the growth of the layer was purely formed by FmocLG and HRP molecules self-assembling at the seeding layer.

3.4 Conclusions
The spontaneous growth of a hydrogel layer on top of an 80 nm thick seeding layer has been proven in this section. The initial seeding layer was grown by electrochemically dropping the pH below 4, with the subsequent templated growth of the top layer occurring in pH 7 solution.

The subsequent growth of the surface film from 80 nm to 1 mm occurred at pH 7. The fact that the bulk pH was considerably above the pKₐ of the free amino acid suggests that protonation of the amino acid and subsequent hydrogen bond formation can occur when a molecule of FmocLG experiences the surface environment. This shows a new mechanism of hydrogel growth, at a pH above the pKa of the amino acid groups, via a templated method. Initially the growth could have been due to excess protons remaining in the 80 nm seeding layer, but it seems unlikely that this could account for the formation of 1 – 2 mm of gel in a pH 7 solution. More likely, the layer on the surface
acts to ‘template’ further growth, possibly by modifying the environment and hence the pKₐ of surface associated molecules.

The ability of a thin layer of FmocLG to template the further self-assembly of a complex fibrous hydrogel is significant as it suggests that gels can form above the pKₐ of the isolated amino acid if the right environment is provided.

4. IMMOBILISING THE HORSE RADISH PEROXIDASE (HRP) ENZYME INTO ELECTROCHEMICALLY FORMED DIPEPTIDE HYDROGELS

4.1 Aim and Introduction
The focus of this section is the use of the electrochemical gelation method to encapsulate the enzyme horseradish peroxidase (HRP) into an FmocLG hydrogel, and to prove the presence of the encapsulated enzyme by measuring its activity using UV-Vis spectroscopy. The hydrogels were measured using SPR and CD. HRP trapped in electrochemically and spontaneously formed hydrogels are compared for activity.

4.2 Results and Discussion
The SPR angle scans showing HRP in FmocLG hydrogel in PBS buffer are shown in Figure 6.12. The film was grown with 20 µA cm⁻² for 3 hours. The parameters obtained from the curves are shown in Table 6.4. The hydrogel formed was approximately 0.5 mm thick, and was clear and homogeneous.
Figure 6.12 shows the increase in minimum and critical angles in the presence of the hydrogel layer with HRP. The reflectivity of the minimum angle also increased, suggesting an increase in the scattering produced by the interaction of the plasmon with the hydrogel.

Table 6.4. Parameters obtained from fitting experimental data in Figure 6.12.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Thickness/nm</th>
<th>$\varepsilon$(real)</th>
<th>$\varepsilon$(imaginary)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prism</td>
<td>$\infty$</td>
<td>3.4117</td>
<td>0</td>
</tr>
<tr>
<td>Gold</td>
<td>51.73</td>
<td>-11.6165</td>
<td>1.4045</td>
</tr>
<tr>
<td>Air</td>
<td>$\infty$</td>
<td>1.0016</td>
<td>0</td>
</tr>
<tr>
<td>Precursor solution</td>
<td>$\infty$</td>
<td>1.786</td>
<td>0</td>
</tr>
<tr>
<td>Gel with HRP</td>
<td>$\infty$</td>
<td>1.803</td>
<td>0.007</td>
</tr>
</tbody>
</table>

The hydrogel layer with HRP in PBS solution had a higher dielectric constant $\varepsilon$(real) than hydrogels formed in NaCl. This may be because the buffer prevented the hydrogen ions from diffusing as far away from the surface, as they were quickly buffered. The hydrogel formed was much thinner than in NaCl. The hydrogel with HRP also had a higher scattering coefficient $\varepsilon$(imaginary), suggesting the layer was less homogenous, due to the presence of the enzyme which is bulky and may have disrupted the structure.
In order to assess the effect of including the HRP enzyme into FmocLG hydrogels, a CD experiment was performed using HRP immobilised inside FmocFF, as a comparison. Three samples were measured, first a 6.94 x 10^{-3} \text{ mol dm}^{-3} \text{ FmocFF} hydrogel, second a solution of HRP at 0.01 mg cm^{-3}, and lastly a combination of HRP and FmocFF at these concentrations, all in 0.1 mol dm^{-3} \text{ NaCl}. The results are shown in Figure 6.13.

The hydrogel with HRP immobilised gave a similar spectrum to the control FmocFF sample, with the spectrum shifted to more positive values, and slightly to the left. This suggests the HRP had some influence in increasing the order of the structure. The negative peak in the FmocFF spectrum at -212 nm shifted to become two peaks in the HRP hydrogel. This showed that on inclusion of HRP into FmocFF hydrogels, an increase in \( \alpha \)- and \( \beta \)- structures are formed.

4.2.i Activity of the HRP enzyme inside an electrochemically grown FmocLG hydrogel

The activity of the encapsulated HRP was carried out as described in Chapter 2, Section 18.4. A calibration curve was first carried out in the UV-Vis cell setup. The final
concentration of the product of the enzyme catalysed reaction with OPD was DAP, which absorbs light at 450 nm. The reaction was monitored by measuring the absorbance peak after 10 min, taken as the average of three data sets. The concentration of OPD added to the immobilised HRP was $7.4 \times 10^{-5}$ mol dm$^{-3}$ (highest concentration on calibration graph). The calibrations curve and results from assay with immobilised HRP in an FmocLG hydrogels are shown in Figure 6.14[a] and [b].

Figure 6.14[a] Calibration curve in buffer solution showing the linear relationship between the concentration of OPD added and the absorbance of the product DAP at 450 nm. Molar extinction coefficient at 450 nm $\epsilon = 11852.9$ mol$^{-1}$ dm$^3$ cm$^{-1}$. [b] UV-Vis kinetics measurement showing production of DAP with time in an FmocLG hydrogel layer with HRP immobilised inside. Taken at 450 nm over 100 min.

Figure 6.14[b] shows the increase in absorbance measured with time, upon addition of OPD and H$_2$O$_2$ to the cell. The molar extinction coefficient $\epsilon$ was obtained and used to find the activity of the HRP enzyme immobilised within the FmocLG hydrogel (growth conditions: 20 $\mu$A cm$^{-2}$ for 3 hours). The reaction and production of DAP was much slower than in solution, caused by the slow diffusion through the hydrogel layer. It is not known how much HRP was encapsulated within the hydrogel layer, but it is assumed that the concentration remained the same as in the initial solution. The results show the maximum absorbance reached around 0.9, which corresponds to a concentration of OPD in solution of $7.0 \times 10^{-5}$ mol dm$^{-3}$, from the calibration graph. The initial concentration of OPD added to the reaction was $7.4 \times 10^{-5}$ mol dm$^{-3}$. Using these two values to obtain a percentage gives the activity of the HRP enzyme to be approximately 93% (at the completion of the reaction after 100 min). This is very similar to in solution, and no oxidation of the product DAP was observed throughout the reaction.
4.2.ii Measuring activity of HRP in a spontaneously formed FmocLG hydrogel

This experiment was used to determine the activity of HRP inside a spontaneously formed FmocLG hydrogel (see Section 3). A thin seeding layer was made by applying 20 µA cm$^{-2}$ current density to a HQ, NaCl and FmocLG solution, for 200 s (details in Chapter 2, Section 2). A thick hydrogel layer containing HRP was allowed to form over 48 hours.

The first layer contained HQ, NaCl and FmocLG. The “buffer solution” used to measure each angle scan was comprised of NaCl and FmocLG. The seeding layer was allowed to form in NaCl and FmocLG rather than PBS as this formed a thicker layer. The fits for each layer are shown in Table 6.5.

Table 6.5. Results from fitting SPR angle scans showing parameters for gel layers.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Thickness / nm</th>
<th>$\varepsilon$(real)</th>
<th>$\varepsilon$(imaginary)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prism</td>
<td>$\infty$</td>
<td>3.4117</td>
<td>0</td>
</tr>
<tr>
<td>Gold</td>
<td>34.61</td>
<td>-11.2191</td>
<td>1.4548</td>
</tr>
<tr>
<td>Air</td>
<td>$\infty$</td>
<td>1.0016</td>
<td>0</td>
</tr>
<tr>
<td>Precursor solution</td>
<td>$\infty$</td>
<td>1.797</td>
<td>0</td>
</tr>
<tr>
<td>First FmocLG gel layer</td>
<td>50</td>
<td>1.806</td>
<td>0</td>
</tr>
<tr>
<td>Gel with HRP after 48 hrs</td>
<td>$\infty$</td>
<td>1.818</td>
<td>0</td>
</tr>
</tbody>
</table>
The first seeding layer was approximately 50 nm thick. The final layer after 48 hours was in excess of 200 nm, and by eye appeared to be approximately 1 mm thick. $\varepsilon(\text{real})$ was measured to be 1.818, with no imaginary coefficient. This suggests a hydrogel with low scattering.

$7.4 \times 10^{-5}$ mol dm$^{-3}$ OPD and H$_2$O$_2$ were introduced to the cell in PBS buffer to measure the activity of the HRP immobilised inside the spontaneously grown hydrogel. As before, the production of DAP was measured at 450 nm. Figure 6.16 shows the UV-Vis kinetics assay, with the kinetic data in [a] and the final spectrum at 100 min in [b]. The cell was sealed to reduce the possibility of the product oxidising to form side products.

![Figure 6.16(a) Kinetic graph showing evolution of the product DAP at 450 nm over time. [b] Absorbance spectrum after 100 min.](image)

The reaction was completed after 1 hour. The total absorbance at 450 nm reached 0.45, which corresponds to $3.5 \times 10^{-5}$ mol dm$^{-3}$ OPD used. From the calibration graph, the absorbance for this concentration of OPD used was 0.9. Here the activity of HRP in the hydrogel was approximately 60%.

The combined results, with the control experiment (no HRP present) are shown in Figure 6.17.
Figure 6.17. Combined results from three FmocLG hydrogels: spontaneously formed FmocLG hydrogel with HRP (blue); electrochemically formed FmocLG hydrogel with HRP (green); control FmocLG hydrogel with no HRP (purple), showing the evolution of DAP over time at 450 nm upon the addition of OPD and \( \text{H}_2\text{O}_2 \), at 18 min.

The results in Figure 6.17 show the activity of the electrochemically formed FmocLG hydrogel with HRP had a higher activity than the spontaneously formed gel. This may have been due to the enzyme becoming denatured during the 48 hours whilst it was in solution, rather than inside the protected environment of a hydrogel. However, as this data came from one experiment for each hydrogel, it is not necessarily possible to draw definitive conclusions to determine which HRP-containing hydrogel gave the best activity. Also, the hydrogels were of different structures and densities, which may have allowed the reactants to move through the gels differently. Further work is required to determine the consistency of these results and to provide more consistent data throughout.

4.3 Conclusions and Future Work
The HRP enzyme was successfully encapsulated into a FmocLG hydrogel at a concentration of \( 5.35 \times 10^{-8} \text{ mol dm}^{-3} \) in PBS buffer. The activity of the enzyme after encapsulation was shown to be retained in the peroxide assay, with both electrochemically and spontaneously formed hydrogels.

The activity of HRP was shown qualitatively here to introduce the concept of immobilisation within electrochemical hydrogels. It was encouraging to find very little
loss of activity in this case for the enzyme, despite being exposed to low pH conditions during the 3 hours of gelation. Conditions for measuring stability and lifetime of enzymes should be carried out under strict biological conditions, in order to accurately compare activities of gelled enzymes compared to free enzymes in solution. The spontaneously formed hydrogel could be compared with this technique. Differences in hydrogel thicknesses could be investigated to find the optimal thickness for diffusion and reaction.

6. ACKNOWLEDGEMENTS

Ursula Potter and John Mitchels at the University of Bath Imaging Suite for TEM images.

7. REFERENCES

CHAPTER SEVEN:
EFFECT OF ADDITIVES ON FMOCFF HYDROGELS

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1. AIM AND INTRODUCTION

The structure and gelation of FmocFF hydrogels has been shown in Chapter 3 to be affected by a wide range of additives. The aim in this section is to investigate the influence of the single amino acids D-Phe and L-Phe and other additives on the hydrogelation process and final hydrogel strength. The effects are measured using a wide range of characterisation techniques, including fluorescence spectroscopy, NMR, TEM, FTIR, DOSY, pH measurements, CD, and Fibre-XRD.

2. RESULTS AND DISCUSSION

2.1 Effect of -Phe on FmocFF hydrogels: Fluorescence measurements

The effect of different enantiomers of amino acids on FmocFF hydrogels was tested. The work here focused on the addition of the D- and L- forms of phenylalanine, D-Phe and L-Phe to the precursor hydrogel solution (Figure 7.1).

![Figure 7.1(a) Structures of D-Phe and (b) L-Phe.]

These enantiomers were added at a range of concentrations, and hydrogel strength and gelation times were measured.

2.1.i Fluorescence measurements: Sample preparation

FmocFF samples for fluorescence were made as described in Chapter 2, Section 8.3, with D / L-Phe at a range of concentrations (up to 0.12 mol dm\(^{-3}\)), and 5 \(\times\) 10\(^{-3}\) mol dm\(^{-3}\) ThT. All samples were made in 1 \(\times\) 10\(^{-2}\) mol dm\(^{-3}\) in HEPES buffer at pH 6.0 to ensure as far as possible the pH was constant and not having an effect on the hydrogels. Each data point represents the average of at least three samples measured. The gradient of each trace was taken and plotted against concentration of D- / L-Phe.
2.1.ii Monitoring the effect of D- / L-Phe on FmocFF gelation kinetics

D/L-Phe was introduced to the precursor solution prior to gelation with FmocFF, at a range of concentrations. Kinetic fluorescence measurements were taken using the fluorescence response of ThT for each hydrogel formed. Figure 7.2 shows the results of these experiments. The highest concentration of D- / L-Phe added is shown in the example kinetics in [a].

![Kinetic ThT fluorescence trace at 494.5 nm, showing hydrogels with 3.47 x 10^{-3} mol dm^{-3} FmocFF control (blue), with L-Phe added (pink) and D-Phe (green). Data points represent average of at least three data sets.

[b] Effect of D- / L-Phe on hydrogelation gradient observed is plotted versus concentration.}

Figure 7.2[a] Kinetic ThT fluorescence trace at 494.5 nm, showing hydrogels with 3.47 x 10^{-3} mol dm^{-3} FmocFF control (blue), with L-Phe added (pink) and D-Phe (green). Data points represent average of at least three data sets. [b] Effect of D- / L-Phe on hydrogelation gradient observed is plotted versus concentration.
It was possible to observe a difference in the gelation kinetics, gelation time, and larger fluorescence increases between FmocFF hydrogels with D-Phe and L-Phe added. Both gave stronger gels and formed more quickly than the control experiment. The presence of D- or L-Phe may have been templating the self-assembly of FmocFF, as it forms micelles in solution, and could provide additional π-π stacking for the dipeptide to form structure around.

2.1.iii Fluorescence measurements: Varying ratio of D- : L-Phe, at constant concentration

To investigate further the differences between adding D- and L-Phe on FmocFF hydrogels, an experiment with $3.47 \times 10^{-3} \text{ mol dm}^{-3}$ FmocFF was performed at a constant overall concentration of D- and L-Phe, varying the ratio of the two. This was to ensure there was no effect from increased concentration of D- / L-Phe. $5.75 \times 10^{-2} \text{ mol dm}^{-3}$ D- / L–Phe was used in a $1 \times 10^{-3} \text{ mol dm}^{-3}$ HEPES buffer solution. All experiments had a starting pH of 6.00. The results are shown in Figure 7.3, with each point representing the average of at least three experiments, plotting the gradients of the ThT trace versus concentration of D-Phe.

![Figure 7.3](image)

Figure 7.3. Graph showing constant total concentration of $3.47 \times 10^{-3} \text{ mol dm}^{-3}$ FmocFF hydrogels with ratios of D-Phe : L-Phe at a total concentration of $5.75 \times 10^{-2} \text{ mol dm}^{-3}$ vs. gradient of ThT trace at 505 nm.

Figure 7.3 shows the effect of increasing the ratio of D-Phe on the gradient of gelation of FmocFF hydrogels. Initially, increasing D-Phe created an increase in the gelation
gradient, after which the hydrogels were relatively unaffected (from 0.02 – 0.04 mol dm$^{-3}$ D-Phe). Then with an increasing ratio of D-Phe the gelation gradient lowered steadily. This shows that the strongest hydrogels were formed at 0.005 – 0.01 mol dm$^{-3}$ D-Phe, between 10 – 20 % D-Phe. It also showed clear differences in hydrogelation between D- and L-Phe.

2.1.iv Fluorescence measurements: Effect of D- / L-Phe on FmocFF at pH 4.8

Figure 7.4 shows the results from using a sodium acetate buffer at pH 4.8 with D / L-Phe added to the precursor solution. The effect of adding -Phe at long times (Figure 7.4[a]), and effect of a range of concentrations at short times [b] was measured.

![Graph](image)

*Figure 7.4[a] ThT response over 18 hours for FmocFF hydrogel control, and with 5.75 x 10$^{-3}$ mol dm$^{-3}$ D / L-Phe added. [b] FmocFF hydrogels formed at pH 4.82 in sodium acetate buffer with D*
Figure 7.4[a] shows the ThT response over 18 hours, showing the initial gelation of FmocFF and restructuring of the hydrogels over long times. The hydrogel with D-Phe had the lowest final ThT response, suggesting this was the hydrogel with the least structure present, although it appeared to gelate more strongly initially. The control FmocFF hydrogel gave the highest final response, showing more fibrous structure, suggesting a stronger or denser network of fibres than with D / L-Phe added. This also suggests a more stable hydrogel over time. Figure 7.4[b] shows the effect of changing concentration of D / L-Phe in sodium acetate buffer on FmocFF gels. Overall under these conditions, the more D / L-Phe added, the stronger the ThT response, and L-Phe hydrogels gave more structure.

2.1.v NMR measurements: Determining position of D- / L-Phe within FmocFF hydrogels

The results of this NMR experiment (described in detail in Chapter 2, Section 14.2) confirmed that D / L-Phe was present in the pores, rather than involved in the fibres, as 99 % D / L -Phe was present in solution at the 0.005 mol dm$^{-3}$ concentration, and 97 % at the higher concentration of 0.05 mol dm$^{-3}$. This suggests D / L-Phe affected the hydrogel structure from solution and remained in the pores after hydrogelation.

This is significant because it suggest the micelles of D- / L-Phe in solution interact differently with the FmocFF. It also begs the question as to which molecules might be incorporated into the hydrogels, and may require computer modelling to design molecules which would become part of the α- and β- structure.

2.1.vi Transmission Electron Microscopy (TEM): FmocFF hydrogels with D / L-Phe

Hydrogels were prepared for TEM as described in Chapter 2, Section 11.2. Images collected are shown in Figure 7.5 to Figure 7.7. 3.47 x 10$^{-3}$ mol dm$^{-3}$ FmocFF hydrogels were allowed to form in MilliQ water, adding 5.75 x 10$^{-2}$ mol dm$^{-3}$ D-Phe and L-Phe, on copper grids (400 mesh). The hydrogels were stained with uranyl acetate.
Figure 7.5[a and b]. TEM images of $3.47 \times 10^{-3}$ mol dm$^{-3}$ FmocFF gels. Scale bars are 100 and 50 nm.

Figure 7.6[a-b]. TEM images of $3.47 \times 10^{-3}$ mol dm$^{-3}$ FmocFF gels with $5.75 \times 10^{-2}$ mol dm$^{-3}$ D-Phe added. Scale bars are 100 and 50 nm.
Figure 7.7[a-b]. TEM images of $3.47 \times 10^{-3}$ mol dm$^{-3}$ FmocFF gels with $5.75 \times 10^{-2}$ mol dm$^{-3}$ L-Phe added. Scale bars are 100 and 50 nm.

TEM of all the hydrogels showed no significant differences in macro-structure, suggesting the fibres were unaffected by adding D-Phe or L-Phe.

2.1.vii Fourier Transform Infra-Red (FTIR) Spectroscopy: FmocFF hydrogels with D-/L-Phe

FTIR was used to measure FmocFF hydrogels with D-/L-Phe added, to test for any measureable difference in the $\alpha$- and $\beta$- structures which would occur in the amide peaks at 1650 and 1688 cm$^{-1}$. The procedure used is described in Chapter 2, Section 6.2. The results are shown in Figure 7.8.
Figure 7.8. [a] FTIR of $3.47 \times 10^{-3}$ FmocFF hydrogels in water (black), with D-Phe added (red), L-Phe (green) and rac-Phe (blue), showing peaks at 1650 and 1688 cm$^{-1}$. These refer to anti-beta sheet structure in the dipeptide hydrogel. [b] Plot of both peak positions for each hydrogel in [a].

This result showed no differences in the peak positions between the control hydrogel and those with D- / L-Phe added. The differences in energy in Figure 7.8[a] were due to slight differences in pressure applied to the samples. This suggests the hydrogel structures were again similar in terms of β-sheet structure.

2.1.viii Diffusion Ordered NMR (DOSY): FmocFF hydrogels with D- / L-Phe

Samples and measurements for DOSY NMR experiments were carried out as described in Chapter 2, Section 5.2. Results of DOSY measurements with D-Phe and L-Phe added to FmocFF hydrogels are shown in Figure 7.9.
As more D- or L-Phe was added to the precursor solution, the diffusion coefficient of water within the hydrogel decreased. An increasing concentration of D / L-Phe gave lower diffusion coefficients, suggesting a more open hydrogel structure. Hydrogels with D-Phe in the system showed consistently higher diffusion coefficients than L-Phe gels, suggesting they had formed denser extended network structures.

2.1.ix Effect of D- / L-Phe on pH changes during FmocFF hydrogelation

pH studies were carried out as follows: An FC200 pH probe with a 6 mm x 10 mm conical tip (Hanna Instruments) suitable for measuring hydrogels was connected to a data logger and clamped into $1 \times 10^{-2}$ mol dm$^{-3}$ pH 6.0 HEPES solution in MilliQ water, or with D- / L-Phe added (sonicated for 1 hour to dissolve). The output voltage was calibrated with a range of known pH solutions.

The starting pH of all the precursor solutions was 6.0. A steady baseline was recorded before the pH meter was removed to add 40 µL of a 100 mg mL$^{-1}$ FmocFF stock solution in DMSO, which was quickly stirred, and the probe replaced to measure the pH drop. Measurements were taken with a concentration of $5.75 \times 10^{-2}$ mol dm$^{-3}$ D-Phe or L-Phe. This made a 2 cm$^3$ volume of hydrogel with a total concentration of FmocFF of $3.47 \times 10^{-3}$ mol dm$^{-3}$. Samples were made in plastic cuvettes. Results are shown in Figure 7.10.
Figure 7.10. pH data log following the kinetics FmocFF of hydrogelation in real time, starting at pH 6.0. The pH is shown on the right axis. FmocFF at $3.47 \times 10^{-3}$ mol dm$^{-3}$ (2 mg mL$^{-1}$) (blue), D-Phe (black) and L-Phe (red) at $5.75 \times 10^{-2}$ mol dm$^{-3}$ (9.5 mg mL$^{-1}$). Data is averaged from at least three data sets, with error bars shown.

This data shows an initial sharp decrease in pH upon addition of FmocFF. Interestingly, those gels with D / L-Phe added to the precursor buffer solution did not reach as low an initial pH as the control hydrogel. There were two distinct sections observed in the pH drop: a constant pH during the cloudy phase, and then a stable pH when the hydrogel had formed in the clear ‘set’ phase. The control hydrogel began to clear at similar times than D- / L-Phe, but took longer to form and to stabilise. D-Phe and L-Phe hydrogels reached a similar final pH value, with the D-Phe hydrogels reaching a very slightly but consistently higher pH than those with L-Phe.

2.1.x Rheology: Effect of D / L-Phe on FmocFF hydrogel strength

Sample preparation for rheology measurements is described in Chapter 2, Section 9.2. Rheological time sweeps were taken over 10 hours and frequency sweeps at a range of D / L-Phe concentrations (each point representing an average of at least three data sets). Figure 7.11[a] used the 9.5 mg mL$^{-1}$ (0.0575 mol dm$^{-3}$) concentration of D-Phe and L-Phe to measure the samples over 10 hours in Milli Q water. Figure 7.11[b] shows the effect of concentration of D-Phe and L-Phe on FmocFF hydrogels after 24 hours.
Figure 7.11[a] 10 hour frequency sweep showing $3.47 \times 10^{-3}$ mol dm$^{-3}$ FmocFF (black), D-Phe (green) and L-Phe (blue) added showing complex viscosity $G'$ (left axis) and shear stress $G''$ (right axis). [b] Plot of concentration of D / L-Phe versus $G'$ at 0.1 % strain, showing FmocFF (red star), D-Phe (green circles) and L-Phe (orange squares).

Figure 7.11[a] and [b] both show the FmocFF hydrogels with L-Phe added were much weaker than those with D-Phe, with time and frequency sweeps. The reproducibility of the $G'$ values obtained for the D-Phe hydrogels was generally lower than for the L-Phe hydrogels, but overall gave higher values for $G'$ over the range of concentrations. The D-Phe hydrogel was changing over long times, becoming more robust with time. L-Phe hydrogels were less strong than the control, which suggested the addition of L-Phe disrupted the structure and did not create as strong links throughout the hydrogel.

2.1.xi Circular Dichroism: Effect of D- / L-Phe on FmocFF hydrogels

Samples were prepared for CD measurements as outlined in Chapter 2, Section 4.2. A $3.47 \times 10^{-3}$ mol dm$^{-3}$ (2 mg mL$^{-1}$) FmocFF hydrogel was measured as the control, and
samples with $5.75 \times 10^{-2}$ mol dm$^{-3}$ D-Phe and L-Phe added to the precursor solution were measured. Scans were taken at intervals between 1 hour and 17 hours. Figure 7.13 shows the CD spectra of D-Phe and L-Phe dissolved in MilliQ water, which were subtracted from the FmocFF hydrogels with D- / L-Phe added, respectively. This graph shows clearly the mirroring between the two spectra, due to the opposite chirality of D-Phe and L-Phe.

Figure 7.12. CD spectra showing D-Phe (green) and L-Phe (red) at 0.05751 mol dm$^{-3}$ in MilliQ water.

LD spectra of the hydrogels were taken by rotating the sample 90°. This was to ensure no artificial alignment was introduced into the samples from the sample cell holder. Figure 7.13 shows the CD spectra and LD spectra of FmocFF with D-Phe and L-Phe incorporated, at 1 hour.
Figure 7.13. [a] CD and [b] spectra of FmocFF hydrogels (blue) with D-Phe (green) and L-Phe (red) added, after 1 hour.

The CD and LD scans appeared to be very similar for the samples with D-Phe and L-Phe at 1 hour, which suggests no artificial alignment was present in the samples. The FmocFF sample showed an extra peak in the LD spectrum, at -198 nm. Figure 7.13 shows peaks at -222 nm, showing $n \rightarrow \pi^*$ transitions, and corresponds to $\beta$-sheet structure. It is interesting that the FmocFF spectrum appears to dominate the spectra, as the sign and chirality of the signals are negative, even in the case of FmocFF with L-Phe which gave a positive signal in water. It is worth noting that the intensity of the peak does not necessarily represent a stronger chirality, as this can be
affected by the preparation of the sample and a possible difference in concentration at the area of the sample which is measured.

At 17 hours (Figure 7.14) there was an additional peak present in the CD and LD scans for D-Phe and L-Phe hydrogels, at -218 nm. These results suggest that overall the D-Phe and L-Phe do not have a direct effect on the hydrogel structure at 1 hour, but by 17 hours there were distinguishable differences between the samples. This suggests the hydrogels are affected differently with the additives at longer times.

Figure 7.14 [a] CD and [b] LD spectra of FmocFF hydrogels (blue) with D-Phe (green) and L-Phe (red) added, after 17 hours.
2.1.xii Fibre X-Ray Diffraction (Fibre-XRD): Effect of D- / L-Phe on FmocFF hydrogels

Samples for Fibre-XRD were measured at the University of Sussex. Details of sample preparation and measurements are given in Chapter 2, Section 7.2. FmocFF hydrogels with D-Phe and L-Phe were measured, with the results shown in Figure 7.15[a-c] and Table 7.1.

![Fibre XRD images in high contrast showing (a) FmocFF (b) FmocFF with D-Phe (c) FmocFF with L-Phe.](image)

**Table 7.1.** Fibre XRD results showing meridional, equatorial and off-meridional signal for FmocFF, FmocFF with D-Phe and L-Phe.

<table>
<thead>
<tr>
<th></th>
<th>FmocFF</th>
<th>FmocFF + D-Phe</th>
<th>FmocFF + L-Phe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meridonal / Å</td>
<td>4.80</td>
<td>4.84</td>
<td>4.70</td>
</tr>
<tr>
<td>Equatorial / Å</td>
<td>12.80</td>
<td>12.80</td>
<td>11.40</td>
</tr>
<tr>
<td></td>
<td>10.30</td>
<td>10.30</td>
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</tr>
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<td></td>
<td>6.43</td>
<td>6.57</td>
<td>6.46</td>
</tr>
<tr>
<td></td>
<td>4.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Off-meridonal / Å</td>
<td>4.13</td>
<td>4.07</td>
<td>-</td>
</tr>
</tbody>
</table>

Each hydrogel gave similar results with values consistent with repetitive spacings of 4.7-8 Å along the fibre axis and ~11-12 Å perpendicular to the fibre axis. These values are very similar to the cross-β patterns seen from amyloid systems. This result corresponds to that of Smith et al² where measurements for FmocFF samples gave a value of 4.3 Å for the spacing between β-strands, and 7.6 Å packing between fluorenyl groups. When compared with the TEM results these show the fibre structure was unaffected by the addition of D- and L-Phe on the level of fine structure on average.
2.2 Fluorescence measurements: Effect of Z-FF on FmocFF hydrogels

Z-diphenylalanine, Z-FF (Bachem, $M_r = 446.5$ g mol$^{-1}$, ) is known to form nanostructures in water (see Figure 7.16).

![Figure 7.16. Z-diphenylalanine, Z-FF.](image)

To measure the effect on hydrogelation of FmocFF with addition of Phe-Phe, a range of concentrations (up to $5 \times 10^{-3}$ mol dm$^{-3}$) were added to MilliQ water prior to the addition of FmocFF. In order to dissolve the Phe-Phe fully the pH of the stock solution was raised to 11 with 1 mol dm$^{-3}$ NaOH. The pH was lowered to 7.0 with addition of 1 mol dm$^{-3}$ HCl just prior to the addition of $3.74 \times 10^{-3}$ mol dm$^{-3}$ FmocFF (from stock solution 100 mg cm$^{-3}$). All points represent the average of at least 3 sets of data. Kinetic scans and ThT gradients were measured (Figure 7.17).
Figure 7.17[a] Selected examples of the kinetic ThT response to FmocFF hydrogelation at 494.5 nm, showing the effect of adding a range of concentrations of Z-FF to the precursor solution. Experiments were carried out in MilliQ water at pH 7. Data is the average of three sets, with error bars added. Gradients of ThT dye series (counts s\(^{-1}\)) are given as m. [b] Plot of ThT gradient (counts s\(^{-2}\)) versus concentration of Z-FF, with linear fit added.

In Figure 7.17[a], the ThT response to several selected Z-FF concentrations are shown. As more Z-FF was added, the ThT gradient increased from around 1 to 12.5 counts s\(^{-2}\), the gelation time became shorter (350 to 100 s), and also the final response of ThT was higher (from around 200 to 375 counts s\(^{-1}\)). This suggests the Z-FF was making the FmocFF organise more quickly, and with stronger interactions, producing stronger hydrogels.
Figure 7.17[b] shows a linear relationship between the gradient (counts s\(^{-1}\)) and concentration of Z-FF. The effect was more marked than with the addition of D / L-Phe, most likely due to the drive for Z-FF self-assemble in pH 7 water.

3. CONCLUSIONS AND OUTLOOK

This Chapter has shown quantitatively the measurable differences between adding D-Phe and L-Phe to FmocFF hydrogels. Data acquired from DOSY, rheology, fluorescence and pH measurements suggest that hydrogels with D-Phe added to the precursor solution gave a denser structure, and formed more quickly than those with L-Phe present. Techniques which measured fibre structure (TEM, Fibre-XRD, CD, and FTIR) showed no difference in the fibrous structure of the hydrogels with D- / L-Phe added. This suggests there was no effect on the structure of the actual fibres of the hydrogels. The addition of D- / L-Phe gave an improvement on gelation time and strength compared with the FmocFF control. Finally, the addition of Z-FF improved gelation time and final ThT response.

There are clearly interesting effects occurring with the addition of single amino acids to the precursor hydrogel solution. That there is a clear difference between the D- and L-forms of -Phe suggests the molecules may template gelation differently, whilst not affecting the fibres but working on other levels within the hydrogels.

4. ACKNOWLEDGEMENTS

At the University of Sussex, Louise Serpell, Tom Williams, Kyle Morris for assistance with TEM, Fibre-XRD and CD data collection and interpretation in this chapter.

5. REFERENCES

CHAPTER EIGHT:
FUTURE WORK AND OUTLOOK

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1. FUTURE WORK

There are a wide range of potential avenues which have been opened in this work which may be subject to further study. There is potential for this work to be used in a range of applications which were discussed extensively in Chapter 1. This work has provided exciting and insightful information about the structures of Fmoc-protected dipeptide hydrogels, how they form and their properties. It has opened up the potential for discovering the effect of additives of over hydrogellation kinetics. This section will introduce some preliminary work undertaken during the course of this study and suggest avenues for further research.
2. AREAS FOR FURTHER RESEARCH

2.1 Furthering work on characterisation of gelation mechanisms

There are many techniques used in the literature to gain a greater understanding into the gelation mechanisms of peptides at the molecular level, and computer modeling has a major role in this study.\(^1,2\) Practically, a wide body of data can be accumulated using commercially available hydrogelators and high throughput experiments. Utilising different salt solutions and additives has been shown throughout this work to affect hydrogelation in a number of ways. Combining this information with computer modeling could provide more accurate methods for predicting potential gelators and achieving specific properties of hydrogels.

The characterisation used in this work could be expanded by utilising Quartz Crystal Microscopy as a technique for the characterisation of electrochemically formed gels, as they can be grown directly onto the gold crystal. This has recently been tested by Ulijn et al.\(^3\) In general, a move away from FmocFF would provide more widely applicable data, as it does not behave like most other dipeptide gelators, due to its high pK\(_a\). Fluorescence data would be more accurate, as pH- or temperature-triggered peptides do not pass through a cloudy phase during the gelation process and the ThT peak would be more prominent throughout.

As mentioned in Chapter 3, Section 1.5 (Figure 3.6), it was noticed that when FmocFF was added to high pH sodium acetate buffer (pH 5.6), a gel formed which shrank to approximately half its original size after 20 minutes. This was not a reversible process, but there is the potential for further environment-responsive materials, and by characterising the driving forces behind the process more understanding would be reached.

Surface Plasmon Resonance (SPR) has proven to be a useful and successful tool for characterising very thin films of dipeptide hydrogels, and Long and Short Range SPR can be utilised to greater effect with nanometre films.

2.2 Addition of amino acids to dipeptide hydrogels

Chapter 7 presented an interesting effect on FmocFF hydrogels with the addition of enantiomers of Phe. A range of other D- and L- forms of other amino acids have been tested with fluorescence measurements and rheology. The preliminary results are shown in Figure 8.1.
Figure 8.1. [a] Fluorescence measurements showing the gradient of the ThT dye trace for a range of D- and L- amino acids, plotted against hydrophobicity. [b] $G'$ values from rheological measurements on FmocFF hydrogels with D- and L- amino acids added.

Figure 8.1[a] is a plot of ThT gradient at 494.5 nm, as described in Chapter 2, Section 8.3, and is plotted versus hydrophobicity of the amino acid. There did not seem to be a particular trend in [a] or [b] (the data was also plotted against the final pH of the hydrogels which did not show a correlation either). However, there were some interesting differences in gelation time and strength with the addition of particular amino acids. The work should be continued using a wider range of self-assembling dipeptides to fully understand how the amino acids or other additives affect gelation.
2.4 Effect of mixing FmocFF with alpha helical proteins

FmocFF hydrogels have been characterised using CD. A study was carried out to investigate the effect of encapsulating an α-helical protein, Sbi₄ (2 mg mL⁻¹) into an FmocFF hydrogel (5 mg mL⁻¹). It was thought the inherent chirality in the hydrogel may alter the helicity of the protein upon gelation. The controls and hydrogels were formed in 0.1 mol dm⁻³ NaCl, and measured using CD.

![Figure 8.2. CD spectrum showing traces for an alpha-helical protein, Sbi₄, incorporated into an FmocFF hydrogel. Showing CD scan for Sbi₄, (blue) 5 mg mL⁻¹ FmocFF (green), and the hydrogel mixture (red).](image)

Here in Figure 8.2, the peak at -210 nm had shifted and diminished significantly from the FmocFF sample to the mixture, as well as at +260 nm. This suggests that the presence of the protein affected the structure of the hydrogel, and is an interesting area for further study.

2.5 Boronic acid sugar sensors

A further method developed within the group for gelating pH-triggered dipeptides utilised the pH drop associated with the reversible complexation of phenylboronic acids to monosaccharides.⁴ ⁵ A 3.47 x 10⁻³ mol dm⁻³ FmocLG solution was added to 1 mol dm⁻³ fructose, 0.1 mol dm⁻³ phenylboronic acid. The result was a pH drop to 3.6, forming a homogenous hydrogel. However, with the same concentration of glucose the pH reached 5.6, which was not low enough to form a gel. Hence the technique may be used for the specific recognition of monosaccharide sugars.
2.6 Enzyme fuel cells: Enzyme Immobilisation and Stabilisation

The work presented in this thesis has provided a platform for the study of immobilised enzymes inside dipeptide hydrogels. The spontaneous gelation of FmocLG and HRP in the presence of a seeding layer (Chapter 6) has given a viable method for creating custom materials, without damaging the enzyme. Before the technology can be used in enzymatic fuel cells, the stability of enzymes in the hydrogels under proper biological conditions must be conducted, studying reaction kinetics and the diffusion of reactants through a range of materials.

Figure 8.3. Schematic of an enzymatic fuel cell for the production of methanol from aqueous carbon dioxide. The cell utilises the photoregeneration of the cofactor, redox mediator NADH to create a fully reversible, self-sustaining process.

Figure 8.3 is a schematic for a proposed enzymatic fuel cell utilising the dehydrogenase enzymes for the production of methanol from aqueous carbon dioxide, where the enzyme is immobilised in a reaction chamber. Aqueous carbon dioxide is present and abundant in chemical waste streams of particular industrial processes, such as the production of ammonia.

2.7 Expanding the scope of the electrochemical gelation technique

The electrochemical (or ‘electroaddressing’) gelation of pH-triggered amino acid gelators can lead to a wide range of applications for peptides in molecular electronics, biosensing, nanobiotechnology, and custom materials, with the potential for creating hydrogels of different shapes and properties. Since our work was published introducing this concept (Johnson et al\(^\text{6}\)) there have been several papers which have utilised the
technique for flexible display technology,\textsuperscript{7} the reversible electro-addressing of amino acid conjugates within microfluidic channels,\textsuperscript{8} and electro-addressing agarose using FmocFF as a temporary scaffold.\textsuperscript{3}

2.8  **Dipeptide hydrogels as 3D cell scaffolds**

As dipeptide hydrogels are a 3D biomimetic environment, there is significant interest in the literature in using these materials to investigate 3D cell culturing.\textsuperscript{9,10} The environment cells are cultured in affects their gene expression patterns and metabolism as they adjust to their surroundings. Replicating the natural cell scaffold \textit{in vitro} by creating an extra-cellular matrix is of importance in medicine.\textsuperscript{11} Fmoc-protected tripeptides have been used to culture human dermal fibroblasts within a 3D culture, which adopted a ‘spindle-like morphology’ compared with the conventional rounded shape.\textsuperscript{12,13}

The electrochemical technique for gelating pH-triggered dipeptides increases the opportunities for the selection of possible shapes of gels, which can be fine tuned by changing the electrode shapes. It will be necessary to explore which peptides can gelate in growth media in order to continue this work.

2.9  **Conducting dipeptide hydrogels for use in algal fuel cells**

Conducting hydrogels have large scope for expanding the research in the area of self-assembling amino acids into electronics, for switches, electron mediators, etc.\textsuperscript{14,15} Conducting hydrogels are useful in the area of fuel cell research.\textsuperscript{16} Their potential for conducting materials rests in the π-π stacking distances between each dipeptide molecule being small enough to enable electron transfer along the fibres, via π-interactions. A novel compound, ferrocene diphenylalanine (FcFF, see Figure 8.5) was synthesised in the Bull group (University of Bath) to investigate its potential stacking with FmocFF molecules, to enhance the conductivity of the system.

The FcFF-FmocFF hydrogel mixture was then used to grow an algal biofilm (Figure 8.4[b]), where the composite hydrogel would act as an electron transfer medium for photosynthesising / metabolising algae to an external circuit, without the need for an electron mediator.
The hydrogels were prepared in pH 8 HEPES buffer, to fully dissolve the FcFF at $2 \times 10^{-4}$ mol dm$^{-3}$. FmocFF was added to gelate at 1.6 mg mL$^{-1}$ mol dm$^{-3}$. Cyclic voltammetry was carried out on hydrogels of various volumes grown directly onto FTO electrodes. The stability of the hydrogels over time is shown in Figure 8.5[a] and [b]. TEM images were also collected, see Figure 8.5[c-e].
The FcFF / FmocFF hydrogels formed over several hours and were less homogenous and cloudier than FmocFF hydrogels. This can be attributed to the insolubility of FcFF, and suggests the compound was not incorporated into the structure of the fibres. However, oxidation and reduction peaks for the ferrocene redox centre were observed, which decreased over time (after 15 minutes the peaks had decreased significantly).
This indicates disruption of the hydrogel, either with FcFF diffusing out, or the gel falling away from the electrode in the KCl solution. Preliminary results from TEM and rheology suggest a fairly robust gel, with $G' = 2060 \text{ Pa s}^{-1}$ (average of three data sets).

Concentrated *Synechococcus* algae in chlorella media was added to the top of the hydrogel, the biofilm was stable for several days and gave a stable photocurrent over this time. To move this work forward, several points should be addressed. The electrode material could be altered to improve adhesion of the gels. A conducting molecule with a similar solubility to FmocFF would improve the likelihood of it becoming incorporated into the fibre structures. Computer modeling would improve the understanding of how molecules fit together and allow for more accurate predictions.

### 3. ACKNOWLEDGEMENTS

For FcFF synthesis: Chris Hotchen, Steve Bull, Tony James, Caroline Walker, and Chris Hall.

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### 4. REFERENCES


(13) Jung, J. P.; Gasiorowski, J. Z.; Collier, J. H. *Biopolymers* 2010, 94, 49.


CONFERENCES

18-23rd September 2011, Hirschegg, Austria: BioNano Workshop
   Oral Presentation

12th January 2011, University of Bath, UK: Microscopy and Analysis
   Poster Presentation (1st Prize)

15th June 2010, University of Bath, UK: ‘Great Western Electrochemistry Meeting’, RSC
   Oral Presentation (2nd Prize)

7-13th February 2010, Hirschegg, Austria: ‘Bionano Workshop,’ AIT
   Poster Presentation (1st Prize) – Chair of Presentation Session


2010, University of Bath, UK: Second Year Presentation

22-23rd September 2009 Manchester, UK: Polymeric and self-assembling hydrogels,’
   Macrogroup, University of Manchester
   Poster Presentation

July 2009 Glasgow, UK: 42nd IUPAC Congress ‘Chemistry Solutions,’ RSC
   Flash poster Presentation, Poster Presentation

2009, University of Bath, UK: Poster Presentation

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   Shane McDonald, Caroline Walker, Charlotte Brown, Neil Poulter, Dave Jamieson,
   Charlie Cummings, Antun Peic, Jim Holdaway

Funding

EPSRC
APPENDIX 1

DOSY DATA

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FmocFF hydrogel

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Appendix 1 Page 213
### D-Phe with FmocFF

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FmocLG hydrogel

SIMFIT RESULTS

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\[ I = I_0 \exp(-D \cdot \text{SQR}(2 \cdot \pi \cdot \text{gamma} \cdot \text{Gi} \cdot \text{LD}) \cdot (\text{BD} - \text{LD}/3) \times 1e4) \]

16 points for Integral 1, Integral Region from 5.249 to 4.371 ppm

Converged after 41 iterations!

Results Comp. 1

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Diff Con. \[ = 2.209e-009 \text{ m}^2/\text{s} \]

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$^1$H Spectrum for FmocLG:
DOSY spectra for FmocLG:

\[
\text{m2/s} \\
\begin{array}{cccccccc}
1.0 \times 10^{-9} & 2.0 \times 10^{-9} & 3.0 \times 10^{-9} & 4.0 \times 10^{-9} & 5.0 \times 10^{-9} & 6.0 \times 10^{-9} & 7.0 \times 10^{-9} & 8.0 \times 10^{-9} \\
9.0 \times 10^{-9} & 1.0 \times 10^{-8} \\
\end{array}
\]

ppm