PROTEIN HYDROGELS AS TISSUE ENGINEERING SCAFFOLDS

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Engineering and Physical Sciences 2015

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LIST OF CONTENTS

LIST OF TABLES ........................................................................................................... 7
LIST OF FIGURES ......................................................................................................... 8
Abstract .......................................................................................................................... 12
DECLARATION ............................................................................................................... 13
COPYRIGHT STATEMENT ............................................................................................... 13
ABBREVIATIONS ........................................................................................................... 14
ACKNOWLEDGMENT ...................................................................................................... 16

Chapter 1  Introduction ................................................................................................. 17
  1.1 Hydrogels definition ............................................................................................. 17
  1.2 Objectives of the study and thesis layout ............................................................... 18

Chapter 2  Literature Review ........................................................................................ 20
  2.1 The importance of tissue engineering ..................................................................... 20
  2.2 Scaffold fabrication ................................................................................................ 21
    2.2.1 The role of scaffolds in tissue engineering ....................................................... 21
    2.2.2 Hydrogels as tissue engineering scaffolds ......................................................... 23
      2.2.2.1 Types of hydrogels ...................................................................................... 23
      2.2.2.2 Composition of hydrogels ........................................................................... 24
      2.2.2.3 Advantages and limitations of using hydrogels ............................................ 25
    2.3 Formation of protein hydrogels ............................................................................ 26
      2.3.1 Protein native structure .................................................................................. 26
      2.3.2 Formation of protein fibrils ............................................................................ 28
        2.3.2.1 Protein stability via disulphide bonds and its unfolding .............................. 28
        2.3.2.2 Lysozyme hydrogels as a tissue engineering scaffold .............................. 30
    2.4 Protein-polymer conjugation ............................................................................... 31
      2.4.1 Introduction ..................................................................................................... 31
      2.4.2 Thermo-responsive poly-N-isopropylacrylamide (PNIPAAm) ....................... 32
2.4.3 RAFT polymerisation .................................................................33

Chapter 3 Materials and methods .........................................................35

3.1 Introduction ..................................................................................35

3.2 Materials used ..............................................................................35

3.3 Sample preparation ........................................................................36

3.3.1 Protein gels .............................................................................36

3.3.2 Protein-polymer conjugates and the final composite gels .............37

3.4 Micro differential scanning calorimetry (MicroDSC) .....................41

3.4.1 Introduction .............................................................................41

3.4.2 Principle ................................................................................41

3.4.3 Experimental procedure ..........................................................42

3.5 Rheology .....................................................................................44

3.5.1 Introduction .............................................................................44

3.5.2 Principle ................................................................................44

3.5.3 Experimental procedure ..........................................................46

3.6 Fourier-transform infrared (FT-IR) spectroscopy .........................47

3.6.1 Introduction .............................................................................47

3.6.2 Principle ................................................................................47

3.6.3 Experimental procedure ..........................................................50

3.7 Microscopy Imaging ................................................................. 51

3.7.1 Transmission Electron Microscopy (TEM) ..............................51

3.7.1.1 Introduction .....................................................................51

3.7.1.2 Principle ..........................................................................51

3.7.1.3 Experimental procedure ....................................................52

3.7.2 Atomic Force Microscopy (AFM) ............................................53

3.7.2.1 Introduction .....................................................................53

3.7.2.2 Principle ..........................................................................53

3.7.2.3 Experimental procedure ....................................................54
3.8 X-Ray Fibre Diffraction (XRD) ........................................... 54
  3.8.1 Introduction ......................................................... 54
  3.8.2 Principle ............................................................ 54
  3.8.3 Experimental procedure ......................................... 55
3.9 Nuclear Magnetic Resonance (NMR) ............................. 56
  3.9.1 Introduction ......................................................... 56
  3.9.2 Principle ............................................................ 57
  3.9.3 Experimental procedure ......................................... 57
3.10 Mass Spectrometry (MS) ............................................. 58
  3.10.1 Introduction ......................................................... 58
  3.10.2 Principle ............................................................ 58
  3.10.3 Experimental procedure ......................................... 61

Chapter 4 Lysozyme gelation studies under reductive and non-reductive conditions ............................. 62
  4.1 Introduction .................................................................. 62
  4.2 Visual observations .................................................... 62
  4.3 Protein secondary structural analysis for HEWL/water and HEWL/DTT samples ..................... 65
    4.3.1 Fourier Transform Infrared Spectroscopy (FTIR) ...................... 65
      4.3.1.1 Effect of heat on lysozyme secondary structure .................. 66
      4.3.1.2 Effect of adding DTT on lysozyme secondary structure ......... 68
      4.3.1.3 Effect of protein concentration on lysozyme secondary structure .......... 70
      4.3.1.4 Effect of varying pDs on lysozyme secondary structure .......... 73
    4.3.2 X-ray fibre diffraction (XRD) studies ............................. 80
  4.4 Thermal behaviour of lysozyme ..................................... 81
    4.4.1 Effect of adding DTT on lysozyme denaturation ..................... 81
    4.4.2 Effect of pH on the denaturation of lysozyme in DTT ............... 84
  4.5 Mechanical properties of HEWL/water & HEWL/DTT gels .................. 87
    4.5.1 Effect of protein concentration and adding DTT on the mechanical behaviour of lysozyme samples .................................................. 87
    4.5.2 Effect of varying pH on the mechanical behaviour of lysozyme samples ...... 90
4.5.3 Mechanical stability of HEWL/water and HEWL/DTT gels .........................94

4.6 Morphology of HEWL/water and HEWL/DTT gels by transmission electron microscopy (TEM) and light microscopy .............................................................95

4.6.1 Morphology at pH 2 by TEM .................................................................95

4.6.2 Morphology at pH 7 by TEM .................................................................99

4.6.3 Fibrillar alignment in crystallised lysozyme gels .....................................100

4.7 Conclusions ...............................................................................................102

Chapter 5 Studies on the effects of different reducing agents on HEWL gelation ......103

5.1 Introduction ...............................................................................................103

5.2 Visual observation ......................................................................................103

5.3 Lysozyme thermal transitions in TCEP and DTT .......................................105

5.3.1 Comparing protein behaviour in TCEP and DTT ..................................106

5.3.2 Effect of pH on HEWL/TCEP samples ................................................107

5.4 Structural analysis of HEWL/DTT and HEWL/TCEP gels .......................108

5.4.1 Effect of pH on the secondary structure of HEWL/TCEP samples ..........108

5.4.2 Effect of TCEP vs. DTT on lysozyme secondary structure .....................112

5.5 Mechanical properties of lysozyme gels formed under non-reductive conditions 115

5.5.1 Effect of pH on HEWL/TCEP gels .......................................................115

5.5.2 Comparing the mechanical properties of HEWL/TCEP and HEWL/DTT gels ......................................................................................................................119

5.5.3 Relationship between the mechanical and structural properties of lysozyme gels ..............................................................................................................121

5.5.4 Measuring the potential of HEWL/TCEP and HEWL/DTT gels for drug delivery applications .................................................................122

5.6 Morphology of HEWL/DTT and HEWL/TCEP gels using electron and light microscopy .......................................................................................................124

5.6.1 Microstructure of the HEWL/TCEP hydrogel formed at pH 4 and pH 5 ....124

5.6.2 Microstructure of the HEWL/TCEP vs. HEWL/DTT gels ......................125

5.6.3 Alignment of fibrils in crystallised lysozyme gels ..................................128

5.7 Conclusions ...............................................................................................129
Chapter 6  Designing temperature-responsive hydrogels from physical mixtures of HEWL and HEWL/PNIPAAm conjugate using thiol-ene click chemistry .................................130

6.1  Introduction.........................................................................................................................130

6.2  Synthesis and characterisation of PNIPAAm polymer ..............................131

6.2.1  PNIPAAm with CTA end (RAFT-PNIPAAm).................................................132

6.2.1.1  Distribution of polymer molecules ..............................................................133

6.2.1.2  Degree of polymerisation for RAFT-PNIPAAm........................................134

6.2.1.3  Thermal behaviour of NIPAAm and RAFT-PNIPAAm in water ........135

6.2.2  Thiol-terminated PNIPAAm (PNIPAAm-SH) ..............................................138

6.3  Formation of bioconjugate and also the composite gel of HEWL/PNIPAAm by disulfide cross-linking .................................................................140

6.3.1  Bioconjugates formation via stable thioether linkages .........................140

6.3.2  Formation and characterisation of the composite HEWL/PNIPAAm gel .....142

6.3.2.1  Visual observation .........................................................................................142

6.3.2.2  Thermal behaviour of composite HEWL/PNIPAAm solution .............143

6.3.3  PNIPAAm attachment onto lysozyme fibrils ..............................................145

6.3.4  Rheological properties of HEWL/PNIPAAm composite hydrogel ........148

6.4  Conclusions....................................................................................................................151

Chapter 7  Conclusions and Future Work.................................................................153

7.1  Conclusions.................................................................................................................153

7.2  Future Work...............................................................................................................154

Chapter 8  References.....................................................................................................155

Word Count: 40,269
LIST OF TABLES

Table 3.1 The concentration of lysozyme used to make samples and the equivalent masses of protein dissolved in 1mL solution. ...........................................................................................................36
Table 3.2 Correlations between common protein structures and amide I frequency of Fourier Transform Infrared (FTIR) Spectroscopy ...........................................................................................................49
Table 4.1 Summary of visual gelation results obtained in the presence of water and DTT.65
Table 4.2 Percentages of secondary structural elements in pD 4 HEWL/DTT samples formed at 2, 3 and 4 mM. ..........................................................................................................................72
Table 4.3 Percentages of secondary structural elements in 4 mM HEWL/D_2O samples formed at pD 2, 3, 4 and 7 ..........................................................................................................................75
Table 4.4 Percentages of secondary structural elements in 4 mM HEWL/DTT samples formed at pD 2, 3, 4 and 7 ..........................................................................................................................78
Table 4.5 A summary of HEWL/DTT denaturation at pH 2, 3, 4 and 7 during the first heating. .................................................................86
Table 5.1 MicroDSC results for 4 mM lysozyme/TCEP/water and lysozyme/DTT/water samples during first and second heating runs ........................................................................................................107
Table 5.2 Percentages of secondary structural elements in 4 mM HEWL/TCEP samples at different pDs. .................................................................................................................................111
Table 5.3 Percentages of secondary structural elements in 4 mM lysozyme samples dissolved in TCEP and DTT solutions. ................................................................................................................114
Table 6.1 Molar ratio of NIPAAm, CTA, AIBN and DMF used to make PNIPAAm at DP of 40 and 60. .................................................................................................................................131
Table 6.2 Mass of monomer, chain transfer agent (CTA), initiator and solvent used in the radical polymerisation process to form RAFT-PNIPAAm at DP=40. ........................................132
Table 6.3 Mass of monomer, chain transfer agent (CTA), initiator and solvent used in the radical polymerisation process to form RAFT-PNIPAAm at DP=60. ........................................132
Table 6.4 Mass of RAFT-PNIPAAm and TCEP and also volume of butylamine used during aminolysis to form thiol-terminated polymers. .................................................................138
LIST OF FIGURES

Figure 2.1 Schematic representation of chemically and physically cross-linked hydrogels from the chemical modification of hydrophobic polymers ................................................................. 24
Figure 2.2 Structure of Hen Egg White Lysozyme (HEWL) in terms of amino acid residues ................................................................................................................................. 26
Figure 2.3 Crystal structure of HEWL showing the presence of α-helical segment (blue), β-sheet (green) and disulphide bridges (yellow) ........................................................................................................... 27
Figure 2.4 Schematic representation of secondary structural element of (a) α-helix, (b) parallel β-sheet and (c) anti-parallel β-sheet ............................................................................................................ 27
Figure 2.5 Two-sequential thiol disulfide exchange reaction for lysozyme in DTT ........... 29
Figure 2.6 Mechanism of disulfides reduction by TCEP in water .................................................................................................................................................................................. 29
Figure 2.7 Schematic representation of lysozyme denaturation and gelation process .... 30
Figure 2.8 Possible routes to protein-polymer conjugation .............................................. 32
Figure 2.9 Examples of reversible phase separation of PNIPAAm (a) linear chains, (b) terminally-grafted PNIPAAm surfaces and (c) multi-point grafted PNIPAAm surfaces ... 33
Figure 2.10 Mechanism for reversible addition-fragmentation grafted PNIPAAm surfaces ... 33
Figure 3.1 Molecular structure of disulfide reductants (a) DTT and (b) TCEP ................. 35
Figure 3.2 Modification of NIPAAm with the initiator AIBN and RAFT agent to form the RAFT-PNIPAAm via free-radical polymerisation ........................................................... 37
Figure 3.3 Molecular structure of the RAFT agent DMP .................................................. 37
Figure 3.4 RAFT-PNIPAAm end group modifications involving amine cleavage followed by phosphine-mediated thiol-ene reactions .................................................................................. 38
Figure 3.5 Schematic representing the click approach to polymer-protein bioconjugate .... 39
Figure 3.6 Molecular structure of BMH with terminal maleimides that can react with sulhydryls between pH 6.5 and pH 7.5 .................................................................................................................. 39
Figure 3.7 Slide-A-Lyzer Dialysis Cassette with MWCO of 10kDa. It is used to remove low molecular weight contaminant which is the unreacted polymer-linker while lysozyme is retained inside the cellulose membrane ...................................................................................... 40
Figure 3.8 A schematic diagram of the microDSC layout .................................................. 42
Figure 3.9 MicroDSC temperature profiles showing the isothermal and also two heating/cooling ramps ................................................................................................................................. 43
Figure 3.10 The oscillating strain and stress response for a viscoelastic gel ....................... 45
Figure 3.11 The AR-G2 Rheometer (TA Instruments) whereby sample was placed on the surface of stage plate and then slowly compressed by the upper plate ................................................................................. 46
Figure 3.12 The basic components of an FT-IR spectrometer .......................................... 48
Figure 3.13 A schematic diagram of the detailed mechanism of FT-IR interferometer ...... 48
Figure 3.14 Specimen-beam interactions that resulted in elastic and inelastic scatterings of electrons leading to an image formation .......................................................................................... 52
Figure 3.15 A schematic diagram of AFM layout showing five components; the sample, cantilever, laser, photo detector and differential amplifier ..................................................................................... 53
Figure 3.16 XRD patterns showing for 4-quadrant symmetry where meridian is along the fibre axis .................................................................................................................................................. 55
Figure 3.17 The stretch-frame alignment of XRD sample .................................................. 56
Figure 3.18 Nuclei spin state in response to an external magnetic field.................................57
Figure 3.19 A schematic diagram of mass spectrometer showing its five essential components..........................................................58
Figure 3.20 Formation of analyte ions in the electrospray ionisation mass spectrometry (ESI-MS) via solvent evaporation and “Coulombic explosion”. .......................59
Figure 3.21 Movement and separation of ions within the Time-of-Flight (TOF) region. ....59
Figure 4.1 Typical photographs of hen egg white lysozyme samples reduced in DTT after heating at 85°C and cooled at room temperature for 45 minutes; starting from left (a) no gelation at pH 2, (b) a weak gel at pH 4 and (c) a strong gel at pH 7.................................64
Figure 4.2 Infrared spectra of 4 mM pD 4 lysozyme dissolved in D2O before (red) and after (green) heating at 85°C for a day and subsequent cooling at room temperature for 45 minutes. ..........................................................65
Figure 4.3 Infrared spectra of 4 mM pD 4 HEWL/D2O solution (green) and pD 4 HEWL/DTT gel (blue) where samples were formed by heating at 85°C for a day and then cooled at room temperature for 45 minutes..........................................................66
Figure 4.4 Smoothed infrared spectra of pD 4 HEWL/DTT sample (light blue) and its smaller component peaks...........................................................69
Figure 4.5 Infrared spectra of pD 4 HEWL/DTT samples at 2 mM (orange), 3 mM (black) and 4 mM (blue) concentration where samples were formed by heating at 85°C for a day and then cooled at room temperature for 45 minutes. ..........................................................71
Figure 4.6 Infrared spectra of 4 mM HEWL/D2O samples prepared at pD 2 (blue), pD 3(pink), pD 4 (green) and pD 7 (orange) by heating them at 85°C for different times and then cooled at room temperature for 45 minutes. ..........................................................73
Figure 4.7 Infrared spectra of 4 mM HEWL/DTT/D2O samples prepared at pD 2 (blue), pD 3 (pink), pD 4 (green) and pD 7 (orange) by heating them at 85°C for different times and then cooled at room temperature for 45 minutes. ..........................................................77
Figure 4.8 Equatorial (left) and meridional (right) signals for pH 2 diluted HEWL/water (blue) and pH 7 HEWL/DTT (red) samples..........................................................80
Figure 4.9 MicroDSC curves showing the first (blue) and second (pink) heating/cooling cycles for 4 mM (a) HEWL/H2O and (b) HEWL/DTT samples prepared at pH 2. ..........82
Figure 4.10 MicroDSC curves showing the first heating of 4 mM lysozyme in DTT at pH 2 (blue), pH 3 (pink), pH 4 (green) and pH 7(orange) with a heating rate of 1 °C min⁻¹. ..........................................................85
Figure 4.11 Average storage, G’ (solid symbols) and loss, G'' (empty symbols) moduli of 2 mM (orange), 3 mM (black) and 4 mM (blue) (a)HEWL/water and (b)HEWL/DTT samples at pH 4 as a function of shear strain obtained at 25°C. ..........................................................88
Figure 4.12 Storage modulus, G’ (red ) and level of β-sheet content (black ) as a function of protein concentration for pH 4 lysozyme samples prepared in the presence of 20 mM DTT. ..........................................................90
Figure 4.13 Average storage, G’ (solid symbols) and loss, G” (empty symbols) moduli of 4 mM (a) HEWL/water and (b) HEWL/DTT samples at pH 2 (blue), pH 3 (pink), pH 4 (green) and pH 7 (orange) as a function of shear strain obtained at 25°C...........................................93
Figure 4.14 Storage modulus, G’(red) and the level of β-sheet content (black) as a function of pH for (a) HEWL/water and (b) HEWL/DTT samples..................................93
Figure 4.15 Average storage, $G'$ (solid symbols) and loss, $G''$ (empty symbols) moduli of pH 2 HEWL/water (blue), pH 3 HEWL/DTT (pink), pH 4 HEWL/DTT (green) and pH 7 HEWL/DTT (orange) hydrogels as a function of shear frequency obtained at 25°C. 

Figure 4.16 TEM micrograph of negatively stained fibrils from a 25-fold diluted HEWL/water hydrogel formed at pH 2 with a magnification factor of 4,800.

Figure 4.17 TEM micrograph of aggregated fibrils from a 25-fold diluted HEWL/DTT sample formed at pH 2 with a magnification factor of 11,000.

Figure 4.18 TEM micrograph of negatively stained fibrils from a 25-fold diluted HEWL/DTT sample formed at pH 2 with a magnification factor of 11,000.

Figure 4.19 TEM micrograph of a 250-fold diluted HEWL/DTT hydrogel formed at pH 7 with a magnification factor of 13,000.

Figure 4.20 TEM micrograph of a 250-fold diluted HEWL/DTT hydrogel formed at pH 7 with a magnification factor of 49,000.

Figure 4.21 Light microscopic images of dried crystalline samples of HEWL/water at pH 2 (left) and HEWL/DTT at pH 7 (right).

Figure 5.1 Phase diagram of HEWL gelation as a function of protein concentration and its pH after samples were heated for 2 hours and then cooled for 45 minutes at room temperature.

Figure 5.2 Typical photographs of 4 mM HEWL/TCEP samples; starting from left strong and clear gels were formed at (a) pH 3.5 and (b) pH 4, followed by (c) strong and slightly opaque gel at pH 4.5, (d) strong and opaque gel at pH 5.5 and (e) protein precipitation at pH 6.

Figure 5.3 MicroDSC first heating runs of lysozyme solutions in TCEP (pH 2.47) and in DTT (pH 3.26) at a scanning rate of 1 °C min⁻¹.

Figure 5.4 Infrared spectra of 4 mM HEWL/TCEP samples at pH 3.5 (brown), pH 4 (red), pH 4.5 (blue), pH 5 (black), pH 5.5 (green) and just below pH 6 (pink).

Figure 5.5 Smoothed infrared spectra of pH 4 HEWL/TCEP sample (light blue) and its small component peaks.

Figure 5.6 Smoothed infrared spectra of 4 mM lysozyme samples including pH 4 HEWL/DTT (blue), pH 4 HEWL/TCEP (red), pH 5 HEWL/TCEP (black) and pH 7 HEWL/DTT (green) gels.

Figure 5.7 Average storage modulus, $G'$ of 4 mM HEWL/TCEP samples as a function of shear strain at 25°C.

Figure 5.8 Average storage, $G'$ (solid symbols) and loss, $G''$ (empty symbols) moduli of 4 mM HEWL/TCEP gels as a function of shear strain at 25°C.

Figure 5.9 Average storage, $G'$ (solid symbols) and loss, $G''$ (empty symbols) moduli of 4 mM lysozyme gels as a function of shear strain at 25°C.

Figure 5.10 Storage modulus, $G'$ (red) and level of β-sheet content (black) as a function of pH for 4 mM lysozyme hydrogels prepared in 15 mM TCEP.

Figure 5.11 Storage modulus, $G'$ as time sweeps for lysozyme gels including pH 4 HEWL/TCEP (red), pH 5 HEWL/TCEP (black), pH 4 HEWL/DTT (blue) and pH 7 HEWL/DTT (green) samples.

Figures 5.12 TEM micrographs of a 250-fold diluted HEWL/TCEP hydrogel formed at (a) pH 4 (left image) and (b) pH 5 (right image) which are magnified 18,500 and 23,000 times respectively.
Figure 5.13 TEM micrograph of a 250-fold diluted HEWL/DTT hydrogel formed at pH 7.

Figure 5.14 TEM micrograph of a 300-fold diluted HEWL/TCEP hydrogel formed at pH 4.

Figure 5.15 Crystallised stalk of pH 4 HEWL/TCEP gel observed under polarised (left) and non-polarised (right) light using the optical microscope.

Figure 6.1 TOF-MS spectrum for RAFT-PNIPAAm40.

Figure 6.2 TOF-MS spectrum for RAFT-PNIPAAm60.

Figure 6.3 $^1$H NMR spectrum, recorded in D$_2$O, of the RAFT-PNIPAAm40 highlighting the presence of end-functional group and the calculation of the absolute degree of polymerisation.

Figure 6.4 MicroDSC curves of RAFT-PNIPAAm47 sample at pH 4 showing the first (blue), second (red) and third (green) heating runs during three heating/cooling cycles at a scanning rate of 1 °C min$^{-1}$.

Figure 6.5 MicroDSC curves of NIPAAm monomer during the first heating run at a scanning rate of 1 °C min$^{-1}$.

Figure 6.6 TOF-MS spectra for (a) RAFT-PNIPAAm47 and (b) PNIPAAm47-SH.

Figure 6.7 MicroDSC curves of PNIPAAm47-SH sample (at pH 3.78) showing the first (blue), second (red) and third (green) heating runs during three heating/cooling cycles at a scanning rate of 1 °C min$^{-1}$.

Figure 6.8 Structure of PNIPAAm-BMH-HEWL conjugate which are cross-linked with two thioether bonds.

Figure 6.9 TOF-MS spectrum for dialysed bioconjugate at DP of 47.

Figure 6.10 Schematic representation of the bioconjugate forming disulphide bonds (circled) with thiol-containing HEWL that constitutes the major protein matrix.

Figure 6.11 Typical photographs of (a) HEWL/TCEP gel and (b) the composite gel containing mixture of HEWL matrix and small amount of bioconjugates and these samples were prepared at pH 4.5.

Figure 6.12 MicroDSC curves of HEWL-PNIPAAm47 composite sample showing the first (blue), second (red) and third (green) heating runs during three heating/cooling cycles.

Figure 6.13 AFM images of lysozyme fibrils found in (a) HEWL/TCEP and (b) composite HEWL/PNIPAAm gels.

Figure 6.14 AFM 3D-imaged of lysozyme fibrils in (a) HEWL/TCEP gel and (b) HEWL/PNIPAAm composite sample with suspected polymer deposits.

Figure 6.15 Mechanism showing the self-assembly of lysozyme (black arrows) and their conjugates with attached polymer chains (blue) that form mesh network of fibrils (black thread-like structure) with polymers lining on its surface within the composite gel.

Figure 6.16 Storage modulus, $G'$ as temperature sweeps for HEWL/TCEP gel (blue) and HEWL/PNIPAAm composite (red) during (a) 1st heating, (b) 1st cooling, (c) 2nd heating and (d) 2nd cooling run at a rate of 1°C min$^{-1}$. 

11
Abstract of thesis entitled “Protein Hydrogels as Tissue Engineering Scaffolds”, submitted by Khairunnisa Nabilah Haji Ruslan for the degree of Doctor of Philosophy on 02/04/2015.

Hydrogels aim to mimic the natural living environment by entrapping large amount of water or biological fluids in their polymeric network. There has been growing interest in the development of peptide and protein hydrogels, due to their improved biocompatibility, biodegradability and biological properties in comparison to purely synthetic polymer hydrogels. Under the appropriate conditions, biomacromolecular protein hydrogels can self-assemble into ordered meso- to macroscopic supramolecules with better resulting networks that promote tissue development. The work presented here mainly focuses on producing protein hydrogels with controlled physical properties useful for tissue regeneration process and drug delivery applications.

Hen egg white lysozyme (HEWL) hydrogels were studied in the presence of water and different reducing agents forming three HEWL systems including HEWL/water, HEWL/DTT and HEWL/TCEP gels. Strong, self-supporting HEWL gels were successfully prepared in the range of pH 2 to 7, using a temperature of 85°C. At pH 2, the protein denaturation in water was relatively slow resulting in a high percentage of turn structure (~50%) that promotes HEWL gelation after 3 days of heating. No lysozyme gelation in water was observed at pH 3, 4 and 7 even after 21 days of heating. A small quantity of DTT (~20 mM) was added to encourage lysozyme unfolding and HEWL/DTT samples formed gels at higher pH including at physiological pH. The pH 2 HEWL/water gel was found to be stronger but more brittle than pH 7 HEWL/DTT gel. It was observed there were some irregularities in the distribution of pH 2 fibrils (~7µm in length) that form large pore sizes within the network. The pH 7 sample contained shorter and stiff fibrils with repetitive polygon-shaped mesh network. The use of TCEP, which is a stronger reductant than DTT, led to the formation of self-supporting HEWL gels between pH 3.5 and 5.5. The highest storage modulus was observed at pH 5, which is related to the high β-sheet content of the sample (~45%).

In addition, a promising strategy has been devised to form thermoresponsive HEWL hydrogels by synthesising and incorporating a small fraction of lysozyme-PNIPAAm bioconjugates into the major protein matrix. Results show the thermoresponsive nature of PNIPAAm was conferred to HEWL protein that exhibits higher storage stability in response to changing temperature.
DECLARATION

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<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>¹H NMR</td>
<td>Proton Nuclear Magnetic Resonance</td>
</tr>
<tr>
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<td>2-(dodecylthiocarbonothioylthio)-2-methylpropanoic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DP</td>
<td>Degree of Polymerisation</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGDMA</td>
<td>Ethylene Glycol Dimethacrylate</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray Ionisation</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administrative</td>
</tr>
<tr>
<td>FSD</td>
<td>Fourier Self Deconvolution</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier-Transform Infrared</td>
</tr>
<tr>
<td>FWHH</td>
<td>Full Width at Half Height</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic Acid</td>
</tr>
<tr>
<td>H₂O</td>
<td>Distilled Water</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>HEMA</td>
<td>Hydroxyethyl Methacrylate</td>
</tr>
<tr>
<td>HEWL</td>
<td>Hen Egg White Lysozyme</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>LCST</td>
<td>Lower Critical Solution Temperature</td>
</tr>
<tr>
<td>MicroDSC</td>
<td>Micro Differential Scanning Calorimetry</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
</tbody>
</table>
MW  Molecular Weight
MWCO  Molecular Weight Cut-Off
NaOH  Sodium Hydroxide
NIPAAm  N-Isopropylacrylamide
NMR  Nuclear Magnetic Resonance
OVA  Ovalbumin
pI  Isoelectric point
PAA  Poly (acrylic acid)
PBS  Phosphate Buffer Saline
PEG  Polyethylene Glycol (PEG)
PID  Proportional, Integral and Differential
PLLA  Poly-(L-lactic) Acid
PNIPAAm  Poly (N-isopropylacrylamide)
PVA  Poly (Vinyl) Alcohol
RAFT  Reversible Addition-Fragmentation Chain Transfer
RDS  Rate Determining Step
SAXS  Small Angle X-ray Scattering
TCEP  Tris-2-carboxyethylphosphine
TEM  Transmission Electron Microscopy
THF  Tetrahydrofuran
TOF  Time-of-Flight
WAXS  Wide-Angle X-ray Scattering
XRD  X-Ray Fibre Diffraction
ACKNOWLEDGMENT

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Chapter 1  Introduction

1.1  Hydrogels definition

Hydrogels are increasingly well-known for their ability to trap large amounts of water or biological fluids within their three-dimensional network of crosslinked polymer [1, 2] and hence, allowing them to mimic the natural living environment of cells inside the human body. This explains the growing interest of their use as a tissue engineering scaffold and also as vehicles for controlled drug delivery [2-6]. Their applications range from contact lenses, disposable baby diapers, wound dressings and in the field of microfluidics [1, 7, 8].

More peptide and protein hydrogels are now being studied due to their improved biological, biocompatible and biodegradable properties in comparison to the traditional synthetic polymer hydrogels. In this work, Hen Egg White Lysozyme (HEWL) is used as a model protein to make hydrogels and study protein gelation. HEWL is one of the most structurally well-characterised proteins and is amenable to biophysical studies [9]. Moreover, the protein is relatively inexpensive and easily available. The importance of studying lysozyme fibrils dates back to the 1930s when familial renal amyloidosis was discovered and identified as the abnormal deposition and accumulation of insoluble fibrils (including lysozyme) that caused harm to human kidneys [10].

The interest in developing the fibrils application as tissue engineering scaffold rapidly grew over the last 30 years [11]. This is partly due to the ever increasing need of organ transplantations and the inability to meet demand [4, 6]. However, protein hydrogels are reportedly used more often for in vitro application such as skin grafting to repair damaged or burnt skin [6, 12].

Proteins can also be covalently modified by attaching synthetic polymers to proteins and this significantly increased the solubility and stability of protein-polymer conjugates [13, 14]. They are useful materials in order to prolong the plasma half-life of therapeutics and also to regulate any enzyme activity through thermal precipitation [13]. One synthetic polymer of interest is poly(N-isopropylacrylamide) which has a critical solution temperature of about 30-34°C just below the physiological temperature of 37°C. For this reason, PNIPAAm has been extensively studied to control the delivery of active substrates from temperature-sensitive drug delivery systems [15].
1.2 Objectives of the study and thesis layout

The first main objective of this work is to improve the properties of HEWL gels by synthesising stronger gels than in previous studies from a fundamental understanding of the gelation under non-reducing and reducing conditions. This is to enable control over the final properties of lysozyme scaffold and hence, the success of its final application. It is also desired to prepare and form strong self-supporting lysozyme gels at a range of pHs using different reducing agents. Hence, the second main aim is to study the effect of reductants on the macroscopic gelation behaviour of HEWL and the morphology of fibrils. The final objective of this work is to design and synthesise a lysozyme-pNiPAAm conjugate in minute quantities and to mix it with lysozyme in reducing agent for tailored control of its thermo-responsive behaviour.

The overall structure of this study comprises of seven chapters. Chapter 1 contains the general background on hydrogels and their potential applications and it also outlines the main objectives of this work. Chapter 2 reviews the background of tissue engineering and the use of hydrogels to fabricate tissue engineering scaffolds. It also considers the formation of protein hydrogels in terms of the gelation process, stabilising forces and conditions that favour their gelation. This chapter also introduces the synthesis route to protein-polymer conjugation. Chapter 3 covers the details relating to the materials used and also the experimental procedures involved for the synthesis and characterisation of the hydrogels. The principles of each analytical technique used in this work are also discussed here.

Chapters 4, 5 and 6 present the research findings and include discussion of the experimental results. Chapter 4 studies the HEWL gelation behaviour in water and the reducing agent dithiothreitol (DTT). Differences between the gels characteristics are discussed here in terms of their secondary structure, thermal, mechanical and morphological properties. In Chapter 5, the addition of the reductant Tris-2-carboxyethyl-phosphine (TCEP) is used to form self-supporting HEWL gels under different conditions. The properties of the reduced gels are examined and compared to lysozyme gels formed in DTT. Chapter 6 presents the synthesis route of thermo-responsive PNIPAAm using RAFT free radical polymerisation leading to control over their molecular weight. The desirable product prepared contains mixture of PNIPAAm and HEWL and its thermal, mechanical and morphological properties are compared to pure lysozyme gelling
system. Chapter 7 summarises the results from previous chapters along with some recommendations for future work.
Chapter 2  Literature Review

This chapter covers the introduction to tissue engineering, followed by the importance of scaffold fabrication in tissue engineering and also the different types of hydrogels used to make the scaffolds. It also focuses on the areas of protein hydrogels formation and synthesis of protein-polymer conjugates.

2.1  The importance of tissue engineering

Tissue engineering has become increasingly important as there is the need to meet the growing demand of human organs and tissues for transplantation [4]. Patients normally suffer from the failure or loss of tissues or organs due to diseases, trauma or congenital anomalies and they can be treated with transplanted tissues or organs [6]. Using tissue engineering, biological substitutes can be developed by manipulating the life sciences and engineering principles in order to maintain, improve or restore the function of tissues or organs [16]. For example, there is an increase in the application of skin substitutes on humans in order to repair damaged or burnt skin [17]. More than 10,000 patients require skin treatments in the US each year [6].

According to the UK National Health Service Blood and Transplant (NHSBT) statistics, 1,320 people have donated organs from 1st April 2013 until 31st March 2014 while at the same time 1,459 people have received transplants and 6,925 people are still waiting for them [18]. This problem of mismatched supply and demand of organs and also the possibilities of organ trafficking and transplant rejection has fuelled the need to potentially engineer man-made organs or tissues [4, 19].

In the field of tissue engineering, the patient’s own cells can be isolated from their tissue and then incorporated into scaffolds before the cells proliferate and differentiate to form the desired tissue [6]. This can help to reduce the chances of organ or tissue rejection since the patient himself/herself is in fact the donor of the organ or tissue. Examples of successful laboratory engineered miniature organs or tissues include the human skin, cartilage, corneas, urinary structures and left mainstream bronchus which have been used in clinical trials [6, 19]. Products for bone regeneration and implants for knee cartilage repair are available in the healthcare market now [19].
Research on tissue engineering spans around these six areas namely; biomaterials, cells, biomolecules, engineering design aspects, biomechanical aspects of design and lastly, informatics to support tissue engineering. This area aims to generate new functional tissues rather than just implanting spare ones [20]. Therefore, this research work focuses on designing and constructing novel biomaterials for tissue engineering scaffolds which can help to direct the adhesion, growth, proliferation and differentiation of cells suitable for healing injured or diseased tissues with major defects [16, 21].

2.2 Scaffold fabrication

2.2.1 The role of scaffolds in tissue engineering

One of the main principles in tissue engineering is to use scaffolds for growing isolated cells and forming larger blocks of desired tissues with predesigned shapes. Scaffolds are biodegradable templates for three-dimensional (3D) tissue growth / differentiation and hence they should emulate the architecture of the natural extracellular matrix (ECM) where cells normally reside in the body [16, 20]. Hydrogels are increasingly used as a tissue engineering scaffold due to the similarities in their properties to that of natural tissue [22].

The scaffold design variables can dictate the success of its final application either as a space-filling agent, as a delivery vehicle for bioactive molecules or for tissue constructs [4]. The relevant design parameters include the physical, mass transport and biological properties of the scaffold materials. Examples of the physical properties are the mechanical characteristics, degradation behaviour and gel formation mechanism and dynamics. The scaffold must exhibit similar mechanical properties to those tissues to be replaced in terms of elasticity, compressibility, tensile strength or failure strain. For example, a scaffold must withstand large stresses for cartilage or bone tissue engineering.

The rate of scaffold degradation should ideally be the same as the rate of new tissue formation, emphasising the need for biocompatible and biodegradable scaffolds [4]. The scaffold must also be highly porous [8]. This helps to maximise the mass transport of gases, nutrients and waste products into and out of the scaffold and hence, ensuring the survival of implanted cells. In addition, the scaffold should promote cellular adhesion and regulate the functions of interacting cells [4].
Due to the remarkable advancement in the fields of materials science and cell biology, it is now possible to fabricate nanofibrous scaffolds with 3D environment that may form a 3D tissue [5]. Molecular self-assembly, electrospinning and thermally induced phase separation are the three methods used to generate nano-fibrous scaffolds [16] and each technique has its own advantages and disadvantage [5, 16].

Molecular self-assembly is a spontaneous process in which self-assembling biomolecules like peptides and proteins form nano-fibrous structures through several non-covalent interactions including hydrogen bonds, van der Waals interactions, electrostatic interactions and hydrophobic interactions [5, 16]. These nanoscale structures are then assemble to form macroscopic gels and this is an example of a bottom-up approach of tissue engineering [23]. The advantages of molecular self-assembly is in the ease of fabrication, its great control over forming 3D-shaped tissues and the built-in biocompatibility associated with using peptides and proteins [5]. There is also a significant advantage in being able to introduce biomaterials into living tissues in a minimally invasive manner i.e. through injection. However, this approach can suffer from poor control over fibres diameter and orientation of the network. It is also difficult to control the pore sizes and the morphology within the scaffold [5]. Using this approach, the sizes of protein fibres formed are estimated in the range of 50 to 500 nm [24].

In addition, there is a growing interest in the latest state-of-the-art 3D printing of hydrogel cell scaffolds. 3D printing offers the advantage to rapidly engineer complex structures by consecutively depositing layer upon layer of materials. An example of its potential application is in the fabrication of aortic valve scaffolds with higher anatomical precision that may replace the defective valve [25]. Another example is the formation of a 3D-printed biomimetic nanocomposite construct in hydrogel as a detoxification device [26].

In summary, a scaffold must possess five key characteristics for its tissue-engineering applications [27], these include:

(1) Excellent biocompatibility with embedded cells;
(2) An open network of pores;
(3) Being suitable for the growth and differentiation of cells;
(4) Exhibiting similar mechanical properties to replacement tissue;
(5) And having a controlled degradation rate.
2.2.2 Hydrogels as tissue engineering scaffolds

Hydrogels are highly hydrated polymer networks that are often swollen in the presence of water [8, 28]. They can absorb up to thousands of time their dry weight in water. The amount of water in a hydrogel will dictate the diffusion of solutes into the gel which relates to the survival of implanted cells. When water enters, the polar groups of the network molecules are hydrated first causing the hydrogel to swell and expose its hydrophobic groups, which are then hydrated [11]. Additional swelling of hydrogels may take place after the polar and hydrophobic groups are hydrated with bound water, whereby free water fills in the space between the network chains driven by osmosis.

2.2.2.1 Types of hydrogels

Hydrogels can be classified into two categories, chemical or physical, depending on the type of cross-links found in the network [11]. Chemical gels are cross-linked by covalent bonding while physical gels are cross-linked by various non-covalent forces.

In the physical gels, molecular entanglements are frequently found within the network and they are held together by secondary forces such as hydrogen bonding, ionic bonding or hydrophobic interactions [11, 29, 30]. These interactions are readily reversible, and as such, physical hydrogels may disintegrate and dissolve by changing the pH, temperature, ionic strength, adding solutes or applying stress. The gels are not homogeneous, with defects in their network due to clusters of molecular entanglement and the presence of free chain ends or chain loops [11]. The mechanical properties of these gels depend on entanglements and the physical branch point that define the network [3, 31].

Chemical gels are formed by cross-linking macromolecules with cross-linkers or UV exposure, which result in permanent covalently-crosslinked networks with ordered arrangement [4, 32, 33]. Their mechanical properties rely on the rigidity of polymer chains, as well as the types and density of cross-linking molecules [6, 34]. Sometimes hydrophobic polymers are chemically converted to hydrophilic polymers with crosslinking to form the hydrogel network [11] and this is illustrated in Figure 2.1.
There are many methods used to form physical and chemical hydrogels. Physical gels can be synthesised by warming or cooling a polymer solution to encourage gelation. For example, agarose and gelatine gels are formed by cooling them in water [11]. In this work, lysozyme from chicken egg white was dissolved in water, heated to a high temperature and subsequently cooled in order to form a gel [2, 35-37].

### 2.2.2.2 Composition of hydrogels

There are three types of hydrogels which have been extensively studied for tissue engineering applications. These include the synthetic polymers, polysaccharides and peptides/proteins hydrogels. This section will briefly discuss the advantages and disadvantages of using these hydrogels.

Synthetic polymer hydrogels were first introduced in the 1960s when Wichterle and Lim produced copolymerization of hydroxyethyl methacrylate (HEMA) with the crosslinker ethylene glycol dimethacrylate (EGDMA) [38]. Further examples of hydrogels from synthetic polymers include poly(acrylic acid) (PAA), poly-N-isopropylacrylamide, poly(ethylene oxide) (PEO), poly(vinyl alcohol) (PVA) and polyphosphazene [6]. The main advantage of synthetic polymer hydrogels is that their properties are controllable and
reproducible due to the advanced state polymer chemistry knowledge. For example, they can be created with specific molecular weights or degradable linkages [4]. However, their limitations come from their lack of bioactivity and that not all of them are Food and Drug Administrative (FDA) approved. In addition, many purely synthetic polymers are known to have high toxicity within the body limiting their applications in tissue engineering.

Polysaccharide gels have been studied for some time due to their biological activity and they are known to be biodegradable by enzymes in our body and produce non-toxic products. Examples of polysaccharide gels include hyaluronic acid (HA), alginate and chitosan [4]. HA can be crosslinked with alginate to form composite hydrogels for drug delivery and cell encapsulation uses [39].

Peptide and protein hydrogels have received increasing interest lately due to their self-assembling ability, which can be exploited to form nano-structured biomaterials [5, 16, 40, 41]. Some examples of the peptide synthesised hydrogels include FEFEFKFK, RADAFI and RADAFII [42, 43] while some of the known protein hydrogels are from collagen, gelatine, fibrin and lysozyme [2, 6, 8, 35-37]. Most of these gels demonstrate good biodegradable/biological properties and promote cell growth and proliferation. However, peptide synthesis can be expensive and time-consuming and there is a risk of pathogen transmission from the use of animal-derived proteins [21].

2.2.2.3 Advantages and limitations of using hydrogels

There are many advantages of using hydrogels as tissue engineering matrices [11]. These include their biocompatibility and ability to protect cells and fragile drugs because of their aqueous environment. Hydrogels are good transporters of nutrients and products into and out of the cells respectively and can be easily injected in vivo as a liquid that gels at body temperature. Their drawbacks as tissue engineering matrices include their mechanical weakness, the difficulties in handling and sterilising them and also problems to load drugs and cells and crosslink them in vitro as a prefabricated matrix [11].
2.3 Formation of protein hydrogels

2.3.1 Protein native structure

Proteins are biological molecules which contain amino acid residues that are linked together by amide (CO-NH) bonds. Native proteins including lysozyme typically have 20 different types of amino acids. The sequence of these amino acids forms the protein primary structure and would also influence its secondary and tertiary structure.

Lysozyme from chicken egg has an isoelectric point (pI) of around 11 and a molecular weight (MW) of 14,000 g mol\(^{-1}\) [44]. It contains a single polypeptide chain with 129 amino acid residues and four disulphide linkages. These disulphide bonds are formed between the cysteine (Cys) residues i.e. Cys 6 and 127, Cys 30 and 115, Cys 64 and 80 and also Cys 76 and 94 which are shown in Figure 2.2. Only carbon, oxygen, nitrogen and sulphur atoms are found in lysozyme.

![Structure of Hen Egg White Lysozyme (HEWL) in terms of amino acid residues.](image)

The protein secondary structure is determined by the conformation of its backbones which are normally stabilised by hydrogen bonding and steric hinderence between side groups. The four main secondary structures of interest in proteins are the \(\alpha\)-helix, \(\beta\)-sheet, turn and random coil [45]. The protein turns are polypeptide chains which are responsible for
connecting the α-helix and β-sheet structural regions. They are also capable of changing direction immediately and linking the β-strands in anti-parallel β-sheets. About half of the structure in native proteins is made of α-helix and β-sheet which is shown in Figure 2.3.

Figure 2.3 Crystal structure of HEWL showing the presence of α-helical segment (blue), β-sheet (green) and disulphide bridges (yellow) [46].

Hydrogen bonding in α-helices are formed between the nth NH group and the (n+4)th CO group while the β-sheets are formed between the NH group and CO group [47]. The differences in their structure are shown in Figure 2.4.

Figure 2.4 Schematic representation of secondary structural element of (a) α-helix, (b) parallel β-sheet and (c) anti-parallel β-sheet [45].

In Figure 2.4(a), the α-helix is filled with backbone atoms while the side chains are pointing outwards. The average length of each helix turn is 5.4 Å with 3-4 amino acid residues inside and their turn is often right-handed [47]. Figures 2.4(b) and (c) show the β-
sheet structures which can be parallel or anti-parallel depending on the peptide backbone that interact and the directionality of \( \beta \)-strands that form the structure [45].

Protein tertiary structure is defined as the folding of secondary structural elements into a higher ordered structure [47]. This tertiary conformation is mainly due to the hydrophobic effect i.e. the ability of non-polar substances to maintain minimal contact with water. For a globular protein like HEWL, such effect origins from the embedded hydrophobic core structure with polar groups on the surface. Meanwhile, quaternary structure of the protein refers to the association of multiple chains within the protein. However, the quaternary conformation could not be found in lysozyme which contains single polypeptide chain [47].

2.3.2 Formation of protein fibrils

2.3.2.1 Protein stability via disulphide bonds and its unfolding

Protein stability arises from the free energy difference between its folded and unfolded (denatured) states [48]. The protein structure is held together by several non-covalent interactions including hydrogen bonding, electrostatic interactions, van der Waal’s forces and hydrophobic effects and it is very stable under physiological condition. Extreme conditions like high temperature, high pH or the addition of organic solvents can easily led to protein unfolding due to changes in its conformation [49, 50].

The main area of interest here is to break the disulfide linkages (S-S) found within the protein which are known to increase its thermal stability. The formation of disulfide bonds is an example of oxidative and reversible reaction [51]. Hence, the addition of a reducing agent such as dithiothreitol (DTT) will promote the break-up of these disulfide bridges. Several studies reported DTT is not capable to reduce all available disulfides within some proteins without denaturants like urea or guanidine [52, 53]. The mechanism of disulfides reduction by DTT in water is shown in Figure 2.5.
In Figure 2.5, the first step of the reaction involves the formation of a mixed disulfide. During the second stage, the mixed disulfide is cleaved by another reductant molecule freeing the sulfhydryl and hence, the reducing agent is oxidised forming an intramolecular ring structure.

Another potential reducing agent is Tris-2-carboxyethylphosphine (TCEP) which is known as a stronger reductant than DTT [54]. Eyles et al. mentioned the temperature of HEWL unfolding was reduced by 24°C when one disulphide bond was broken in TCEP [55]. The addition of TCEP is expected to break more disulphide bridges and promote higher degree of protein unfolding. The mechanism of reduction of disulfides in TCEP is shown in Figure 2.6 and the cleavage of disulfide bond is known as the rate determining step (RDS) [56].

Figure 2.5 Two-sequential thiol disulfide exchange reaction for lysozyme in DTT (adapted from [54]).

Figure 2.6 Mechanism of disulfides reduction by TCEP in water (adapted from [54]).
In Figure 2.6, TCEP reacts with biological disulfides in order to cleave the S-S bond. The phosphine is oxidised to form stable phosphine that contains the oxide bond and also the formation of reduced disulfides. Hence, this suggests the presence of hydrophilic and hydrophobic interactions between reducing agent molecules and the interior of protein patches.

### 2.3.2.2 Lysozyme hydrogels as a tissue engineering scaffold

It is proposed to continue the work on developing lysozyme protein hydrogels for tissue engineering application that had been previously conducted by Yan et al. [2, 8, 35-37]. Yan et al. reported the formation of a thermoreversible lysozyme hydrogel using HEWL/water/DTT system [2, 8, 35-37, 47]. Lysozyme has a critical gelation concentration of 3 mM and its gelation can be achieved by heating the protein solution up to 85°C and then slowly cooling them down to room temperature. The lysozyme protein denatures and forms β-sheet fibrils that further entangled into a gel network in Figure 2.7.

![Figure 2.7 Schematic representation of lysozyme denaturation and gelation process [35].](image)

Upon heating, the lysozyme hydrogel is reversed to a solution. The small globular protein lysozyme is reported to be cytocompatible to living fibroblast cells suggesting lysozyme hydrogels may work successfully as cell scaffolds for tissue engineering. Their rheological properties revealed the relation of storage modulus to lysozyme concentration using a power law model [8, 35]. The HEWL hydrogels elastic modulus was found to scale as 2.43 above the critical gelation concentration indicating their network was densely cross-linked where segment length approximates the mesh size.
Most of the work carried out by Yan et al. focus on making lysozyme samples at pH 2, 7 and 12 [2, 35-37, 47]. It will be interesting to find out the mechanism and gelation behaviour of lysozyme in between pH 2 and 7 with and without dithiothreitol since this region is nearing to the physiological pH. Lysozyme is known to carry positive charges within this pH region.

Proteins and peptides are capable of self assembling in vivo and in vitro to form protein misfolding (in disease) and cell scaffolds respectively [57]. The use of X-ray diffraction (XRD) can help to describe the 3D internal structure of self-assembled proteins and peptides and also for better understanding of their self-assembling mechanisms [57]. Examples of XRD include small-angle X-ray scattering (SAXS) and wide-angle X-ray scattering (WAXS).

Some hydrogels have the ability to respond quickly to changes in temperature, pH, electric field and light by changing their swollen volume and mechanical properties [58]. As a result, these hydrogels are applied in the areas of biosensor development, microfluidics, tissue engineering and drug delivery due to their unique swelling, mechanics, permeability and optical properties. Hydrogels that respond to temperature change (thermoreponsive hydrogels) are reportedly used in biomedical applications; as cell carriers for tissue regeneration to cover wound and also for drug and gene delivery [28]. They can be engineered for faster rate of therapeutics release and its degradation.

2.4 Protein-polymer conjugation

2.4.1 Introduction

It has been identified that there is a lack of interactions between the seeded cells and the matrix (scaffold) especially when guiding the cells to form the desired tissue [59]. One way to tackle this problem is to conjugate bioactive molecules to hydrogels and hence, forming bioconjugate gels. This new combination enhances the ability to control the physical and biological properties of the gel system through the synthetic and biological components respectively [60].

Synthetic material like polyethylene glycol (PEG) and polysaccharides such as hyaluronic acid (HA) and heparin are examples of biomolecules that can activate the desired cell
functions [59]. Oss-Ronen and Seliktar have developed a novel hydrogel system containing both mono-PEGylated albumin and PEGylated fibrinogen. Albumin acts as the drug carrier while fibrinogen promotes tissue regeneration at the same time in a minimally invasive manner [60]. Another study reveals the reaction between PEG and heparin and their conjugation with RGD peptide actually formed a gel matrix with controlled elasticity, cell adhesion and survival [61].

Heredia and Maynard reported there are two possible routes to protein-polymer conjugation and they are shown in Figure 2.8 [13, 14].

![Figure 2.8 Possible routes to protein-polymer conjugation](image)

These include the postpolymerisation modification strategies and the new methods that synthesise the polymers directly from protein initiation sites. For examples, the pre-formed polymers can be modified to become amine-reactive, thiol-reactive or even used for click conjugate formation via crosslinkers to form well-defined conjugates with pre-determined properties such as molecular weight. The new method also results in the in-situ formation of well-defined protein-polymer conjugates at a faster rate.

### 2.4.2 Thermo-responsive poly-N-isopropylacrylamide (PNIPAAm)

PNIPAAm is one of the most widely studied polymers due to its lower critical solution temperature (LCST) nearing to the physiological temperature. LCST is defined as the temperature at which polymers exhibit phase transition since their solubility normally increases with decreasing temperature and vice versa [62]. PNIPAAm in water has a LCST of around ~32°C [6]. Below the LCST, PNIPAAm is hydrophilic and has a coil conformation. Above the LCST, the polymer undergoes coil-to-globule transition as the intermolecular hydrogen bonding between polymer and water breaks. This causes the
hydrophobic interactions between hydrophobic polymer segments to increase [63] and as a result, the polymer chains collapsed and aggregate to form two phases that is polymer suspended in water.

Figure 2.9 Examples of reversible phase separation of PNIPAAm (a) linear chains, (b) terminally-grafted PNIPAAm surfaces and (c) multi-point grafted PNIPAAm surfaces (adapted from [42, 64]).

Several studies show the use of PNIPAAm and its co-polymers to form thermo-responsive conjugates suitable for its desired end application [28]. These include for drug and vaccine carriers and also tissue engineering. Lowe et al. reported the formation of PNIPAAm and poly-(L-lactic) acid (PLLA) conjugates that formed nanoparticles in aqueous solution and used as therapeutic delivery agents to tumour tissues [65].

2.4.3 RAFT polymerisation

PNIPAAm can be synthesised from its monomers, NIPAAm using different polymerisation techniques including the Reversible Addition Fragmentation Transfer (RAFT) polymerisation [66-69]. RAFT polymerisation is a reversible deactivation radical polymerisation that focuses on the transformation or removal of polymers using the thiocarbonylthio (S=C(Z)S-R) group found in the RAFT agent [68]. It is relatively cheap and straightforward to implement and also offers good control over the radical polymerisation of monomers and high compatibility with different reaction conditions. The mechanism for RAFT process is shown in Figure 2.10.
Figure 2.10 Mechanism for reversible addition-fragmentation chain transfer (RAFT) [67].
Chapter 3  Materials and methods

3.1 Introduction

This chapter introduces the materials used to make the lysozyme hydrogels and lysozyme-PNIPAAm conjugates, their preparation methods and also the experimental techniques involved in the characterisation of these hydrogels.

3.2 Materials used

Powdered hen egg white lysozyme (HEWL, cat# L6876), dithiothreitol (DTT, cat# 43817) and tris-2-carboxyethylphosphine (TCEP, cat# C4706) were bought from Sigma and used as received without further purification. The molecular weight (MW) of HEWL, DTT and TCEP are 14,300 g mol$^{-1}$, 154.25 g mol$^{-1}$ and 286.65 g mol$^{-1}$ respectively. Deuterium oxide (D$_2$O, cat# 151882), 12.39 M hydrochloric acid (HCl, cat# 320331) and sodium hydroxide pellets (NaOH, cat# S5881) were also purchased from Sigma and used to adjust the pH of the lysozyme samples.

The reducing agents DTT and TCEP have a MW of 154.25 and 250.19 respectively with their molecular structure shown in Figure 3.1.

![Molecular structure of disulfide reductants](image)

Figure 3.1 Molecular structure of disulfide reductants (a) DTT and (b) TCEP.

The differences in the reaction of DTT and TCEP with biological disulfides are explained in Figures 2.5 and 2.6.
To synthesise the lysozyme-PNIPAAm conjugates the following reagents were used; N-Isopropylacrylamide (NIPAAm, cat# 415324), 2-(dodecylthiocarbonothioylthio)-2-methylpropanoic acid (DMP, cat# 723010), azobisisobutyronitrile (AIBN, cat# 441090), bismaleimidohexane (BMH, cat# 22330), butylamine (cat# 471305), diethyl ether (DEE, cat# 309966), dimethylformamide (DMF, cat# D4551), dimethyl sulfoxide (DMSO, cat# D1435), hexane (cat# 296090), phosphate buffer saline (PBS, cat# PBS1) and tetrahydrofuran (THF, cat# 270385). All reagents were purchased from Sigma except for BMH which was supplied by Thermo Scientific. NIPAAm was purified by recrystallisation from hexane and stored in the freezer at -20°C until needed.

### 3.3 Sample preparation

#### 3.3.1 Protein gels

Most HEWL samples were prepared at 2, 3 and 4 mM concentration as below.

<table>
<thead>
<tr>
<th>Lysozyme concentration (mM)</th>
<th>Lysozyme concentration (mg mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>28.6</td>
</tr>
<tr>
<td>3.0</td>
<td>42.9</td>
</tr>
<tr>
<td>4.0</td>
<td>57.2</td>
</tr>
</tbody>
</table>

HEWL was dissolved in distilled or deuterated water to form non-reductive samples at 2, 3 and 4 mM. Meanwhile, 20 mM DTT and 15 mM TCEP solutions were prepared and then used to dissolve the powdered protein to form HEWL/DTT and HEWL/TCEP solutions respectively. The resulting protein solutions were agitated using a vortex mixer for 100 seconds. A few drops of 1.2 M HCl and 0.5 M NaOH were added to the samples as necessary to obtain the required pH. These were checked using a pH meter.

The samples were again agitated before being placed in a heating block and heated at 85°C for different times. The length of heating time to form gels varied for different pH. For example, HEWL/water samples at pH 2 were heated for 3 days while pH 3 and pH 4 HEWL/DTT samples were heated for 1 day and pH 7 HEWL/DTT sample for 1 hour in
order to form gels. After heating, the samples were left to cool down at room temperature for about 45 minutes and they were then immediately used for characterisation experiments.

3.3.2 Protein-polymer conjugates and the final composite gels

To prepare the lysozyme-PNIPAAm gels, the synthetic polymer PNIPAAm is first synthesised with end-group modification and then mixed with excess linker and lysozyme to form the conjugate. A small fraction of the conjugate will be further mixed with gelling lysozyme to form the final composite gel. The overall step-by-step mechanism involved in the preparation of this bioconjugate is explained below.

The first step (Figure 3.2) is the free-radical polymerisation of NIPAAm to form PNIPAAm using RAFT agent and this is to allow control over the polydispersity of the polymer [66, 68-70].

![Figure 3.2 Modification of NIPAAm with the initiator AIBN and RAFT agent to form the RAFT-PNIPAAm via free-radical polymerisation.](image)

The RAFT agent used (Figure 3.3) is 2-(dodecylthiocarbonothioylthio)-2-methylpropanoic acid (DMP) which contains trithiocarbonates next to its C=S functionality.

![Figure 3.3 Molecular structure of the RAFT agent DMP.](image)
In Figure 3.2, DMF was degassed for 30 minutes with the nitrogen gas. NIPAAm and RAFT agent were weighed and added into a schlenk tube together with a small magnetic stirrer. The content was then carefully purged with nitrogen even after the addition of degas DMF later. While waiting for the reaction mixture to equilibrate at 70°C, the required AIBN was prepared in a separate schlenk tube placed over ice. 1 mL of degas DMF solution was added to it under nitrogen gas. Using a syringe purged with nitrogen, the AIBN solution was added to the monomer/RAFT solution when the temperature stabilised at 70°C. The final mixture was purged with nitrogen again for 5 minutes before being sealed and left to react for at least 8 hours.

After 12 hours, the reaction mixture was exposed to air to quench further radical process. The mixture was evaporated using rotator evaporator to remove the DMF solution. The residue left behind was re-dissolved in THF solution to form a concentrated and non-viscous solution. Using a plastic pipette, few drops of the latter solution were slowly added onto cold DEE solution to precipitate out the RAFT-PNIPAAm. The total volume of DEE solution used was 10 times more than THF and placed in at least 4 centrifuge tubes which were refrigerated for an overnight. RAFT-PNIPAAm was then filtered from DEE and allowed to dry in the fumehood to form a yellow solid.

The second step (Figure 3.4) is the aminolysis of RAFT-PNIPAAm to produce thiol-terminated PNIPAAm (PNIPAAm-SH) [68, 69].

![Diagram of RAFT-PNIPAAm and PNIPAAm-SH](image)

Figure 3.4 RAFT-PNIPAAm end group modifications involving amine cleavage followed by phosphine-mediated thiol-ene reactions.

The result of this reaction was visually followed whereby yellow RAFT-PNIPAAm dissolved in DMF solution became colourless after aminolysis. Initially DMF solution was degassed with nitrogen for 30 minutes and the heater was set at 30°C. RAFT-PNIPAAm
and TCEP were weighed in a schlenk tube with the addition of a magnetic stirrer and the tube was degassed with nitrogen. DMF was added to the tube content and continued to be purged with nitrogen for 10 minutes. Using a nitrogen-purged micropipette, the required butylamine was added and the content was degassed for another 10 minutes. The reaction was sealed and allowed to react at 30°C for 12 hours before a transparent solution was obtained. The polymer was recovered by evaporating DMF first and subsequently precipitation in cold DEE solutions twice. PNIPAAm-SH was filtered from DEE and dried overnight to form a white solid.

The third step (Figure 3.5) is the crosslinking of PNIPAAm-SH to lysozyme-SH via a homobifunctional crosslinker BMH at a very small quantity. This was followed by their dialysis against water to remove the unwanted products.

![Figure 3.5 Schematic representing the click approach to polymer-protein bioconjugate.](image)

1. Add BMH in DMF to PNIPAAm in PBS

2. Add mixture to lysozyme-TCEP, 25°C and 12 h

PNIPAAm-SH

PNIPAAm-S-BMH-S-lysozyme

The cross-linker used (Figure 3.6) is Bismaleimidohexane (BMH) which has a MW of 276.29 [71]. It contains a non-cleavable, 6-atom spacer between terminal maleimide groups that create a 16.1Å bridge between conjugated macromolecules.

![Figure 3.6 Molecular structure of BMH with terminal maleimides that can react with sulphydryls between pH 6.5 and pH 7.5.](image)
In Figure 3.5, HEWL, aminolysed PNIPAAm and BMH were weighed separately in 2 mL vials. The required amount of HEWL was dissolved in 15 mM TCEP solution and it was immediately incubated in the fridge at 4°C for at least 2 hours. The needed amount of BMH linker was then dissolved in 0.5 mL of DMF solution while PNIPAAm-SH was dissolved in PBS solution.

The BMH solution was added to the PNIPAAm solution with the former at a concentration of 2 or 3-fold in molar excess than the latter. The mixture was then vortexed and incubated at the room temperature for an hour. The HEWL-TCEP solution was later mixed with the PNIPAAm-BMH solution in a 15 mL centrifuge tube. This final mixture was allowed to react for an overnight and it was immediately subjected to water dialysis for 2 days in order to remove the excess and unreacted polymer-maleimide linker. A dialysis cassette of slide-a-lyzer type (Figure 3.7) with a molecular weight cut-off (MWCO) of 10kDa was used. It can hold a sample volume between 0.5 mL and 3 mL which is suitable for mixing the bioconjugate.

![Slide-A-Lyzer Dialysis Cassette with MWCO of 10kDa.](image)

Figure 3.7 Slide-A-Lyzer Dialysis Cassette with MWCO of 10kDa. It is used to remove low molecular weight contaminant which is the unreacted polymer-linker while lysozyme is retained inside the cellulose membrane.

The final step involves in further physical mixing of bioconjugate formed in Figure 3.5 with lysozyme in TCEP solution prepared in Section 3.3.1. The final mixture was heated at 85°C for different times depending on the sample pH to form the final composite gel.
3.4 Micro differential scanning calorimetry (MicroDSC)

3.4.1 Introduction

MicroDSC experiments were carried out to examine the effect of different protein concentration, pH and DTT addition on the thermal behaviour of the lysozyme protein. Its theory and the experimental method involved are discussed here.

3.4.2 Principle

Thermal analysis is a study of the correlation between a sample’s physical property and its temperature while the sample is heated or cooled in a controlled manner [72]. The heat flow rate is measured as a function of the temperature when using a DSC. To be more precise, it is the difference in the heat flow rate (or electrical power difference) that travelled into a sample and a reference material that is measured [72] while they are being subjected to a temperature-controlled programme [73].

MicroDSC rather than DSC is used for the investigation of lysozyme thermal behaviour due to microDSC’s higher sensitivity in scanning samples with small enthalpies of transition [47]. Modern microDSCs try to measure the precise amount of heat energy taken in or released during the experiment which in this case reflected the phase transitions of the lysozyme protein [73]. These phase transitions include the folding and unfolding of the protein [47]. In addition, microDSC also records the characteristic temperatures of the transition and the partial heats developed within a temperature interval [73].

A twin calorimeter (Figure 3.8) which is electrically operated is used to reduce the influence of disturbances such as heat leaks [74]. It contains two identical cylindrical measuring systems; one for the reference and the other for the sample which are separated from each other but positioned in the same block. Each measuring system is connected to a heater to heat the reference and sample separately. Heat exchange must first take place before heat flow can be measured. The temperature of the surroundings inside the block remains constant [73] due to gilts cylinder which act as the thermal buffer and also the heat insulated material used [47]. This type of calorimeter belongs to power compensation DSC whereby the heat to be measured is compensated with electric energy [73].

41
In a power-compensated DSC, both sample and reference are maintained at the same temperature by varying the power input to the two furnaces [75, 76]. The difference in thermal power (energy) needed to maintain them at the same temperature is measured and plotted as a function of temperature [75]. This energy is a direct measurement of the enthalpy change, $\Delta H$ of the sample holder relative to the reference [76]. In Figure 3.8, the power-compensated DSC is equipped with proportional, integral and differential (PID) temperature control of the sample holder. The output signal exhibits temperature delay and an overshoot [77] which formed peak areas when $\Delta H$ is plotted against temperature. Tanaka (1992) demonstrated that the peak area recorded from a PID temperature controlled sample holder is exactly equal to the enthalpy change which is shown in Equation 3.1 [77].

$$\Delta H = \int_{0}^{\infty} p(t)dt \quad \text{--- Equation 3.1}$$

$p(t)$= output signal as a function of time

### 3.4.3 Experimental procedure

A SETARAM micro differential scattering calorimeter III was used to run the microDSC experiments. Both reference and sample cells were filled with 0.5 mL solutions (not gels) using a plastic pipette. If the sample was protein/DTT/water then the reference would be DTT/water. It is important to ensure the presence of an identical mass of solution in both cells before and after each experiment. This would help to measure the lysozyme thermal contribution accurately and also to show any loss of materials during the experiment.
through evaporation. The cells lids were tightened closely using a metal spanner and the samples were placed in their respective compartments.

Several parameters were set before running the experiment including a scanning rate of 1°C min⁻¹ with temperatures ranging between 15°C and 85°C. Each sample experienced two cycles of heating and cooling that lasted approximately 7 hours as shown in Figure 3.9.

![MicroDSC temperature profiles showing the isothermal and also two heating/cooling ramps.](image)

Figure 3.9 MicroDSC temperature profiles showing the isothermal and also two heating/cooling ramps.

Samples were initially cooled down from 21°C to 15°C at a cooling rate of 1°C min⁻¹. This was followed by an isotherm delay at 15°C for 900 seconds. Samples were then heated from 15°C to 85°C at a heating rate of 1°C min⁻¹. They remained at 85°C for 900 seconds before being cooled down again to 15°C. These whole processes were repeated for a second and third cycle. In the end, the temperature inside the microDSC was restored to 21°C.

Due to limited time, some of the microDSC experiments were only repeated twice not thrice in order to show reproducibility. The microDSC thermographs obtained were then analysed using the supplied SETARAM Soft 2000 software and the results recorded include the peak temperature, the onset point temperature and also the associated enthalpy.
3.5 Rheology

3.5.1 Introduction

The mechanical properties of the lysozyme solutions or gels were investigated by performing rheological studies. The principle of rheology and the experimental method used are explained here.

3.5.2 Principle

Rheology involves studying the deformation of materials when they are subjected to a small or large force [78]. This force is normally referred to as the stress (force per unit area). The sample is placed in between two plates; the lower and upper plates. The lower plate is fixed while a shear stress is applied to move the upper plate. As a result, the sample in between would experience a change in velocity. A viscous liquid sample would flow and dissipate most of the extra energy as heat energy. Meanwhile, an elastic solid sample would deform and store most of this energy until the removal of the force and then would return to its original shape.

Hydrogels are a good example of materials that can be considered as both solid and liquid and are termed as viscoelastic. These gels are mainly comprised of water trapped in an entangled network even though the gels looked elastic. It is the proportion of their solid-like (elastic) and liquid-like (viscous) behaviour which are of interest and can be measured using the rheological technique. An oscillating strain at a given frequency is applied to the sample. After the sample is constrained, an oscillating stress developed as a response to the strain. Strain is defined as the relative deformation or deformation per unit length [78].
Three important parameters are derived from the two waveforms in Figure 3.10 namely the phase angle $\delta$, the storage modulus $G'$ and the loss modulus $G''$. The phase angle is the difference between the peak value of the strain $a_o$ and the peak value of the stress $b_o$. In theory, it is equal to zero for an elastic solid and has a value of $90^\circ$ for a liquid.

The storage modulus $G'$ is sometimes also referred to as the elastic modulus. It measures the stored energy representing the elastic aspect of the gel behaviour and is defined by Equation 3.2.

$$G' = \left| \frac{a_o}{b_o} \right| \cos \delta \quad \text{--- Equation 3.2}$$

Meanwhile, the loss modulus $G''$ is also referred to as the viscous modulus which measures the energy dissipated as heat, representing the viscous aspect of the gel behaviour. The loss modulus is defined by Equation 3.3 below.

$$G'' = \left| \frac{a_o}{b_o} \right| \sin \delta \quad \text{--- Equation 3.3}$$
3.5.3 Experimental procedure

The AR-G2 magnetic bearing rheometer (TA Instruments) shown in Figure 3.11 was used for the rheological experiments. It had two plates running parallel to each other with a gap size of 250nm. The peltier plate acted as the stage or bottom plate and it is in a fixed position. An integrated magnetic cylinder was inserted to a shaft and hence attached to the equipment to become the upper plate. The upper component was rotated by a torque produced by an induction motor. The computer software configured the unique geometry of this cylinder which was identified as 20mm stainless steel flat plated geometry. 1 mL of sample was then pipetted and loaded onto the peltier plate for every experiment. The upper steel plate was then slowly lowered until the desired gap between plates was reached. Any excess sample was wiped off to reduce evaporation.

![Image](image1)

Figure 3.11 The AR-G2 Rheometer (TA Instruments) whereby sample was placed on the surface of stage plate and then slowly compressed by the upper plate.

There were four types of rheological experiments carried out at 25°C namely amplitude (or strain), oscillatory frequency, time and temperature sweep. For the amplitude sweep, the % strain was set between the values of 0.01 and 100 with a steady frequency of 1 Hertz (Hz). Frequency sweeps were then carried out on the samples by varying the frequency from 1 to 100 Hz while controlling the strain at either 0.5% or 1%. The chosen % strain must lie within the mid-section of linear viscoelastic region derived from the amplitude sweep results.
For time sweep experiments, the samples were subjected to pre-shear, shearing and subsequently post-shear stages in order to study their recovery behaviour. During the second stage, samples were sheared at 150% strain for several minutes and their ability to restore $G'$ was monitored for its possible application as an injectable material.

The temperature sweep tests were conducted to compare the response of lysozyme-PNIPAAm and lysozyme gels to an increase or decrease in temperature by measuring their moduli. Samples were heated and cooled twice within the temperature range of 25-85°C with 2°C increment/decrement. For these experiments, a thin layer of paraffin oil was placed around the sample peripheral together with a solvent trap in order to minimise evaporation. All experiments were repeated three times to ensure reproducibility.

### 3.6 Fourier-transform infrared (FT-IR) spectroscopy

#### 3.6.1 Introduction

The main aim of doing FT-IR spectroscopy is to study in detail the secondary structural transition that lysozyme experienced before and after heating. The effect of varying the protein concentration, pH and also the addition of DTT on the composition of the protein’s secondary structure was also investigated. FT-IR theory and the method involved in carrying out this experiment are discussed in this section.

#### 3.6.2 Principle

Infrared (IR) spectroscopy utilised the vibrations of the atoms of a molecule [79] for the measurements of emission intensity which are recorded as infrared spectra. The term vibrations include the stretching and bending movements of the molecules. An example of a modern infrared spectrometer is the FT-IR spectrometer which made use of the mathematical method Fourier transformation. FT-IR spectroscopy offered quick and reliable FT-IR data processing, conversion and measurement due to the recent tremendous improvement in computing capability.
An FT-IR spectrometer works according to the mechanism in Figure 3.12 where the source generated an infrared light which is forced to pass through an interferometer and converted into a signal before reaching the sample and being detected. The signal is amplified and converted to a digital form by an analogue-to-digital converter and transferred to the computer where Fourier transformation occurred.

![Diagram of FT-IR spectrometer components](image)

**Figure 3.12** The basic components of an FT-IR spectrometer. Diagram adapted from [79].

The processes that take place at the interferometer are illustrated in Figure 3.13 and are the most crucial ones as they set apart the mechanism of FT-IR spectrometer from other types of infrared spectroscopy.

![Diagram of FT-IR interferometer mechanism](image)

**Figure 3.13** A schematic diagram of the detailed mechanism of FT-IR interferometer. Diagram adapted from a Michelson interferometer [79] and an optical schematic of an IR interferometer [80].
A single beam of infrared light is produced from a hot wire (the source) and it is then split into two; a transmitted beam and an incidence beam upon reaching the beam splitter. These two beams are reflected from the mirrors and returned to the beam splitter to recombine and then split again shortly afterwards to form another two new beams.

The first beam returns to the source while the second beam emerges from the splitter at a perpendicular position to the first beam. This second beam (also called interferogram) is dependent on the distance between the moving mirror and the beam splitter and it is eventually detected in FT-IR spectrometry, amplified and converted using Fourier transformation. Since a single beam is used to generate an FTIR spectrum, the background and sample spectra must be collected separately.

The most sensitive spectral region for the analysis of protein secondary structural composition and conformational changes is the amide I band (1700-1600 cm⁻¹) [81]. This IR absorption band arises from the C=O stretch vibrations of the peptide linkages. In the amide I region, each type of secondary structure leads to a different C=O stretching frequency because of unique molecular geometry and hydrogen bonding pattern[81] and their correlations are shown in Table 3.2.

Table 3.2 Correlations between common protein structures and amide I frequency of Fourier Transform Infrared (FTIR) Spectroscopy (adapted from [82]).

<table>
<thead>
<tr>
<th>Structure</th>
<th>Amide I frequency (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>antiparallel β-sheet / aggregated strands</td>
<td>1675-1695</td>
</tr>
<tr>
<td>3₁₀-helix</td>
<td>1660-1670</td>
</tr>
<tr>
<td>α-helix</td>
<td>1648-1660</td>
</tr>
<tr>
<td>unordered</td>
<td>1640-1648</td>
</tr>
<tr>
<td>β-sheet</td>
<td>1625-1640</td>
</tr>
<tr>
<td>aggregated strands</td>
<td>1610-1628</td>
</tr>
</tbody>
</table>
3.6.3 Experimental procedure

FTIR spectra were collected in transmission mode using a Nicolet 5700 spectrometer manufactured by Thermo Electron Scientific Instrument Corporation. The protein samples were dissolved in deuterium oxide (D$_2$O) rather than distilled water (H$_2$O) because the O-H bend in distilled water will overlap in the same region as the amide I band of the protein [82].

1 mL of sample was placed in the sample holder using a plastic pipette or spatula for scanning. Each sample was scanned 256 times with a resolution of 4 cm$^{-1}$ and was subjected to attenuated total reflectance (ATR) correction. Spectra of pure D$_2$O and DTT/D$_2$O were first collected as backgrounds and later subtracted from the sample spectra of lysozyme/D$_2$O and lysozyme/DTT/D$_2$O respectively. All of the collected spectra had absorbance (measured in arbitrary units) as the y-axis and wavenumbers (measured in cm$^{-1}$) as the x-axis.

Using the OMNIC software version 7.2, the spectra were smoothed and their baselines were corrected. Fourier Self Deconvolution (FSD) was performed by adjusting the bandwidth and enhancement so that the recorded FSD trace matched the original spectrum. FSD-curve fitting was then performed on the original spectrum with the aim to mathematically create individual peaks that summed up to the original data[83].

The same profile was chosen for all peaks including the use of combined Gaussian-Lorentzian curves, constant baseline and the value of full width at half height (FWHH) was set as 10. The number of peaks on the original spectrum was manually located and set to the number suggested by the structures in Table 3.2. The peaks were resolved to produce a good fit to the protein spectrum with a standard error of less than 7%. The ratio of peak areas within the amide I region was used[83] to estimate the percentages of α-helix, β-structure, turns and unordered structures in the protein secondary structure [79] as a function of protein concentration, pH and the absence or presence of reducing agent.
3.7 Microscopy Imaging

3.7.1 Transmission Electron Microscopy (TEM)

3.7.1.1 Introduction

TEM experiments were performed to explore the fibrillar structure of protein hydrogels formed under different conditions. The TEM theory and its experimental procedure are explained here.

3.7.1.2 Principle

TEM is well-known for its ability to magnify nanoscale materials with sizes ranging from 1nm to 100nm [84] using a magnification factor of $10^3$ to $10^6$ [85]. This technique involves in imaging and measuring of matters which will be useful for the nano-characterisation of biomaterials [84] such as fibrils in protein hydrogels.

Electrons are needed to create the TEM images [86]. A beam of highly energetic electrons are first produced by the tungsten filament cathode found in an electron gun chamber. This beam of electrons is accelerated by the anode also found in the gun chamber [86] and then focussed onto the thin specimen using several vertical electromagnetic lenses that formed a lens column [85]. The specimen is placed on the specimen stage which allows it to be moved or held in a stationary position.

The electrons from the beam would be transmitted to the transparent part of specimen [84] and the rest of electrons are scattered out of the sample. The difference between transmitted and scattered electrons would result in contrast for the individual areas of specimen [86]. With the help of negative staining, a final image of the sample is formed on the monitor of an electronic camera system [85].

The detailed interactions between electrons in the beam and atoms in the specimen that resulted in scattering electrons are exemplified in Figure 3.14. Flegler et al. reported that the electrons interact with the positive nuclei of atoms in the sample to produce elastically scattered electrons that are scattered through wide angles with no energy loss [86].
Inelastic scattered electrons are formed when electrons in the beam interacted with electrons in the specimen atoms resulting in slight deviation in its path. Sometimes unscattered electrons that pass through the specimen are also produced [86].

![Diagram of specimen-beam interactions](image)

Figure 3.14 Specimen-beam interactions that resulted in elastic and inelastic scatterings of electrons leading to an image formation. Diagram adapted from [86].

Williams and Carter mentioned that one of the major limitations in using TEM is the need to produce and use thin specimens since it could largely affect the final imaging structure [84]. A thinner specimen is suspected to be better since it allows transmission of sufficient electrons and hence enough intensity falling onto the screen. This would result in a good image that can be easily interpreted within a reasonable amount of time.

### 3.7.1.3 Experimental procedure

TEM experiments were carried out using a Joel 1220 TEM operating at 100 keV. All lysozyme samples were agitated vigorously especially the gels and then diluted between 25-fold and 300-fold. Carbon-coated copper grids were ionised for 30 s. The shiny surface of the grid was placed on a 10 µL drop of the resulting sample solution for 10 s. The grid was then placed on a 10 µL drop of distilled water for 10 s and blotted against a filter paper. This washed grid was subsequently placed on a 10 µL drop of 2% (w/v) uranyl acetate solution for negative staining for about 60 s before being blotted again. The sample was transferred into the TEM chamber for imaging.
3.7.2 Atomic Force Microscopy (AFM)

3.7.2.1 Introduction

AFM provides individual particle imaging with three-dimensional (3D) display and direct measurements of height and volume at a lower cost than TEM [87, 88]. It also requires minimal sample preparation at ambient temperature. This technique is used in Chapter 6 for studying the surface morphology of lysozyme fibrils and to confirm the presence of cross-linking polymers onto these fibrils surface. Its theory and sample preparation technique used are also discussed.

3.7.2.2 Principle

AFM works by measuring force between the probe (cantilever) tip and the sample when they are brought to close proximity but never in contact with each other. In Figure 3.15 upon receiving a laser beam, the cantilever deflects and the reflected laser beam strikes onto a photo detector which is position sensitive. Photo detector measures the lateral and vertical deflections of the cantilever and these signals will go to the amplifier for data collection and also being feedback to the sample stage to control sample position [88-90].

Figure 3.15 A schematic diagram of AFM layout showing five components; the sample, cantilever, laser, photo detector and differential amplifier [89, 90].
3.7.2.3 Experimental procedure

A Multimode-Picoforce Bruker Atomic Force Microscope was used for imaging protein fibrils. The sample was diluted 25-fold and vortex for few minutes before being deposited onto freshly cleaved mica. An adhesive tape is used to fix the sample on a substrate. It is noted that AFM particle imaging requires the fibrils to be rigidly adhered to and dispersed on a substrate (mica). The substrate was then placed onto the sample stage and image-scanned. The image was retrieved and analysed using NanoScope Analysis software.

3.8 X-Ray Fibre Diffraction (XRD)

3.8.1 Introduction

XRD aims to offer more information on the internal structure of self-assembled HEWL fibrils from X-ray scattering data which is not obtainable from other analytical techniques. Often the fibre diffraction pattern of amyloid fibrils showed a cross-β characteristic with signals at 4.7Å and 10Å [91]. This characteristic will be checked for lysozyme samples under reductive and non-reductive conditions and also to show whether their intensity signals are correlated to the level of β-sheet content or the effects of adding HCl or NaOH. The principle of XRD and its sample preparation are also mentioned here.

3.8.2 Principle

X-ray diffraction studies use Bragg’s Law to examine and predict the structure of crystals and molecules. According to the Bragg’s Law in Equation 3.4, the X-ray beam directed onto a plane surface will reflect at the same angle $\theta$.

\[ n\lambda = 2d\sin\theta \quad \text{--- Equation 3.4} \]

$n = \text{an integer}$

$\lambda = \text{the wavelength of the X-ray}$

$d = \text{the spacing between different layers of atoms in a crystal}$

$\theta = \text{the angle between incident ray and the scatter plane}$

Fibrous and polymeric molecules have a three-dimensional lattice with ordered and repetitive arrangement along a fibre axis. Such molecular organization of fibrous
molecules resulted in X-ray diffraction scattering with a special characteristic of 4-quadrant symmetry which is shown in Figure 3.16.

Figure 3.16 XRD patterns showing for 4-quadrant symmetry where meridian is along the fibre axis.

3.8.3 Experimental procedure

4 mM gels were used to ensure the presence of enough fibrils for structural analysis. Some samples were purposely diluted for better results of alignment and fibre diffraction pattern. A 10 µL droplet of solution was rested between two ends of wax-tipped capillary tubes which were placed with a gap of about 0.5-2mm on a plastic Petri-dish (Figure 3.17(a)). The sample drop was slowly stretched to increase the fibrils alignment as illustrated in Figure 3.17(b) and left to air-dry overnight at room temperature to form a fibrous crystal.
Figure 3.17 The stretch-frame alignment of XRD sample. (a) A drop of fibre-containing solution was placed between the ends of two capillaries using a pipette. (b) Fibre-axes orientation (i) Fibrils were initially in random direction (ii) Upon sample-stretching, fibrils started to re-arrange themselves and became closer and (iii) After drying, fibrils were perfectly aligned with each other and ready for scan. Adapted from [91].

The aligned sample was then mounted on a goniometer head and tested on a Rigaku CuKα (\(\lambda = 1.5419\text{Å}\)) rotating anode generator with VariMax-HF mirrors and a Saturn 944+ CCD detector with exposure times of 10-400 s and a specimen-to-detector distance of 50nm or 100nm. Mosflm was used to display the diffraction data which were then converted into TIFF file for further pattern analysis using CLEARER.

3.9 Nuclear Magnetic Resonance (NMR)

3.9.1 Introduction

NMR spectroscopy is a powerful technique for getting the chemical, physical and structural information on molecules. In this work, \(^1\text{H}\) NMR spectroscopy (proton spectroscopy) is used to determine the molecular structure of synthesised PNIPAAm polymer with respect to hydrogen-1 nuclei and most importantly for estimation of its chain length. The principle of NMR and its experimental procedure are also explained here.
3.9.2 Principle

NMR is based on the concept of spin in nuclei. Nuclei experience no spin, integer spin and half-integer spin based on the number of protons or neutrons they have. In the absence of a magnetic field, the nuclei spin randomly. Nevertheless, they will arrange themselves with or against the larger applied field when there is a magnetic field. Nuclei will adapt either the $\alpha$-spin or $\beta$-spin state as illustrated in Figure 3.18.

![Figure 3.18 Nuclei spin state in response to an external magnetic field.](image)

The $\alpha$-spin state has a lower energy than $\beta$-spin state. Their energy difference increases as the strength of applied magnetic field increases. The emitted energy difference led to the formation of an NMR signal.

3.9.3 Experimental procedure

A 400 MHz Bruker NMR spectrometer was used for carbon and proton detection in the sample. 10 mg of polymer was dissolved in 2 mL D$_2$O or preferably DMSO solution and the sample was vortex for 100 s before being transferred into the NMR tube. The tube was placed in a spinner and its position was corrected using depth gauge. It was then inserted in the auto sampler carousel and scanned 64 times. SpinWorks 3.1.8.1 software was used for NMR analysis.

A NMR spectrum typically exhibits the shape of the signals and their chemical shifts in ppm on the horizontal scale. The spectrum was processed by performing a good phasing adjustment on the signal which affects the rise and fall of the baseline, followed by fully automatic baseline correction and integrating spectrum[92]. The region of interest is the
multiplets in the range of 3.6-4.5ppm reflecting the protons from the protein backbone. Two integrals were recorded within this region and one of these integrals has the value of 1. They were then compared with each other to generate the relative integral size that estimates the degree of polymerisation of synthesised PNIPAAm polymer.

3.10 Mass Spectrometry (MS)

3.10.1 Introduction

Mass spectrometry (MS) is a useful technique for measuring masses of particle in a sample. Time-of-Flight (TOF) MS is frequently used in Chapter 6 that covers work on synthesising thermo-responsive lysozyme-PNIPAAm conjugate whereby it is necessary to monitor the mass distribution of polymer particles and also for conjugate mass determination. Its theory and the experimental technique used are also discussed here.

3.10.2 Principle

A mass spectrometer is the equipment used to carry out MS experiments. It must have these five basic components shown in Figure 3.19 in order to operate; the sample inlet, ion source, mass analyser, ion detector and data collection.

![Figure 3.19 A schematic diagram of mass spectrometer showing its five essential components. Adapted from [93].](image)

The sample inlet and ion source are effectively combined in one step since electrospray ionisation (ESI) mass spectrometer is used. This ionisation technique involves in the spray of analyte solution (sample to be analysed) through a narrow capillary forming a spray of charged droplets. These droplets are immediately dried by heated counter-current nitrogen gas flow. In Figure 3.20 the charge density on the droplets surface is further increased as solvent evaporation occurs. When repulsion of like charges overcomes the surface tension of solvent droplets, “Coulombic” explosion happens resulting in ions formation.
Figure 3.20 Formation of analyte ions in the electrospray ionisation mass spectrometry (ESI-MS) via solvent evaporation and “Coulombic explosion”. Adapted from [93].

Meanwhile, a modern type of mass analyser called Time-of-Flight (TOF) mass analysis is used in the mass spectrometer. Figure 3.21 shows the separation of ions by the TOF analyser based on the kinetic energy and velocity of these ions.

Figure 3.21 Movement and separation of ions within the Time-of-Flight (TOF) region. Adapted from [94].
Ions enter TOF region in Figure 3.21 with constant kinetic energy shown in Equation 3.5.

\[ KE = \frac{1}{2}mv^2 = zV \quad \text{--- Equation 3.5} \]

- \( m \) = mass of the ion
- \( v \) = velocity of the ion
- \( z \) = charges on the ion
- \( V \) = voltage from the source

They will travel at different speed and arrive at the detector at different times based on their molecular mass. The time of flight, \( T_f \) is the time taken for an ion to travel through TOF region and is defined in Equation 3.6.

\[ T_f = \frac{L}{v} \quad \text{--- Equation 3.6} \]

- \( L \) = length of the drifting zone
- \( v \) = velocity of the ion

From Equations 3.5 and 3.6,

\[ T_f = L \sqrt{\frac{m}{z} \frac{1}{2} V} \quad \text{--- Equation 3.7} \]

The time of flight is directly proportional to the square root of mass-to-charge ratio (Equation 3.7). All of the ions produced in the source are detected and converted for data collection. As a result, a list of ions with different mass to charge ratio (m/z) is produced by using the TOF analyser.

It is known that the peaks in ESI spectrum represent the molecular ions and the charge (n) varies by 1 between neighbouring peaks. Equation 3.8 shows the relation between the charge state and the m/z [93].

\[ n_2 = \frac{m_1-1}{m_1-m_2} \quad \text{--- Equation 3.8} \]

- \( m_1 \) and \( m_2 \) = m/z of ions 1 and 2 in ESI spectrum
- \( n_1 \) and \( n_2 \) = number of charges on ions 1 and 2

The molecular weight (M) of the protein sample is determined by the arithmetic series shown in Equation 3.9 [93]. However, this method is useful when there are limited components in the feed mixture such as dialysed PNIPAAm-BMH-HEWL conjugate.

\[ M = n_2(m_2 - 1) \quad \text{--- Equation 3.9} \]
3.10.3 Experimental procedure

0.5 mg of powdered synthesised polymer or protein was dissolved in 1.5 mL of High Performance Liquid Chromatography (HPLC) water. The mixture was then vortex and subjected to filtration using a 0.20µm filter. The filtered solution was transferred into a 2 mL glass vial with perforated lid. The vial was then placed in the sample compartment for the Electrospray Ionisation Mass Spectrometer (ESI-MS) and subjected to scanning.
Chapter 4   Lysozyme gelation studies under reductive and non-reductive conditions

4.1 Introduction

Protein gelation is known to be influenced by a number of factors such as temperature, pH and the solvent used. This chapter compares the gelation behaviour of Hen Egg White Lysozyme (HEWL) in the presence of water and with the addition of a denaturing agent dithiothreitol (DTT). It was found out in previous studies of Yan et al. that lysozyme in water formed strong gels at pH 2 and in the presence of DTT strong gelation took place at pH 7 [2]. Hence, it is interesting to investigate the difference in protein behaviour within the range of pH 2 to 7 with and without DTT that has led to some gelation results in this work. Another important main aim of this study is to synthesise stronger lysozyme gels than in previously reported work and thus, improve their physical properties from a fundamental understanding of the gelation process. The design of the scaffold is highly important in determining the success of the gel to create and maintain a space for tissue development [6].

In Section 4.3, FTIR recorded changes in the protein secondary structural elements at different sampling conditions and further structural analysis were performed using XRD. The thermal behaviour of lysozyme in water and DTT were monitored using microDSC in Section 4.4 while the gels mechanical properties were studied using small shear oscillatory rheometry in Section 4.5. Using TEM, the difference in morphological network of heated and reduced protein fibres were observed. All results are correlated to give an insight of the physical properties of lysozyme gels formed in water and DTT.

4.2 Visual observations

It is known that the critical gelation concentration of reduced lysozyme is 3 mM [2]. Therefore, samples in this work were purposely prepared at 2, 3 and 4 mM in order to study the relationship between the protein secondary structure and the gels mechanical properties at different protein concentration. pH 2, 3, 4 and 7 HEWL/water and HEWL/DTT/water solutions were initially heated at 65°C for 15 minutes and later cooled at room temperature for 45 minutes to allow gelation to take place. The sample vials were
subsequently inverted to determine the nature of the matrix inside. A free-flow liquid after heating would indicate no gel formation.

At 65°C, no gels were formed under both reductive and non-reductive conditions. This is expected for lysozyme in water samples as the protein has a denaturation temperature of 75°C. The heating temperature was then adjusted and increased to 85°C to ensure protein denaturation. Heating can cause disruption to the hydrogen bonds and non-polar hydrophobic interactions that formed the protein secondary and tertiary structure. The hydrophobic patches in the core are also exposed such that they aggregate since they do not like to be in water. The native α-helix structure of protein has now uncoiled into a random shape and formed β-sheet fibrils causing the macroscopic gelation of samples.

The time taken to heat samples and form gels at different pH levels was varied by trial and error. It was found that strong colourless HEWL/water gels were formed at pH 2 after 3 days of heating at 85°C using 3 and 4 mM concentration. However, Yan et al. reported the formation of transparent gels at pH 2 after 2 days of incubation at 65°C [37]. This difference might originate from the final quality of powdered HEWL bought which could have different percentage purity of targeted protein. The non-reduced samples at pH 3, 4 and 7 remained as solutions after being incubated for more than 21 days. This is consistent with previous work, for example, Yan et al. observed no gelation for the pH 7 lysozyme-water sample even after 14 days of incubation [37].

Strong transparent gels were produced at pH 7 in the presence of DTT after 1 hour of incubation at 85°C (Figure 4.1(c)) except at 2 mM concentration where no gel formation occurred. Only weak gels were observed at pH 3 and 4 after 24 hours of heating under reductive conditions (Figure 4.1(b)). At pH 2, the reduced samples remained liquid-like after one day of heating and small traces of gels were only formed after 3 days of incubation (Figure 4.1(a)).
Figure 4.1 Typical photographs of hen egg white lysozyme samples reduced in DTT after heating at 85°C and cooled at room temperature for 45 minutes; starting from left (a) no gelation at pH 2, (b) a weak gel at pH 4 and (c) a strong gel at pH 7.

Hence, DTT addition has encouraged lysozyme gelation as the protein backbone became flexible due to disruption of their disulphide bridges. All of the lysozyme/DTT/water samples were later prepared using a standard procedure whereby pH 2, 3, 4 and 7 samples were heated at 85°C for 3 days, 1 day (both pH 3 and 4) and 1 hour respectively. The length of heating time used reflects the minimal time required to observe gels formation at each pH in the presence of DTT. The pH seems to affect lysozyme gelation behaviour under non-reductive and reductive conditions. All of these results are summarised in Table 4.1.
Table 4.1 Summary of visual gelation results obtained in the presence of water and DTT.

<table>
<thead>
<tr>
<th>pH</th>
<th>Length of heating time</th>
<th>Without DTT</th>
<th>DTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 2</td>
<td>3 days</td>
<td>2 mM- No gelation 3 &amp; 4 mM- Strong gels</td>
<td>Highly viscous liquids for all concentrations</td>
</tr>
<tr>
<td>pH 3</td>
<td>1 day</td>
<td>No gel formation</td>
<td>2 mM- No gel formation 3 mM- Weak transparent gel 4 mM- Weak transparent gel</td>
</tr>
<tr>
<td>pH 4</td>
<td>1 day</td>
<td>No gel formation</td>
<td>2 mM- No gel formation 3 mM- Weak transparent gel 4 mM- Weak transparent gel</td>
</tr>
<tr>
<td>pH 7</td>
<td>1 hour</td>
<td>No gel formation</td>
<td>2 mM- Highly viscous liquid 3 mM- Strong transparent gel 4 mM- Strong transparent gel</td>
</tr>
</tbody>
</table>

4.3 Protein secondary structural analysis for HEWL/water and HEWL/DTT samples

4.3.1 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR is employed to probe the secondary structure of lysozyme which consisted of $\alpha$-helix, $\beta$-sheet, turn and unordered structures that may vary in percentages depending on the sampling condition [78]. Fourier self deconvolution (FSD) helped to predict how much $\beta$-sheet fibrils were present in each sample which will be linked to the rheological, thermal and morphological results.
4.3.1.1 Effect of heat on lysozyme secondary structure

FTIR spectra were recorded for a pH 4 lysozyme sample without DTT both before and after heating at 85°C (Figure 4.2). Samples were dissolved in pure water (D\textsubscript{2}O) rather than H\textsubscript{2}O. H\textsubscript{2}O is a poor IR solvent with very intense absorbance in the 830-1540 cm\textsuperscript{-1} range including the amide I region [80]. To avoid the O-H bend in the amide I region, D\textsubscript{2}O was used instead [78].

Figure 4.2 Infrared spectra of 4 mM pH 4 lysozyme dissolved in D\textsubscript{2}O before (red) and after (green) heating at 85°C for a day and subsequent cooling at room temperature for 45 minutes.

Figure 4.2 shows the spectra of both samples were dominated by the amide I region of the protein, where a single peak was observed with a maximum absorption at ~1643 cm\textsuperscript{-1}. This result is consistent with the findings of Yan and also Byler and Susi who recorded a maximum absorption at 1653 cm\textsuperscript{-1} and 1654 cm\textsuperscript{-1} respectively for the native lysozyme at room temperature [35, 36, 47, 95].
The results in Figure 4.2 indicate non-reduced lysozyme still retained its $\alpha$-helix structure even after heating for 24 hours at 85°C. The sample spectrum became broader after heating especially within amide I region suggesting an increase in the formation of unordered structure (1620-1630 cm$^{-1}$). However, heating the protein for 24 hours at pD 4 would not guarantee its complete denaturation and aggregation into $\beta$-sheet structure. No visible peak was formed in the range of 1615-1625 cm$^{-1}$ to indicate $\beta$-sheet fibrils formation.

The amide II band (~1535 cm$^{-1}$) corresponds to N-H vibrations while the amide II’ band (~1450 cm$^{-1}$) corresponds to N-D vibrations. The addition of D$_2$O promotes the exchange of hydrogen to deuterium atom in the existing N-H protein bonds. However, not all hydrogen atoms were accessible for this exchange process because they were buried deep in the core structure of the protein. This explains the appearance of amide II band of the protein at ~1535 cm$^{-1}$. The absorbance of green spectrum was higher than the red one for amide II’ band which means more N-D bonds were present for heated sample compared to non-heated one. It can be concluded that heating the sample actually promotes the exchange of hydrogen to deuterium atom especially for the solvent-accessible hydrogens that are located on the surface of the protein.

Meanwhile, two small absorption bands were also observed at 1515 cm$^{-1}$ and 1585 cm$^{-1}$ which correspond to tyrosine residues and the ionised carbonyl group vibration respectively. Tyrosine is located in the hydrophobic core of a protein and its presence indicates the lysozyme hydrophobic core is not interrupted.

It is also observed that after the heated sample was cooled to room temperature its spectrum did not overlap with the non-heated sample spectrum. This indicates a low possibility for the lysozyme to renature and return to its native structure even after being heated without DTT. To summarise, heating a sample without DTT causes the protein to unfold to a certain extent that led to the formation of an unordered but not $\beta$-sheet structure. Consequently, DTT was added to encourage such structural transition and the formation of lysozyme gels.
4.3.1.2 Effect of adding DTT on lysozyme secondary structure

This section aims to study the effect of adding DTT on the percentages of lysozyme secondary structural elements especially on β-sheet fibrils formation. Protein gelation is known to be promoted by these fibrils formation. FTIR spectra were collected for pD 4 lysozyme samples dissolved in D$_2$O and DTT (Figure 4.3). Both samples were prepared by heating at 85°C for a day and subsequently cooled at room temperature for 45 minutes.

![Infrared spectra of 4 mM pD 4 HEWL/D$_2$O solution (green) and pD 4 HEWL/DTT gel (blue) where samples were formed by heating at 85°C for a day and then cooled at room temperature for 45 minutes.](image)

Figure 4.3 Infrared spectra of 4 mM pD 4 HEWL/D$_2$O solution (green) and pD 4 HEWL/DTT gel (blue) where samples were formed by heating at 85°C for a day and then cooled at room temperature for 45 minutes.

Figure 4.3 shows the presence of a new peak at ~1619 cm$^{-1}$ indicating the formation of β-sheet fibrils. This is in line with the weak gelation observed at pD 4 with DTT addition. It is also noticed that lysozyme still retained part of its native α-helix structure (1643 cm$^{-1}$) even after DTT addition. There was a significant decrease in tyrosine residues at 1515 cm$^{-1}$ for HEWL/DTT sample showing a higher degree of disruption to the inner core of protein which led to protein unfolding and later gelation. Less N-D protein bonds were formed in the amide II’ band for HEWL/DTT sample. This suggests that DTT focussed more on disrupting the disulphide bridges and hydrophobic interactions between amino acids in the interior rather than hydrogen bonding on the protein outer surface.
An iterative curve fitting procedure using a combination of Gaussian and Lorentzian curves was performed on both spectra with the help of Fourier Self Deconvolution. Yan reported the consistency in her results from FTIR curve fitting for dissolved lysozyme with previous X-ray data for crystalline lysozyme [47]. In Figure 4.4, each spectrum is deconvoluted to form smaller decomposed peaks within the range of 1400-1800 cm⁻¹ and these peaks often represent the different protein structural features based on the position of the peaks.

![Smoothed infrared spectra of pD 4 HEWL/DTT sample (light blue) and its smaller component peaks. The dotted line is the summation of the decomposed curves which tried to mimic the smoothed spectra with a standard error of ±7%.](image)

By calculating the area underneath each component curve, it is quantified that at pD 4 reduced lysozyme in DTT is consisted of 10% α-helix, 14% unordered, 27% turns, 38% intermolecular β-sheet and 12% anti-parallel β-sheet structures. Meanwhile pD 4 lysozyme in water contains 11% α-helix, 19% unordered, 42% turns, 22% intermolecular β-sheet and 6% anti-parallel β-sheet structures. The uncertainties in the estimation of secondary content from FTIR measurements in Figure 4.3 were calculated as 4.4% and 4.2% for lysozyme in water and DTT respectively.
From these results, it is observed that the addition of DTT promoted a higher degree of α-helix to β-sheet structure in comparison to α-helix to unordered and α-helix to turns transitions. More protein turns (27%) rather than coiled structure (14%) was found within the HEWL/DTT sample implying more linkages have been developed between α-helix and β-sheet strand in the presence of a reducing agent.

The HEWL/water sample still retained one-tenth of its native structure with no indication of peak formation at ~1619 cm⁻¹ in Figure 4.3. However, using FSD it is estimated that the heated lysozyme in D₂O contains a total of 28% β-sheet structure. Heating the protein without DTT has encouraged a high transition of α-helix to turns and unordered structure rather than α-helix to β-sheet structure. This confirms that heating can only disrupt the bonding interactions on the outer surface of protein causing a limited degree for protein to unfold, denature, aggregate and form fibrils. To summarise, the addition of DTT to pD 4 lysozyme has promoted more α-helix to β-sheet structural transition. The pD 4 HEWL/DTT sample contains 48% β-sheet structure which was 10% higher than the non-reduced lysozyme at pD 4.

4.3.1.3 Effect of protein concentration on lysozyme secondary structure

Another area of interest is the effect of protein concentration on the degree of structural transition and β-sheet formation for reduced samples. pD 4 HEWL/DTT samples were prepared at 2, 3 and 4 mM and heated at 85°C for a day. Weak gels were formed at 3 and 4 mM concentration. FTIR spectra of these three samples were collected and plotted with baseline corrections in Figure 4.5 and they were subsequently analysed using FSD in Table 4.2.
Figure 4.5 Infrared spectra of pH 4 HEWL/DTT samples at 2 mM (orange), 3 mM (black) and 4 mM (blue) concentration where samples were formed by heating at 85°C for a day and then cooled at room temperature for 45 minutes.

Figure 4.5 shows the formation of β-sheet structure (~1617 cm⁻¹) in all samples even at 2 mM which was below the critical gelation concentration of lysozyme. This implies heating the pH 4 samples with DTT for 24 hours led to the destabilisation of native protein due to the broken disulphide bonds. In Figure 4.5, it is also observed that there was a significant increase in the overall band intensity for the 4 mM sample. It is not known why the absolute values for absorbance do not scale with concentration but finding ways to normalise the curves would allow better and clear comparisons.

In Table 4.2 the quantitative results from iterative curve fitting procedure showed at 3 mM the lysozyme secondary structure contains 8% α-helix, 20% unordered, 36% turns, 32% intermolecular β-sheet and 5% anti-parallel β-sheet structures. For the 2 mM HEWL/DTT sample, the protein secondary structural elements include 15% α-helix, 25% unordered, 23% turns, 31% intermolecular β-sheet and 7% anti-parallel β-sheet structures. The uncertainties in the FSD estimation of lysozyme secondary content in Figure 4.5 were calculated as 1.4-1.5% at 2 and 3mM.
Table 4.2 Percentages of secondary structural elements in pD 4 HEWL/DTT samples formed at 2, 3 and 4 mM.

<table>
<thead>
<tr>
<th>Secondary structure</th>
<th>Amide I frequency (cm⁻¹)</th>
<th>2 mM</th>
<th>3 mM</th>
<th>4 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermolecular β-sheet</td>
<td>1614-1616</td>
<td>30.9</td>
<td>31.6</td>
<td>37.6</td>
</tr>
<tr>
<td>Unordered</td>
<td>1632-1635</td>
<td>25.2</td>
<td>19.6</td>
<td>14.2</td>
</tr>
<tr>
<td>α-helix</td>
<td>1643-1645</td>
<td>14.6</td>
<td>7.6</td>
<td>9.9</td>
</tr>
<tr>
<td>Turns</td>
<td>1655-1657</td>
<td>22.6</td>
<td>36.1</td>
<td>26.5</td>
</tr>
<tr>
<td>Anti-parallel β-sheet/Aggregated strands</td>
<td>1677-1681</td>
<td>6.7</td>
<td>5.2</td>
<td>11.9</td>
</tr>
<tr>
<td>Total % of β-sheet Structure</td>
<td></td>
<td>37.6</td>
<td>36.8</td>
<td>49.5</td>
</tr>
<tr>
<td>Ratio of β-sheet to α-helix</td>
<td></td>
<td>2.6</td>
<td>4.8</td>
<td>5.0</td>
</tr>
<tr>
<td>FSD Standard Error</td>
<td></td>
<td>1.49</td>
<td>1.44</td>
<td>4.19</td>
</tr>
<tr>
<td>FSD F-statistic</td>
<td></td>
<td>0.02</td>
<td>0.02</td>
<td>0.18</td>
</tr>
</tbody>
</table>
The results in Table 4.2 revealed the quantities of β-sheet structure at 2 and 3 mM were almost similar (~37-38%) and its level had increased to 48% at 4 mM. Less coiled structure was found as the protein concentration increased while more α-helix to protein turns transition had occurred instead. The protein turns could be in the form of reverse turns that connect the anti-parallel β-sheet strands together causing their moderate amount (about 5-10%) in HEWL/DTT samples. In summary, higher protein concentration promotes more α-helix to turns and β-sheet structural transitions for samples with DTT.

### 4.3.1.4 Effect of varying pDs on lysozyme secondary structure

This section aims to investigate the optimal pD for breaking the S-S bonds and hence, the maximum formation of β-sheet within the studied range of pDs (between pD 2 and 7). 4 mM samples were prepared with and without DTT at pD 2, 3, 4 and 7 and they were heated at 85°C for different times as shown in Table 4.1. The samples were scanned and their FTIR spectra were collected and analysed in Figures 4.6 and 4.7.

![Infrared spectra of 4 mM HEWL/D₂O samples prepared at pD 2 (blue), pD 3 (pink), pD 4 (green) and pD 7 (orange) by heating them at 85°C for different times and then cooled at room temperature for 45 minutes.](image)

Figure 4.6 Infrared spectra of 4 mM HEWL/D₂O samples prepared at pD 2 (blue), pD 3 (pink), pD 4 (green) and pD 7 (orange) by heating them at 85°C for different times and then cooled at room temperature for 45 minutes.
Figure 4.6 shows most protein chains in HEWL/D\textsubscript{2}O samples were still in their native α-helix state (at $\sim 1645 \text{ cm}^{-1}$) even after heating at 85°C and subsequent cooling. This is supported when most samples remained as viscous liquids except at pD 2. Strong HEWL/D\textsubscript{2}O gel was formed at pD 2 after 3 days of heating. However, there was no peak formation at $\sim 1619 \text{cm}^{-1}$ to indicate fibrils formation. Quantitative analysis on lysozyme secondary structural elements was then performed on all HEWL/D\textsubscript{2}O samples and the results were shown in Table 4.3.
Table 4.3 Percentages of secondary structural elements in 4 mM HEWL/D$_2$O samples formed at pD 2, 3, 4 and 7.

<table>
<thead>
<tr>
<th>Secondary structure</th>
<th>Amide I frequency (cm$^{-1}$)</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pD 2</td>
</tr>
<tr>
<td>Intermolecular β-sheet</td>
<td>1616-1617</td>
<td>21.3</td>
</tr>
<tr>
<td>Unordered</td>
<td>1632-1633</td>
<td>13.1</td>
</tr>
<tr>
<td>α-helix</td>
<td>1642-1644</td>
<td>9.5</td>
</tr>
<tr>
<td>Turns</td>
<td>1655-1657</td>
<td>49.9</td>
</tr>
<tr>
<td>Anti-parallel β-sheet /Aggregated strands</td>
<td>1678</td>
<td>6.3</td>
</tr>
<tr>
<td>Total β-sheet Structure</td>
<td></td>
<td>27.6</td>
</tr>
<tr>
<td>Ratio of β-sheet to α-helix</td>
<td></td>
<td>2.9</td>
</tr>
<tr>
<td>FSD Standard Error</td>
<td></td>
<td>4.43</td>
</tr>
<tr>
<td>FSD F-statistic</td>
<td></td>
<td>0.20</td>
</tr>
</tbody>
</table>
Table 4.3 revealed lysozyme at pH 2 contains higher percentages of turns (49.9%) and anti-parallel β-sheet strands (6.3%) in comparison to other samples. The protein turns might form permanent link between α-helix and β-sheet and also between anti-parallel β-strands themselves to encourage fibrils formation and sufficient gelation to take place. Arnaudov and de Vries mentioned the uniqueness of pH 2 in promoting the fibrillar aggregation of HEWL by heating the sample at 57°C for two days [96]. They also reported that changes in lysozyme secondary were quite slow and continuous upon heating [96]. This implies the low stability of protein molecule towards heat treatment at pH 2 and that prolonged heating at 85°C can cause further protein unfolding which led to fibrils formation after three days.

In Table 4.3 it is also noticed that the level of intermolecular β-sheet increased while the anti-parallel β-sheet and number of protein turns decreased at higher pDs. The total β-sheet structures at pH 7 (31%) was higher than at pH 2 (27.6%). However, this did not guarantee the process of gelation at pH 7. The pH 7 sample was even heated at 85°C for 21 days but remained as a liquid. Hence, this implies the importance of high number of protein turns in promoting the fibrils formation in HEWL/D₂O sample. In summary, strong gelation occurred at pH 2 for lysozyme in water due to prolonged heat treatment and continuous changes in its secondary structure resulting in high percentages of protein turns (~ 50%) and anti-parallel β-strands.

Samples of pH 2, 3, 4 and 7 were also prepared in the presence of DTT and they were heated for the same time as HEWL/D₂O samples. Weak gels were formed at pH 3 and 4 after a day of heating at 85°C while strong pH 7 gel was formed after one hour of heating. Their FTIR spectra with corrected baselines were shown in Figure 4.7.
Figure 4.7 Infrared spectra of 4 mM HEWL/DTT/D$_2$O samples prepared at pH 2 (blue), pH 3 (pink), pH 4 (green) and pH 7 (orange) by heating them at 85°C for different times and then cooled at room temperature for 45 minutes.

Figure 4.7 shows the formation of a new peak at $\sim$1619 cm$^{-1}$ at pH 3, 4 and 7 indicating the presence of $\beta$-sheet fibrils within these samples. This is in line with their visual gelation results and implies that fibrillation is necessary for gel formation to occur. At pH 2, the reduced lysozyme still retained part of its $\alpha$-helix structure even after heating at 85°C for three working days. This confirms that changes in lysozyme secondary structure were relatively slow but continuous at pH 2 even after the addition of DTT.

The quantity of $\alpha$-helix, $\beta$-sheet, turn and unordered structures present in samples with DTT were determined using FSD and shown in Table 4.4. A combination of Gaussian and Lorentzian curves was used to find the area of the peaks that reflected each secondary structural element.
Table 4.4 Percentages of secondary structural elements in 4 mM HEWL/DTT samples formed at pD 2, 3, 4 and 7.

<table>
<thead>
<tr>
<th>Secondary structure</th>
<th>Amide I frequency (cm(^{-1}))</th>
<th>Percentage (%)</th>
<th>pD 2</th>
<th>pD 3</th>
<th>pD 4</th>
<th>pD 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermolecular β-sheet</td>
<td>1614-1616</td>
<td></td>
<td>20.5</td>
<td>31.9</td>
<td>37.6</td>
<td>34.9</td>
</tr>
<tr>
<td>Unordered</td>
<td>1632-1635</td>
<td></td>
<td>18.2</td>
<td>14.4</td>
<td>14.2</td>
<td>15.7</td>
</tr>
<tr>
<td>α-helix</td>
<td>1643-1645</td>
<td></td>
<td>9.6</td>
<td>9.5</td>
<td>9.9</td>
<td>5.5</td>
</tr>
<tr>
<td>Turns</td>
<td>1655-1657</td>
<td></td>
<td>45.4</td>
<td>35.6</td>
<td>26.5</td>
<td>36.4</td>
</tr>
<tr>
<td>Anti-parallel β-sheet/Aggregated strands</td>
<td>1677-1681</td>
<td></td>
<td>6.4</td>
<td>8.6</td>
<td>11.9</td>
<td>7.5</td>
</tr>
<tr>
<td>Total β-sheet structure</td>
<td>26.9</td>
<td></td>
<td>40.5</td>
<td>49.5</td>
<td>42.4</td>
<td></td>
</tr>
<tr>
<td>Ratio of β-sheet to α-helix</td>
<td>2.8</td>
<td></td>
<td>4.3</td>
<td>5.0</td>
<td>7.7</td>
<td></td>
</tr>
<tr>
<td>FSD Standard error</td>
<td>4.33</td>
<td></td>
<td>2.57</td>
<td>4.19</td>
<td>6.76</td>
<td></td>
</tr>
<tr>
<td>FSD F-statistic</td>
<td>0.19</td>
<td></td>
<td>0.07</td>
<td>0.18</td>
<td>0.46</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.4 shows the secondary content of reduced lysozyme at pD 2 includes 9.6% α-helix, 20.5% intermolecular β-sheet, 6.4% anti-parallel β-sheet, 45.4% turn and 18.2% unordered structures. At pD 7, the secondary structure of 4 mM lysozyme in DTT contains 5.5% α-helix, 34.9% intermolecular β-sheet, 7.5% anti-parallel β-sheet, 36.4% turn and 15.7% unordered structures. The standard error of these FSD measurements was found to be 4.33% and 6.76% for pD 2 and pD 7 sample respectively.

As the pD increased, more α-helix to β-sheet structural transition took place causing an increase in total β-sheet content and a decrease in turn and unordered structures. One possible explanation for this is that lysozyme becomes less positively charged at higher pDs allowing more hydrophobic-hydrophilic interactions between protein and DTT molecules. As a result, the degree of protein unfolding increased as pD increased. This is evident at pD 3 and 4 that contained higher percentages of intermolecular and anti-parallel β-sheet in comparison to pD 2 sample.

Another observation is that the pD 4 HEWL/DTT sample contains more intermolecular and anti-parallel β-sheet structures than the pD 7 sample. This is in contrast to their macroscopic gelation behaviour whereby the sample forms a weaker gel at pD 4 in comparison to at pD 7. It can be concluded that the ratio of β-sheet to α-helix structure within the sample is a better indicator for the degree of disulphide bonds breakage and to estimate fibrils formation. The strong gelation at pD 7 could also be attributed by the relatively high number of protein turns (36.4%) which connect the aggregated strands together. This further illustrates the importance of turns in encouraging fibrils formation.

The quantitative results in Tables 4.3 and 4.4 also revealed insignificant difference in the percentages of structural elements between non-reduced and reduced pD 2 samples. This implies that there is a difference in the protein unfolding pathways and the mechanism of fibrils formation between the addition of a denaturant and heat treatment. TEM experiments were performed in Section 4.6.1 to confirm β-sheet fibrils formation for reduced and non-reduced pD 2 samples. To conclude, strong gelation occurred at pD 2 for lysozyme in water and at pD 7 with the addition of DTT due to their high ratio of β-sheet to α-helix content and also the presence of many protein turns.
4.3.2 X-ray fibre diffraction (XRD) studies

The main aim of this section is to analyse the internal structure of self-assembled lysozyme fibrils and also to confirm the fibrils formed with or without DTT were β-sheet structure. Dried crystalline samples of pH 2 HEWL/water and pH 7 HEWL/DTT were prepared as in Figure 4.22. The samples were scanned and x-ray fibre diffraction patterns were formed due to the ordered arrangement of protein molecules [91]. These diffraction patterns were analysed using CLEARER tool analysis to give the reflexion on meridian and equator in Figure 4.8 [91].

![Figure 4.8](image)

Figure 4.8 Equatorial (left) and meridional (right) signals for pH 2 diluted HEWL/water (blue) and pH 7 HEWL/DTT (red) samples.

Figure 4.8 reveals the spacing between β-strands that ran perpendicular to the fibre axis as ~4.6 Å. This reflects the presence of hydrogen bonding (typically 4.7 Å) between these strands that formed a series of layer along the fibre axis [91]. Meanwhile, the β-sheets that ran parallel to the fibre axis are separated by 10 Å. This is in line with the expected range of 10-12 Å that originates from the size of side-chain associations between the β-sheets [91]. Hence, these results showed that lysozyme fibrils formed in water and DTT have similar characteristics with amyloid fibrils derived from diseased tissue [97].

It is also observed that the relative intensity of pH 7 sample was higher than the one recorded by pH 2 at the positions of 4.6 Å meridional and 10 Å equatorial. The relative
intensities at these positions represent the percentages of anti-parallel intra-strands β-sheet and also parallel inter-strands β-sheet found in a sample. FTIR results in Tables 4.3 and 4.4 reveal lysozyme at pH 7 contained 7.5% anti-parallel and 34.9% intermolecular β-sheets which were higher than those found at pH 2 (6.3% anti-parallel and 21.3% intermolecular). This explains the broader peak and also increasing intensity at pH 7 in comparison to at pH 2.

Figure 4.8 also shows the formation of two shoulder peaks for equatorial signal; the first peak was found within the range of 18-22 Å and the second peak lies at 25 Å which were suspected to be the protein turns and α-helix respectively. The protein turns are meant to connect the α-helix to β-sheet structure of the protein. There was a large difference in the quantity of protein turns between pD 2 (49.9%) and pD 7 (36.4%) based on their FTIR deconvolution results which might led to the formation of shoulder peaks 1.

Another slight shoulder peak was observed at 25 Å for the pH 2 sample which might represent its high α-helix content (9.5%). This peak was almost invisible at pH 7 because of its low α-helix content (5%). In summary, the fibre diffraction results confirmed HEWL formed fibrils with intra- and inter β-sheet spacings of 4.6 Å and 10 Å respectively under both reductive and non-reductive conditions.

4.4 Thermal behaviour of lysozyme

MicroDSC experiments allowed evaluation of the small amount of energy taken in and released when heating and cooling the lysozyme respectively. From these enthalpies the thermal transitions associated with the gelation process were identified and further correlate with the structural changes shown by FTIR results. The protein gelation process typically involved in the denaturation of protein and also the aggregation process due to the intermolecular association between denatured proteins [98, 99]. MicroDSC experiments also helped to recognise the range of temperature when these transitions took place.

4.4.1 Effect of adding DTT on lysozyme denaturation

This section aims to study the thermal transitions of lysozyme in water at pH 2 and also the effect of adding DTT on the protein denaturation temperature at this pH. Fresh 4 mM
lysozyme samples were prepared at pH 2 with and without DTT and they were studied using a heating/cooling rate of 1 °C min⁻¹ in the temperature range of 20-85°C. The thermographs for the first and second heating and cooling runs of both samples were shown in Figure 4.9. The peak areas under the curves were integrated using the software supplied with the instrument to estimate the associated enthalpies.

Figure 4.9 MicroDSC curves showing the first (blue) and second (pink) heating/cooling cycles for 4 mM (a) HEWL/H₂O and (b) HEWL/DTT samples prepared at pH 2.

In Figures 4.9(a) and 4.9(b), a single endothermic peak is observed at ~57 ± 0.5°C during the first heating of lysozyme in water and lysozyme in DTT at pH 2. This first endothermic transition is normally associated with the denaturation (partial unfolding) of lysozyme [2] since heat is needed to disrupt the hydrogen bonds and non-polar hydrophobic interactions which are responsible for the protein secondary structure. Lysozyme solution prepared with the reductant has a lower first endothermic enthalpy of 1305 ± 30 mJ g⁻¹ in
comparison to the pure lysozyme solution, 1409 ± 30 mJ g⁻¹. This indicates the protein has a lower stability in the presence of DTT.

Gosal and Ross-Murphy believed that protein denaturation could start anytime between the onset and peak temperatures [100]. It is thought that lysozyme with and without DTT at pH 2 could denature within the temperature range of 44 ± 0.5°C to 57 ± 0.5°C during the first heating. For these experiments the peak of first heating curve is assumed to be the denaturation temperature of lysozyme which is at 57°C. Arnaudov and de Vries also reported that lysozyme dissolved in water has a denaturation temperature of 57°C at pH 2 [96]. Meanwhile, Yan et al. reported the denaturation temperature to be ~65°C [37]. The difference in results might originate from the quality or purity of protein used or experimental errors had occurred while running the microDSC scans. Despite being predicted to have a lower denaturation temperature, lysozyme in water at pH 2 still failed to gel even after three days of heating at 65°C. As a result, the heating temperature for the lysozyme samples was increased to 85°C as discussed in Section 4.2.

During the first cooling of pure lysozyme solution, an exothermic transition is observed in Figure 4.9(a) at the same peak temperature, 57 ± 0.5°C. This first exotherm has a smaller enthalpy of 1144 ± 30 mJ g⁻¹ in comparison to the denaturation enthalpy during the first heating indicating a large portion of the protein has not been disrupted by the first heat treatment. Meanwhile, two transitions were observed at 38 ± 0.5°C and 60 ± 0.5°C during the second heating. It is suspected that at these temperatures, the melting of pre-formed aggregates and also the denaturation of residual native protein occurred [2, 35] leading to an even higher denaturation temperature of lysozyme (60 ± 0.5°C) with an associated enthalpy of 1089 ± 30 mJ g⁻¹. Hence, only a small portion of lysozyme in water is thought to be denatured irreversibly during each heating run.

In Figure 4.9(a), the two transitions observed during second heating were also present on second cooling. This clearly indicates the thermally induced lysozyme has a slow denaturation process and repetitive sample cooling promotes the reversible protein aggregation which could be indicated at 38 ± 0.5°C. FTIR results in Table 4.3 show the high percentages of turn structure and aggregated strands found in the pH 2 HEWL/water gel when the first heating was prolonged to three consecutive days. The high number of protein turns is believed to connect the aggregate strands together leading to fibrils formation and hence, the macroscopic gelation of lysozyme in water at pH 2. Gosal and
Ross-Murphy also reported protein aggregation normally leads to the formation of fine stranded network and hence, a physical gel [100].

Figure 4.9(b) shows the addition of DTT encouraged most lysozyme to denature irreversibly which may be due to disruption of their disulphide bridges and also an increase in the protein backbone flexibility [2]. This is evident when a considerably small exotherm enthalpy (170 ± 40 mJ g⁻¹) was observed during the first cooling. The second denaturation transition occurs at 38 ± 0.5°C with an enthalpy of 312 ± 40 mJ g⁻¹ representing the melting of pre-formed aggregates. There is a decrease in the heat energy and temperature required to denature the protein prepared in DTT as compared to a pure lysozyme solution during the second heating.

A single exothermic transition is observed at 38 ± 0.5°C during the subsequent cooling of lysozyme in DTT which is approximately 22°C lower than the one observed for pH 2 HEWL/water sample. This implies that the addition of DTT promotes a higher degree of α-helix to β-sheet structural transitions during the first and second heating. However, hydrolysis of fibrils might occur because of the prolonged heating of sample which might lead to more α-helix to unordered structural transition instead. At pH 2, lysozyme in DTT contains 18.2% unordered structure which is higher than in pure lysozyme solution (13.1%). In conclusion, the addition of DTT lowers the protein stability at pH 2 causing a significant decrease in lysozyme denaturation temperature especially during the second heating run.

4.4.2 Effect of pH on the denaturation of lysozyme in DTT

To understand the role of pH on the unfolding of lysozyme in DTT, 4 mM HEWL solutions were prepared at pH 2, 3, 4 and 7. The sample pH was adjusted by adding a small amount of HCl or NaOH solution. The effect of different pH on the denaturation temperature of lysozyme is to be investigated. These samples were subjected to first heating for about 85 minutes at a scanning rate of 1 °C min⁻¹ in Figure 4.10.
Figure 4.10 MicroDSC curves showing the first heating of 4 mM lysozyme in DTT at pH 2 (blue), pH 3 (pink), pH 4 (green) and pH 7 (orange) with a heating rate of 1 °C min\(^{-1}\).

Figure 4.10 shows a single endothermic transition occurred at pH 2, 3 and 4 for lysozyme in DTT which resulted in the formation of an endothermic peak at different temperatures. This endothermic peak has been previously associated with the denaturation of protein [2, 98] indicating lysozyme in DTT has the lowest denaturation temperature at pH 2 which occurred at 57°C. At pH 3 and 4, the protein denatures around 74°C. This is in good agreement with Yan (2008) who reported the denaturation peak at 77.6 ± 0.5°C for lysozyme in water at pH 4 [47].

Lysozyme experiences great intramolecular protein electrostatic repulsion at lower pH due to its high positive charges [101]. As a result, a high temperature is needed for lysozyme to denature which is visible at pH 3 and 4. However, this is not the case for the pH 2 sample which further highlights the uniqueness of lysozyme unfolding mechanism at this pH with and without DTT.

In Figure 4.10, two endothermic transitions were observed at pH 7; the first denaturation peak was found at 67°C while the second one occurred at 78°C. These results are comparable with the findings of Yan et al. who reported these transitions at 67.8 ± 0.5°C.
and 74.5 ± 0.5°C respectively [2, 36]. The low temperature transition peak suggests the denaturation of the protein while the high temperature transition peak could be related to the melting of gel aggregates formed during the denaturation process or further denaturation of the protein which are not affected by DTT.

It is expected that at high pH the repulsive intramolecular electrostatic force would decrease and hence, more hydrophobic-hydrophilic interactions between protein and DTT molecules occur causing more protein to unfold leading to gelation [2]. In addition, the results in Figure 4.10 also explained the need to use a higher heating temperature (~85°C) to prepare lysozyme samples in this work. This is to ensure the denaturation of lysozyme and macroscopic gelation of samples to take place in order to allow the gels characterisation.

The transition temperature and enthalpy during the first heating were calculated by integrating peak areas using the software supplied with the instrument and their results are summarised in Table 4.5.

<table>
<thead>
<tr>
<th>pH</th>
<th>Peak temperature / °C</th>
<th>Onset temperature / °C</th>
<th>Enthalpy / mJ g⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>56.8 ± 0.5</td>
<td>44.5 ± 0.5</td>
<td>1300 ± 30</td>
</tr>
<tr>
<td>3</td>
<td>73.4 ± 0.5</td>
<td>64.0 ± 0.5</td>
<td>2810 ± 30</td>
</tr>
<tr>
<td>4</td>
<td>73.7 ± 0.5</td>
<td>66.7 ± 0.5</td>
<td>2530 ± 30</td>
</tr>
<tr>
<td>7 (1st transition)</td>
<td>66.5 ± 0.5</td>
<td>59.7 ± 0.5</td>
<td>630 ± 40</td>
</tr>
<tr>
<td>7 (2nd transition)</td>
<td>78.3 ± 0.5</td>
<td>72.2 ± 0.5</td>
<td>110 ± 40</td>
</tr>
</tbody>
</table>

Table 4.5 clearly indicates the exceptional denaturation behaviour of HEWL at pH 2 from its small denaturation enthalpy during the first heating, 1300 ± 30 mJ g⁻¹ in comparison to pH 3 and 4 samples. There was also a decrease in the endothermic enthalpy as the pH is increased from pH 3 to pH 7. This explains the strong lysozyme gelation at pH 7 after an hour of heating at 85°C as seen in Figure 4.1(c). It is also observed that the denaturation enthalpy at pH 3 and 4 were approximately 4-5 times greater than at pH 7 causing an increase in the lag time needed for fibrils formation [96]. As a result, the pH 3 and 4 samples were heated at 85°C for one day before they formed weak gels. However, it is not
known if adding DTT would completely disrupt all the four disulphide bridges of lysozyme after a single heating run. In summary, lysozyme in DTT has a lower denaturation temperature of 67°C at pH 7 in comparison to at pH 3 and 4 (74°C) which explains the time required for their heat treatment and final gelation results.

4.5 Mechanical properties of HEWL/water & HEWL/DTT gels

Dynamic rheological experiments were performed at 25°C using small oscillatory shear rheometry in order to characterise the mechanical properties of HEWL solutions/gels and correlate them with their FTIR results. The storage and loss moduli, $G'$ and $G''$ were measured and compared at different conditions.

4.5.1 Effect of protein concentration and adding DTT on the mechanical behaviour of lysozyme samples

Firstly, the effect of different protein concentrations on the viscoelastic behaviour of lysozyme dissolved in water and DTT were compared in Figure 4.11. This is followed by investigating the mechanical behaviour of sample with the addition of DTT. pH 4 samples were prepared at 2, 3 and 4 mM similar to the ones in Section 4.3.1.3 by heating at 85°C for a day and subsequently cooled at room temperature. Weak lysozyme gels were formed at 3 and 4 mM in the presence of DTT.
Figure 4.11 Average storage, $G'$ (solid symbols) and loss, $G''$ (empty symbols) moduli of 2 mM (orange), 3 mM (black) and 4 mM (blue) (a)HEWL/water and (b)HEWL/DTT samples at pH 4 as a function of shear strain obtained at 25°C.

Figures 4.11(a) and 4.11(b) show an increase in $G'$ as the lysozyme concentration increased for pH 4 samples in water and in DTT respectively. Nevertheless, the $G'$ was considerably low (3 to 5 Pa) for lysozyme in water with a very small difference between $G'$ and $G''$. This explains why the samples in Figure 4.11(a) flowed freely upon inversion of their vials (a characteristic of structured liquids) after their heating and cooling processes. Another observation is the formation of more viscous solutions as the protein concentration increased for pH 4 samples in water.

On the contrary, the HEWL/DTT samples in Figure 4.11(b) exhibited higher $G'$ values of 15, 45 and 650 Pa at 2, 3 and 4 mM lysozyme respectively. The magnitude of difference between $G'$ and $G''$ increased at higher protein concentrations resulting in weak macroscopic gelation at 3 and 4 mM. To illustrate, the $G'$ (650 Pa) was almost an order of magnitude greater than the $G''$ (80 Pa) for 4 mM HEWL/DTT sample. However, there was a significant decrease in the moduli difference at 3 mM whereby the magnitude of $G'$ (45 Pa) was just thrice larger than $G''$ (15 Pa). This could be related to the lower β-sheet content at 3 mM (37%) in comparison to at 4 mM (50%) suggesting the formation of stronger gels at higher protein concentrations.
In Figure 4.11(b), the linear viscoelastic region (LVR) increased beyond 5% strain with the addition of DTT. This implies the reduced samples can withstand higher strain and that the hydrogels can be stretched further without exceeding the cross-over point of strain and return to their original state. FTIR results reveal these samples contained high amount of β-sheet fibrils (37-50%) which explained the increase in their elasticity. It is also noted that the point of cross-over between $G'$ and $G''$ was found at a lower percentage strain for the 4 mM sample and this might be related to the stiffness of fibrils which formed the gel network as reported by Yan [35, 36, 47]. In conclusion, higher protein concentrations and also the addition of the reducing agent DTT promoted the formation of stronger and more elastic pH 4 lysozyme gels.

A summary of the relationship between the lysozyme concentration, the storage modulus $G'$ and also the level of β-sheet content within the reduced samples was shown in Figure 4.12.

Figure 4.12 Storage modulus, $G'$ (red) and level of β-sheet content (black) as a function of protein concentration for pH 4 lysozyme samples prepared in the presence of 20 mM DTT.

Figure 4.12 shows a large increase in $G'$ between 3 and 4 mM due to the relatively high percentage of β-sheet structure found within the sample (between 38 and 50%). This indicates the elasticity of HEWL/DTT gels above the critical gelation concentration of lysozyme was dependent on the amount and morphology of their fibrils. However, this is not the case for samples between 2 and 3 mM.
Similar percentages of intermolecular (31-32%) and anti-parallel (5-7%) β-sheet structure were found at 2 and 3 mM. Yet more α-helix to turn transition took place at 3 mM causing its low α-helix content (8%) but high number of protein turns (36%) in comparison to at 2 mM (15% α-helix and 23% turn). Hence, this suggests the high percentage of protein turns played an important role in connecting the anti-parallel β-sheet strands and causing sufficient gelation to take place at 3 mM. This also explains the sharp increase in $G'$ from 2 to 3 mM. To conclude, both $G'$ and β-sheet content of a sample increased when the protein concentration is increased above its critical gelation concentration of 3 mM.

4.5.2 Effect of varying pH on the mechanical behaviour of lysozyme samples

This section aims to study the effect of varying pH on the mechanical properties of lysozyme solutions/gels (Figure 4.13). 4 mM HEWL/water and HEWL/DTT samples were prepared at pH 2, 3, 4 and 7 in accordance to Table 4.1. The pH of samples was purposely varied in order to encourage fibrils formation and consequently gelation especially for lysozyme in DTT. Weak gels were formed at pH 3 and 4 while strong gelation occurred at pH 7 in the presence of DTT.

![Figure 4.13](image)

Figure 4.13 Average storage, $G'$ (solid symbols) and loss, $G''$ (empty symbols) moduli of 4 mM (a) HEWL/water and (b) HEWL/DTT samples at pH 2 (blue), pH 3 (pink), pH 4 (green) and pH 7 (orange) as a function of shear strain obtained at 25°C.
Figure 4.13(a) illustrates the formation of a strong gel at pH 2 with a $G'$ of 6 kPa for lysozyme dissolved in water. The gel was visually observed to be rather brittle and this is supported with its short linear viscoelastic region (LVR) and low cross-over point at 3% strain. The pH 3, 4 and 7 samples recorded extremely low $G'$ values in the range of 3 to 5 Pa. They were behaving as structured liquids that flowed upon the application of stress and experienced shear thinning (becoming less viscous) after shearing at higher rates[78]. These results demonstrate that even a small change in pH (especially between pH 2 and 3) could cause a significant difference on the gelation behaviour of lysozyme in water.

Arnaudov and de Vries reported the pH has a large influence on the stability of HEWL in water and that the protein became less stable at acidic pH [96]. It is suspected that the low stability of lysozyme at pH 2 reduces the lag time to form fibrils and hence, heating the sample at 85°C for three consecutive days was sufficient to cause its gelation. The conditions used slightly differed from Arnaudov and de Vries and Yan et al. who had reported gel formation at pH 2 after two days of heating at 57°C and 65°C respectively [37, 96].

FTIR results in Table 4.3 reveal that the pD 2 lysozyme was still partly unfolded with 9.5% α-helix content even after three days of heating at 85°C. More α-helix to turn and also anti-parallel β-sheet structural transitions took place at pD 2 resulting in less unordered structure (13%) as compared to the ones found in pD 3, 4 and 7 (16-19%). Arnaudov and de Vries also found that their thermally induced pH 2 HEWL fibrils still have a coiled structure with an interval of 30 nm and also exhibited conformation defects after every 4-5 turns [96].

The high level of α-helix and unordered structures found within the pD 3, 4 and 7 samples indicate their high stability towards heat treatment. It is quantified in Table 4.3 that these samples contained similar or even a higher percentage of intermolecular β-sheet than the pD 2 sample but lower content of turns and anti-parallel β-sheet structures. This shows the importance of high number of protein turns and anti-parallel β-sheet in promoting fibrils formation for lysozyme in water.
It is also suspected that there is a distinct lag time to form fibrils for lysozyme in water at different pH. However, no gelation was observed at pH 3, 4 and 7 even after the samples were heated at 85°C for 21 days. Arnaudov and de Vries and Yan et al. also reported no gel formation after heating pH 3 and 4 samples for several days [37, 96]. These results clearly suggest the uniqueness of the pH 2 condition in encouraging the gelation of HEWL/water sample.

Meanwhile, Figure 4.13(b) indicates the formation of strong HEWL/DTT gels at pH 4 and 7 with $G'$ of 650 and 750 Pa respectively. The pH 7 gel is found to be quite elastic in nature due to its long linear viscoelastic region that extended until 10% strain with a crossover point at 100% strain. This result slightly differs from Yan et al. who reported the magnitude of $G'$ as 1.5 kPa for 4 mM HEWL/DTT sample with a shorter linear viscoelastic region [35, 36, 47]. One possible reason for this is the difference in time taken to heat the lysozyme samples at 85°C. Yan et al. mentioned the samples were heated for 10 minutes leading to the formation of rigid β-sheet fibrils [35, 36, 47]. So there is perhaps a difference between fibres formed at pH 2 and pH 7 which shall be explored in Section 4.6.

Another observation is that the HEWL/DTT sample forms a weaker and more brittle gel at pH 4 as compared to the pH 7 gel. However, both samples showed a similar magnitude difference between their $G'$ and $G''$ in Figure 4.13(b). It is quantified in Table 4.4 that the ratio of β-sheet to α-helix structure at pH 4 was lower than the pH 7 sample. Lysozyme becomes more positively charged at lower pH leading to more repulsion between protein molecules in the sample. As a result, the degree of lysozyme partial denaturation is restricted at pH 4 causing its higher α-helix content (9.9%) in comparison to at pH 7 (5.5%). This explains why the gel is weak and breaks easily at pH 4.

Lower $G'$ values (around 15 to 29 Pa) were observed for pH 2 and 3 samples. This is in line with the viscous solution observed at pH 2 and also the weak gel formed at pH 3. At lower pH, the electrostatic repulsion between the positively charged protein molecules is suspected to be greater than the attractive interactions between hydrophilic DTT and hydrophobic patches of protein. Hence, this forms a limitation for the lysozyme to unfold causing an even lower ratio of β-sheet to α-helix structure at pH 2 and 3 in comparison to at pH 4. The pH 2 fibrils formed in the presence of DTT were compared to those fibrils formed in water in Section 4.6.
The relationship between the sample pH, the storage modulus \( G' \) and its \( \beta \)-sheet content for HEWL/water and HEWL/DTT samples were shown in Figure 4.14.

![Figure 4.14](image)

Figure 4.14 Storage modulus, \( G'(\text{red}) \) and the level of \( \beta \)-sheet content (black) as a function of pH for (a) HEWL/water and (b) HEWL/DTT samples.

Figure 4.14(a) shows the magnitude of \( G' \) does not depend on the level of \( \beta \)-sheet structure found within lysozyme samples in water. The quantity of \( \beta \)-sheet structure remained almost linear as the sample pH increased. It is suspected that the high percentage of protein turns (about 50% of protein secondary structure) helped to connect the aggregated fibrils strands causing HEWL/water gelation at pH 2.

However, the strength of the gel network formed with DTT is dependent on both the sample pH and the percentage of \( \beta \)-sheet content. Figure 4.14(b) shows a sharp increase in \( G' \) between pH 3 and 4 due to increasing \( \beta \)-sheet content. The \( G' \) continued to slightly increase despite a drop in the level of \( \beta \)-sheet structure. This confirms that lysozyme experienced less electrostatic repulsion at pH 7 and more interactions occurred between hydrophilic DTT and hydrophobic protein patches causing higher degree of protein unfolding and the relatively low \( \alpha \)-helix content (5.5%) at pH 7 [2]. In summary, HEWL gelation is greatly affected by the sample pH and the solvent used to dissolve the protein [99]. Strong and brittle lysozyme gels were formed at pH 2 in the presence of water. The addition of DTT increases the elasticity of lysozyme gels with their gelation occurring at higher pH.
4.5.3 Mechanical stability of HEWL/water and HEWL/DTT gels

Another area of interest is to examine the ability of lysozyme gels to resist deformation when they are subjected to increasing frequency. This indirectly relates to measuring the strength of existing permanent junctions found in the hydrogels network. In this experiment, the upper plate of the rheometer was rotating at a faster rate causing an increase in the rate of material deformation. The response of the lysozyme gels to increasing frequency was monitored at a constant strain and temperature and it was shown in Figure 4.15. The chosen samples include the pH 2 HEWL/water gel and also HEWL/DTT gels at pH 3, 4 and 7. All samples were at 4 mM concentration.

![Graph showing the mechanical stability of HEWL/water and HEWL/DTT gels](image)

Figure 4.15 Average storage, $G'$ (solid symbols) and loss, $G''$ (empty symbols) moduli of pH 2 HEWL/water (blue), pH 3 HEWL/DTT (pink), pH 4 HEWL/DTT (green) and pH 7 HEWL/DTT (orange) hydrogels as a function of shear frequency obtained at 25°C.

Figure 4.15 shows the $G'$ and $G''$ remained unchanged at lower frequencies (6 to 100 rads$^{-1}$) for the pH 2, 4 and 7 samples. Hence, these samples do not exhibit any changes in their behaviour in response to the applied stress indicating the presence of many permanent junctions from the physical cross links between fibrils within the hydrogels network.
At higher angular frequencies, the $G'$ decreased slightly earlier at pH 7 as compared to pH 2. This implies that the number of cross-links/entanglements and arrangement of fibrils might be different between the non-reduced pH 2 and reduced pH 7 samples which will be checked with TEM in Section 4.6. It is also noticed that the pH 4 sample showed more resistant to the stress in comparison to the pH 7 sample. This could be related to the higher percentage of $\beta$-sheet structure found at pH 4 (49.5%) rather than at pH 7 (42.4%). Higher number of fibrils might lead to the formation of more cross links/associations within the network making the gel system more stable.

In Figure 4.15, the $G'$ and $G''$ of the pH 3 sample increased steadily at higher angular frequencies. It is suspected that the pH 3 gel initially contains temporary physical entanglements that led to its weak gelation. However, these entanglements were broken at or above 20 rads$^{-1}$ causing the sample to flow at higher frequencies and display a decrease in viscosity after the end of experiment. To summarise, lysozyme hydrogels at pH 2, 4 and 7 exhibit longer viscoelastic relaxation behaviour at lower frequencies. This could be due to the formation of permanent cross links in the hydrogels network which contribute to the resilience of lysozyme gels.

4.6 Morphology of HEWL/water and HEWL/DTT gels by transmission electron microscopy (TEM) and light microscopy

The main aim of using TEM is to determine the morphology of lysozyme gels formed under reductive and non-reductive conditions, on the micrometer to nanometer length scales. Hydrogels are quite difficult to analyse using electron microscopy due to their delicate nature and the need for diluting the samples. Therefore, TEM could only reveal the size and structure of lysozyme fibrils but not the network of the gel itself as sample would have been diluted and dried.

4.6.1 Morphology at pH 2 by TEM

The morphology of transparent HEWL/water gel (Figures 4.16 and 4.17) and HEWL/DTT solution (Figure 4.18) formed at pH 2 were initially investigated. Both 4 mM samples were diluted 25-fold based on the studies conducted by Yan et al. who had diluted pH 2 lysozyme gels 20-fold [37].
Figure 4.16 TEM micrograph of negatively stained fibrils from a 25-fold diluted HEWL/water hydrogel formed at pH 2 with a magnification factor of 4,800. The scale bar represents 0.5µm.

Figure 4.16 shows the formation of long and flexible lysozyme fibrils that were capable to bend and stretch from one end to the other within the micrograph. These fibrils were estimated to be 13nm in diameter and 7µm in length. The diameter was within the expected range of 6 to 13nm for protein fibres. Yan also reported the formation of pH 2 fibres which were several microns in length for lysozyme heated at 65°C without DTT [47].

In Figure 4.16, at least 3 to 6 strands of these fibrils would entwine and met at one junction point before separating and forming many other physical entanglements. The arrangement of these fibrils might explain its formation as a strong physical gel. However, the fibrils were not evenly distributed and tend to be dispersed from each other. The empty space in between could contribute to the brittleness of its structure which explains its short linear viscoelastic region from the rheological studies in Figure 4.13(a). To conclude, there was only a moderate degree of fibrillar entanglement for non-reduced pH 2 lysozyme sample.
Figure 4.17 TEM micrograph of aggregated fibrils from a 25-fold diluted HEWL/water hydrogel formed at pH 2 with a magnification factor of 30,000. The scale bar represents 100nm.

Figure 4.17 shows that some fibrils were more densely stained (in white) than others revealing their twisting structure at pH 2 without DTT. Another observation is the apparent increase in the fibril’s diameter causing it to become thicker when it was heavily stained. This suggests the possibility of fibrils aggregation whereby two or more fibrils were sticking to each other and appeared as a single strand under the electron microscope. FTIR results in Table 4.3 showed the highest formation of aggregated strands (6.3%) occurred at pD 2 for lysozyme in water.
Figure 4.18 TEM micrograph of negatively stained fibrils from a 25-fold diluted HEWL/DTT sample formed at pH 2 with a magnification factor of 11,000.

Figure 4.18 reveals that only a small number of fibrils were found in the reduced pH 2 lysozyme sample. This explains its non-gelation even after 3 days of heating at 85°C and implies that gelation occurs due to the formation of mesh network of fibrils within the sample. These fibrils were relatively shorter than the ones in Figure 4.16 with an estimated length of 1.5µm and 10nm in diameter.

FTIR results in Table 4.4 indicate the HEWL/DTT sample contained less β-sheet structure than HEWL/water gel at pD 2. This implies that the addition of DTT still promoted the partial unfolding of native lysozyme structure which led to fibrils formation at pD 2. Linden and Foegeding mentioned the importance of chemical changes in protein in order to promote the formation of fibrils [102].

It is suspected that the prolonged heating of HEWL/DTT sample at a very high temperature has caused the hydrolysis of the intact lysozyme together with the fibrils formed earlier on. Mishra et al. reported the importance of hydrolysis to cause HEWL fibrillisation at lower pH and also the possibility of remaining unhydrolysed lysozyme slowing down this fibrillisation process [103].
Figures 4.16 and 4.18 show some irregularities and disparities in the formation of fibrillar networks within the pH 2 samples. This has also been reported by Arnaudov and de Vries who observed some irregularities on lysozyme fibrils formation based on their atomic force microscopy results and they referred to this process as shaving [96]. In summary, the formation of β-sheet fibrils was observed in both non-reduced and reduced samples at pH 2 with some degree of disparities due to prolonged heating for 3 days.

4.6.2 Morphology at pH 7 by TEM

This section aims to investigate the effect of increasing pH on the microstructure of HEWL/DTT gel especially at pH 7 where strong gelation occurred. 4 mM HEWL/DTT sample was used and diluted to 250-fold in order to obtain clear images of the fibrils as illustrated in Figures 4.19 and 4.20.

Figure 4.19 TEM micrograph of a 250-fold diluted HEWL/DTT hydrogel formed at pH 7 with a magnification factor of 13,000.

Figure 4.19 shows the formation of a dense polygon-shaped fibrillar network at pH 7 and these fibrils were found to be evenly distributed throughout the micrograph. The fibrils were measured as 13.5nm in diameter and 457nm in length. They were shorter, stiffer and more persistence in nature as compared to the pH 2 fibrils in HEWL/water gel but formed a highly entangled fibrillar network. This is supported by the FTIR results in Tables 4.3 and 4.4 which revealed a higher formation of intermolecular and anti-parallel β-sheet at
pH 7 rather than at pH 2 for the non-reduced sample. Yan et al. also reported the formation of short and stiff pH 7 fibrils but they were found to be in the range of 4-6 nm in diameter in the presence of DTT [37].

Figure 4.20 TEM micrograph of a 250-fold diluted HEWL/DTT hydrogel formed at pH 7 with a magnification factor of 49,000.

Figure 4.20 shows some parts of the fibrils were quite granular and aggregated such that they formed thicker strands. This aggregation could be resulted from the presence of many permanent network junctions or that these fibrils were overlapping several times before forming a 3D porous network. It is noted that a higher dilution factor (300-fold or more) might be needed to image individual pH 7 fibrils. To summarise, lysozyme fibrils formed at pH 7 were shorter, stiffer and denser than pH 2 fibrils formed with and without DTT.

4.6.3 Fibrillar alignment in crystallised lysozyme gels

The alignment of lysozyme fibrils in pH 2 HEWL/water and pH 7 HEWL/DTT gels were further observed using light microscope in Figure 4.21. The samples were prepared as in Section 3.8.3 and dried overnight to form a crystal stalk.
Figure 4.21 Light microscopic images of dried crystalline samples of HEWL/water at pH 2 (left) and HEWL/DTT at pH 7 (right).

Figure 4.21 reveals the formation of a wavy and twisted-like fibre within the pH 7 sample. This agrees with the TEM results in Figures 4.19 and 4.20 that showed the formation of a highly entangled fibrillar network at pH 7 with DTT. Jiminez et al. suggested the models of lysozyme amyloid fibrils in the form of six circular protofilaments which were wavy in nature based on cryo-electron microscopy results [104]. On the contrary, the insignificant fibrils formation within the crystallised pH 2 sample confirms their irregular pattern of distribution and formation in Figure 4.16.
4.7 Conclusions

HEWL samples were heated at 85°C for different times at different pH and subsequently cooled at room temperature for 45 minutes. Strong, colourless gels were formed at pH 2 after 3 days of heating the lysozyme in water and at pH 7 after an hour of heating in the presence of DTT. Weak HEWL/DTT gels were also observed at pH 3 and 4 after 24 hours of heating at 85°C. The physical properties and morphology of these gels were studied using several characterisation techniques.

FTIR results revealed the high percentages of protein turns structure (~50%) and anti-parallel β-strands (6.3%) formation at pH 2 for pure lysozyme in water. It is also quantified that by adding DTT more α-helix to β-sheet structural transitions occurred between pH 3 and 7. The highest β-sheet to α-helix ratio was observed at pH 7 indicating high number of fibrils formation which promotes its strong gelation. MicroDSC results confirmed lysozyme becomes less stable with the addition of DTT and increasing pH resulting in smaller denaturation enthalpies in comparison to lysozyme in water. However, the reduced protein still experienced a moderate degree of renaturation on first cooling especially at pH 3 and 4 explaining their weak gelation.

At pH 2, the protein denaturation in water was observed to be slow but continuous due to prolonged heating. This resulted in high number of protein turns which connect the aggregated strands together and encouraged the sample gelation. The pH 2 HEWL/water sample exhibits a high storage modulus of 6 kPa with shorter LVR suggesting the gel is strong but brittle in nature. Its TEM result indicates the presence of long, flexible fibrils which were several microns in length with some irregularities in their distribution which might contribute to the gel’s delicate state. On the contrary, the pH 7 fibrils were found to be shorter and stiffer forming a dense polygon-shaped porous network with a storage modulus in the range of 0.75 to 1 kPa. Hence, the addition of a small quantity (20 mM) of DTT encouraged HEWL gelation at higher pH resulting in differences between heating and reduced protein fibres.
Chapter 5  Studies on the effects of different reducing agents on HEWL gelation

5.1 Introduction

Chapter 5 discusses HEWL gels formation in the presence of two reducing agents, DTT and TCEP and it compares comprehensively on the behaviour, properties and structure of these reduced gels. The main aim of this work is to synthesise stronger lysozyme gels and improve their physical properties than in previous studies from a fundamental understanding of the gelation process. At first, the visual results of lysozyme gelation in DTT and TCEP and the necessary conditions that promoted them will be discussed in Section 5.2. These results are then linked to the protein thermal behaviour in DTT and TCEP from microDSC experiments in Section 5.3.

Structural changes linked with the thermal transitions are then correlated to FTIR results in Section 5.4 which predict the content of protein secondary structural elements. In Section 5.5 the mechanical properties of HEWL/DTT and HEWL/TCEP gels were measured to find their true potential as tissue engineering scaffolds and for drug delivery vehicles. The results of TEM in Section 5.6 will allow further studies on the microstructure of reduced HEWL hydrogels. The results of these findings will reveal whether the choice of reductant is application specific and can cause significant differences to the physicochemical properties of HEWL gels and changes on the fibrils morphology and mesh size.

5.2 Visual observation

The interest in developing self-supporting HEWL/TCEP hydrogels rapidly grows when preliminary weak gels were formed in the presence of 15 mM and 20 mM TCEP at pH 2.5, after two days of heating samples at 85°C and cooling them for 45 minutes at room temperature. The samples were prepared at 2 mM (28.6 mg mL⁻¹), 3 mM (42.9 mg mL⁻¹) and 4 mM (57.2 mg mL⁻¹) which were below, at and above the critical gelation concentration of lysozyme respectively. It was noticed that samples quickly reached their pI (below pH 5) in the presence of 20 mM TCEP. Hence, the 15 mM TCEP solution is preferred for further gelation work in this chapter.
In order to examine how TCEP influences the macroscopic gelation behaviour of lysozyme, samples at 2, 3 and 4 mM were then dissolved in 15 mM TCEP solution between pH 2 and 6 to encourage their gelation under mild conditions. They were heated for 2 hours and then cooled for 45 minutes at room temperature. The phase diagram of HEWL gelation in TCEP was shown in Figure 5.1.

![Figure 5.1 Phase diagram of HEWL gelation](image)

Figure 5.1 Phase diagram of HEWL gelation as a function of protein concentration and its pH after samples were heated for 2 hours and then cooled for 45 minutes at room temperature. Black points represent experimental data for 4 mM HEWL/TCEP samples. By trial and error, the minimum length of heating time to cause gelation for the strong gels was 1.5 hours.

In Figure 5.1, it is observed that lysozyme sample in 15 mM TCEP precipitated out when nearing to pH 6 and reached its isoelectric point faster than in DTT (pI 8). Lysozyme that is directly dissolved in TCEP solution (pH 2.5) was more acidic than in DTT solution (pH 3.3). It is expected on the addition of NaOH the positive and negative charges on protein quickly became equal in TCEP rather than in DTT explaining the difference in their measured pI.

TCEP is also known as a stronger reductant than DTT and hence, the former is likely to cause near complete reduction of lysozyme disulfide bonds. This correlates to the formation of strong and clear HEWL/TCEP gels at pH 3.5 and 4 when samples were prepared at 4 mM in Figures 5.2(a) and 5.2(b) respectively. At the critical gelation
concentration of lysozyme, strong gels began to form at pH 4. These results were in contrast to the formation of weak HEWL/DTT gels at pH 4 and strong clear gels at pH 7 in DTT.

Figure 5.2 Typical photographs of 4 mM HEWL/TCEP samples; starting from left strong and clear gels were formed at (a) pH 3.5 and (b) pH 4, followed by (c) strong and slightly opaque gel at pH 4.5, (d) strong and opaque gel at pH 5.5 and (e) protein precipitation at pH 6.

In Figure 5.2 strong and opaque HEWL/TCEP gels were also observed at pH 4.5, 5 and 5.5 after one heating and cooling cycle. Lysozyme becomes less positively charged when nearing to the pI and there should be less electrostatic repulsion between the protein molecules. It is suspected that less lysozyme molecules dissolved in TCEP solution at these pHs and participated in the attractive interactions between hydrophilic TCEP and hydrophobic protein patches. However, their attraction was large enough to unfold the protein and allowed fibrils formation leading to the gels turbid appearance.

5.3 Lysozyme thermal transitions in TCEP and DTT

In this section, microDSC experiments were conducted in order to understand the role of the reductants TCEP and DTT on the unfolding behaviour of lysozyme which subsequently affected its denaturation temperature. The effect of varying pH on the heating and cooling behaviour is also to be studied for HEWL/TCEP samples.
5.3.1 Comparing protein behaviour in TCEP and DTT

4 mM lysozyme solutions were prepared in both DTT/water and TCEP/water mixtures without any pH adjustment while their reference was the reductant itself in water. The samples were then scanned and subjected to first heating using microDSC in Figure 5.3. Hamaguchi mentioned that lysozyme exhibits many interesting features in terms of denaturation [105].

Figure 5.3 MicroDSC first heating runs of lysozyme solutions in TCEP (pH 2.47) and in DTT (pH 3.26) at a scanning rate of 1 °C min⁻¹.

In Figure 5.3, it is observed both protein samples showed a single endothermic peak. The results suggest lysozyme in TCEP denatures at ~58.4 ± 0.5°C while the protein in DTT denatures at ~73.6 ± 0.5°C. There was a relatively small free energy change in lysozyme denaturation for HEWL/TCEP sample indicating a higher degree of selective reduction of disulfides by TCEP rather than DTT in order to maintain free sulphydryl groups. The transition temperature and enthalpy during the first and second heating/cooling cycles were calculated by integrating peak areas using the software supplied with the instrument and their results are summarised in Table 5.1.
Table 5.1 MicroDSC results for 4 mM lysozyme/TCEP/water and lysozyme/DTT/water samples during first and second heating runs.

<table>
<thead>
<tr>
<th></th>
<th>1st heating run</th>
<th>2nd heating run</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TCEP</td>
<td>DTT</td>
</tr>
<tr>
<td>pH</td>
<td>2.47</td>
<td>3.26</td>
</tr>
<tr>
<td></td>
<td>2.47</td>
<td>3.26</td>
</tr>
<tr>
<td>Peak temperature / °C</td>
<td>58.4 ± 0.5</td>
<td>73.6 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>53.0 ± 0.5</td>
<td>60.7 ± 0.5</td>
</tr>
<tr>
<td>Onset temperature / °C</td>
<td>49.5 ± 0.5</td>
<td>66.7 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>45.2 ± 0.5</td>
<td>42.4 ± 0.5</td>
</tr>
<tr>
<td>Enthalpy / mJ g⁻¹</td>
<td>588 ± 40</td>
<td>1465 ± 30</td>
</tr>
<tr>
<td></td>
<td>179 ± 40</td>
<td>260 ± 40</td>
</tr>
</tbody>
</table>

Table 5.1 shows the denaturation enthalpy of HEWL in TCEP was smaller than its denaturation enthalpy in DTT during first and second heating. For example, the endothermic enthalpies for samples in TCEP and DTT were measured as 588 ± 40 mJ g⁻¹ and 1465 ± 30 mJ g⁻¹ respectively on first heating. The results in Table 5.1 further confirmed that TCEP is a stronger and more stable reducing agent than DTT causing the smaller endothermic enthalpy. Burns et al. reported the ability of TCEP to reduce oxidised DTT at pH 5 when at room temperature [56].

5.3.2 Effect of pH on HEWL/TCEP samples

From the results in Figure 5.2, it was clear that lysozyme gelation is largely dependent on pH. Fitzsimons et al. mentioned that there are two processes involved in a gelation; the protein denaturation and also the association process between denatured proteins [106]. Fresh 4 mM HEWL/TCEP samples were prepared at various pHs and subjected to two heating/cooling cycles at a scanning rate of 1°C min⁻¹ between 20 and 85°C. The main aim of these experiments is to find the relationship between pH and the denaturation temperature of lysozyme.

It is observed that the thermographs baseline from microDSC results was not properly levelled for pH 3.5, 4, 4.5, 5 and 5.5 HEWL/TCEP samples. This could be due to the strong reducing agent TCEP that is able to rapidly reduce the protein disulphides even at 30°C causing instability to the baseline. Another possible reason is the presence of noise during the experiment and problems with maintaining the refrigerated cooler’s temperature within the desired range. Thus, it was difficult to estimate the exact transition temperature and enthalpy of these HEWL/TCEP samples. However, all of the samples exhibited a denaturation peak between 47 and 62°C, explaining their gelation after being heated at 85°C for 2 hours and cooled at room temperature for at least 45 minutes.
5.4 Structural analysis of HEWL/DTT and HEWL/TCEP gels

FTIR is a very useful method in studying and probing the secondary structure of a protein. It measures the emission intensity of a sample based on the vibrations of their atoms and recorded them as infrared spectra [78, 80]. In this work, FTIR was used to investigate the secondary structural transitions of lysozyme dissolved in TCEP at the various pHs. The detailed composition of α-helix, β-sheet and unordered structures in a sample was further revealed using the OMNIC Fourier Self-Deconvolution (FSD). Another objective of this work is to compare the amount of β-sheet fibrils present in HEWL/TCEP and HEWL/DTT samples and then link them to results from other characterisation techniques.

5.4.1 Effect of pH on the secondary structure of HEWL/TCEP samples

It was found that the gelation behaviour of lysozyme is pH-dependent from the results in Figures 5.1 and 5.2. Using FTIR the degree of protein unfolding and fibrils formation could now be estimated. 4 mM lysozyme samples were prepared in TCEP/D$_2$O solution and heated at 85°C for two hours to form gels at different pDs. The samples were scanned and their FTIR spectra were collected and analysed in Figure 5.4.
Figure 5.4 Infrared spectra of 4 mM HEWL/TCEP samples at pD 3.5 (brown), pD 4 (red), pD 4.5 (blue), pD 5 (black), pD 5.5 (green) and just below pD 6 (pink).

Figure 5.4 shows lysozyme was still in its α-helical form at pD 3.5 due to the single peak at ~1645 cm\(^{-1}\). Above pD 3.5 the formation of β-sheet fibrils was detected within the frequency range of 1605-1620 cm\(^{-1}\) for all gels. This implies the TCEP addition promoted the structural transition of native α-helix to β-sheet within these samples. Some α-helix to random-coiled transition also occurred which is indicated by the shoulder peaks at ~1645 cm\(^{-1}\) and ~1635 cm\(^{-1}\) respectively, suggesting that lysozyme was not completely unfolded with the addition of TCEP.

To quantify the percentages of α-helix, β-sheet and unordered structures present, the sample spectrum was subjected to an iterative Gaussian-Lorentzian curve fitting procedure. An example of the curve fitting of a spectrum was shown in Figure 5.5.
Figure 5.5 Smoothed infrared spectra of pD 4 HEWL/TCEP sample (light blue) and its small component peaks. The dotted line is the summation of the decomposed curves which tried to mimic the smoothed spectra with a standard error of ±5%.

In Figure 5.5 the areas underneath smoothed curve that depict the desired structural components of the protein (Amide I band) were calculated. For example, the secondary structural content of lysozyme in TCEP at pD 4 is 51% α-helix, 4% unordered and 45% β-sheet. It is noted the intermolecular and anti-parallel β-sheet were accounted for the total content of fibrils within the sample. Quantitative analysis on lysozyme secondary structural elements was then performed on HEWL/TCEP samples between pD 3.5 and 6 and their results were summarised in Table 5.2. The standard error of their FSD measurements was found between 2 and 4.6%.
Table 5.2 Percentages of secondary structural elements in 4 mM HEWL/TCEP samples at different pDs.

<table>
<thead>
<tr>
<th>Secondary structure</th>
<th>Amide I frequency (cm⁻¹)</th>
<th>Percentage (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pD 4</td>
<td>pD 4.5</td>
<td>pD 5</td>
<td>pD 5.5</td>
<td>near pD 6</td>
</tr>
<tr>
<td>Intermolecular β-sheet</td>
<td>1614-1616</td>
<td>24.8</td>
<td>30.3</td>
<td>24.0</td>
<td>24.2</td>
<td>7.4</td>
</tr>
<tr>
<td>Unordered</td>
<td>1627-1634</td>
<td>4.4</td>
<td>3.8</td>
<td>1.9</td>
<td>11.8</td>
<td>25.8</td>
</tr>
<tr>
<td>α-helix</td>
<td>1643-1646</td>
<td>50.6</td>
<td>51.7</td>
<td>53.1</td>
<td>47.8</td>
<td>55.9</td>
</tr>
<tr>
<td>Anti-parallel β-sheet</td>
<td>1673-1677</td>
<td>20.1</td>
<td>14.2</td>
<td>21.0</td>
<td>16.3</td>
<td>10.9</td>
</tr>
<tr>
<td>Aggregated strands</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total % of β-sheet structure</td>
<td></td>
<td>44.9</td>
<td>44.5</td>
<td>45.0</td>
<td>40.5</td>
<td>18.3</td>
</tr>
<tr>
<td>Ratio of β-sheet to α-helix</td>
<td></td>
<td>0.9</td>
<td>0.9</td>
<td>0.8</td>
<td>0.8</td>
<td>0.3</td>
</tr>
<tr>
<td>FSD Standard Error</td>
<td></td>
<td>4.64</td>
<td>4.57</td>
<td>4.31</td>
<td>2.00</td>
<td>4.17</td>
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<tr>
<td>FSD F-statistic</td>
<td></td>
<td>0.22</td>
<td>0.21</td>
<td>0.19</td>
<td>0.04</td>
<td>0.17</td>
</tr>
</tbody>
</table>
Table 5.2 shows a high amount of fibrils were present in pH 4, 4.5 and 5 gels with approximately 45% of β-sheet content, implying these conditions favour the α-helix to β-sheet structural transitions. As the pH increased, more α-helix to random-coiled transition occurred instead which could be seen for pH 5.5 and 6 samples. This led to a higher percentage of the protein unordered structure rather than fibrils formation. It is expected the interactions between hydrophobic protein and hydrophilic TCEP molecules were large enough to cause the protein backbone to become flexible and hence, lysozyme chains were in unordered state at higher pHs.

In summary, TCEP addition promotes lysozyme unfolding to a certain extent. About 47 to 53% of the protein native structure was retained between pH 4 and 5.5. The pH of the sample played an important role in dictating the protein structural transitions and fibrils formation based on the quantitative results in Table 5.2.

5.4.2 Effect of TCEP vs. DTT on lysozyme secondary structure

This section compares the effect of adding different reducing agents on lysozyme secondary structure particularly on the β-sheet fibrils formation. A moderate concentration of the reductant (15 mM TCEP or 20 mM DTT) was added to encourage lysozyme gelation. Both HEWL/TCEP and HEWL/DTT samples formed gels at pH 4. These samples were selected for further comparisons on their structural components. In addition, the pH 5 HEWL/TCEP and pH 7 HEWL/DTT samples were also compared since they formed relatively strong gels after 1-2 hours of heating at 85°C and subsequent cooling at room temperature. The samples were scanned and their FTIR spectra were collected and shown in Figure 5.6.
Figure 5.6 Smoothed infrared spectra of 4 mM lysozyme samples including pD 4 HEWL/DTT (blue), pD 4 HEWL/TCEP (red), pD 5 HEWL/TCEP (black) and pD7 HEWL/DTT (green) gels. The spectra were later deconvoluted and curve-fitted in order to quantify the fractions of protein chain present as α-helix, β-sheet and unordered structures.

Figure 5.6 shows the formation of β-sheet fibrils at~1615 cm\(^{-1}\) within all samples. Quantitative results in Table 5.3 reveals more fibrils were formed in pD 7 gel (with ~50% of β-sheet content) in comparison to other samples that showed 42-45% of β-sheet structure. Hence, more α-helix to β-sheet transitions took place at pD 7 with DTT addition rather than at pD 5 in the presence of TCEP. However, the level of aggregated strands was higher at pD 5 (21%) than pD 7 (14.3%) which implies the fibrils would frequently overlap to form stronger strands and also more stable junction points. This could be linked to their rheological and TEM results in Sections 5.5 and 5.6 respectively. The level of aggregated strands at pD 4 (20.1%) is similar to pD 5 (21%) in the presence of TCEP. This indicates the addition of TCEP has encouraged the formation of anti-parallel β-sheet rather than intermolecular β-sheet interactions.
Table 5.3 Percentages of secondary structural elements in 4 mM lysozyme samples dissolved in TCEP and DTT solutions.

<table>
<thead>
<tr>
<th>Secondary structure</th>
<th>Amide I frequency (cm⁻¹)</th>
<th>PDE 4</th>
<th>PDE 4</th>
<th>PDE 5</th>
<th>PDE 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HEWL/DTT</td>
<td>HEWL/TCEP</td>
<td>HEWL/TCEP</td>
<td>HEWL/TCEP</td>
<td>HEWL/DTT</td>
</tr>
<tr>
<td>Intermolecular β-sheet</td>
<td>1614-1616</td>
<td>27.9</td>
<td>24.8</td>
<td>24.0</td>
<td>35.5</td>
</tr>
<tr>
<td>Unordered</td>
<td>1631-1634</td>
<td>11.6</td>
<td>4.4</td>
<td>1.9</td>
<td>6.0</td>
</tr>
<tr>
<td>α-helix</td>
<td>1643-1650</td>
<td>46.6</td>
<td>50.6</td>
<td>53.1</td>
<td>44.2</td>
</tr>
<tr>
<td>Anti-parallel β-sheet/Aggregated strands</td>
<td>1673-1676</td>
<td>13.9</td>
<td>20.1</td>
<td>21.0</td>
<td>14.3</td>
</tr>
<tr>
<td><strong>Total % of β-sheet structure</strong></td>
<td></td>
<td>41.8</td>
<td>44.9</td>
<td>45.0</td>
<td>49.8</td>
</tr>
<tr>
<td><strong>Ratio of β-sheet to α-helix</strong></td>
<td></td>
<td>0.9</td>
<td>0.9</td>
<td>0.8</td>
<td>1.1</td>
</tr>
<tr>
<td><strong>FSD Standard Error</strong></td>
<td></td>
<td>3.82</td>
<td>4.64</td>
<td>4.31</td>
<td>8.03</td>
</tr>
<tr>
<td><strong>FSD F-statistic</strong></td>
<td></td>
<td>0.15</td>
<td>0.22</td>
<td>0.19</td>
<td>0.65</td>
</tr>
</tbody>
</table>
Table 5.3 shows lysozyme in TCEP contained a very small percentage of unordered structure that lies within the range of 2-4%. It is also evident that lysozyme experienced more α-helix to unordered transition in the presence of DTT rather than TCEP at pD 4. To quantify this, the unordered structure increases from 4% in TCEP to 12% in DTT at pD 4. This confirms that DTT is weaker than TCEP in reducing the protein disulfide bonds causing the former to promote a higher degree of partial unfolding of lysozyme. The α-helix to β-sheet transition also occurred at the same time whereby the total β-sheet content increases from 42% in DTT to 45% in TCEP at pD 4. In summary, DTT addition promotes a higher degree of α-helix to unordered protein structure while TCEP addition led to the formation of more aggregated fibril strands.

5.5 Mechanical properties of lysozyme gels formed under non-reductive conditions

Rheological studies were performed on 4 mM HEWL/DTT and HEWL/TCEP gels in order to measure and compare their mechanical properties and also explore the suitability of the hydrogels for different applications. These experiments are intended to find the abilities of the gels to stretch without breaking and also to recover to their original condition after being sheared. Two parameters of the gels were closely monitored and measured over time; their storage, $G'$ and loss, $G''$ moduli.

5.5.1 Effect of pH on HEWL/TCEP gels

Lysozyme samples were dissolved in 15 mM TCEP solution and the pH of the samples was varied between pH 3.5 and 6 using an increment of pH 0.5. Samples were heated at 85°C for 2 hours and cooled for at least 45 minutes at room temperature. Here the $G'$ of HEWL/TCEP gels at a variety of pH’s were measured at 25°C and then compared in Figure 5.7.
Figure 5.7 Average storage modulus, $G'$ of 4 mM HEWL/TCEP samples as a function of shear strain at 25°C. Using the symbols, ■ represents pH 3.5, ▲ represents pH 4.0, ● represents pH 4.5, ▼ represents pH 5.0, ● represents pH 5.5 and ▲ represents just below pH 6.0. The average value was derived from three experimental runs of amplitude sweep for each sample.

In Figure 5.7, the highest storage modulus for 4 mM HEWL/TCEP sample was recorded at pH 5 which measures around ~4.7 kPa. This was subsequently followed by the sample nearing to pH 6 with its $G'$ of 3 kPa. Both pH 4 and 4.5 samples exhibited almost a similar mechanical strength of 1.7 kPa while pH 5.5 gel has a $G'$ of 1.2 kPa. Most of these HEWL/TCEP gels showed a relatively high storage modulus (greater than 1 kPa) except for pH 3.5 gel which has a $G'$ of 0.35 kPa.

At lower pH, it is expected the strong electrostatic repulsion between positively charged lysozyme molecules overcame the hydrophobic-hydrophilic interactions of protein-TCEP molecules and hence, there was a limitation for the protein to unfold. This is evident when the pH 3.5 gel exhibited the lowest storage modulus amongst the HEWL/TCEP samples in Figure 5.7.
Above pH 3.5, more protein molecules were involved in the thiol-disulphide exchange reactions causing a higher degree of protein unfolding and fibrils formation. This is reflected by the higher storage modulus of HEWL/TCEP samples between pH 4 and 6. The difference in their elasticity could also be attributed to the number, size and structure of fibrils that formed the gel’s physical network. This will be checked using TEM and further discussed in Section 5.6.

In Figure 5.7, most HEWL/TCEP samples (except for pH 3.5 gel) exhibited a long linear viscoelastic region (LVR) which stretched near or above 10% strain. This implies the HEWL/TCEP gels were quite elastic in nature when compared to HEWL/water gels in Chapter 4. Gels at pH 4.5, 5 and 6 were able to stretch beyond 10% strain while pH 4 and 5.5 gels could only stretch till 5% strain before the gels break. On the contrary, the pH 3.5 gel showed the least ability to stretch (i.e. until 2% strain) which could be related to its low $G'$ and less fibril formation within the gel.

Few of the HEWL/TCEP samples were selected for further studies on the magnitude difference of their $G'$ and $G''$. The $G'$ and $G''$ of HEWL/TCEP gels at pH 3.5, 4.5 and 5 were measured at 25°C and shown in Figure 5.8.
Figure 5.8 Average storage, $G'$ (solid symbols) and loss, $G''$ (empty symbols) moduli of 4 mM HEWL/TCEP gels as a function of shear strain at 25°C. Using the symbols, ▼ represents pH 3.5 HEWL/TCEP, ■ represents pH 4.5 HEWL/TCEP and ▲ represents pH 5.0 HEWL/TCEP samples.

From Figure 5.8, it is estimated the $G'$ was 4-5 times higher than $G''$ especially at pH 4.5 and 5. Meanwhile, the pH 3.5 HEWL/TCEP gel exhibited its solid-like behaviour an order of magnitude greater than its liquid-like behaviour despite its low storage modulus of 0.35 kPa. It is also observed the samples in Figure 5.8 appeared more elastic in nature with smaller difference in magnitude between their $G'$ and $G''$. These include the pH 4.5 and 5 gels that showed a high crossing-over point (i.e. at 100% strain) to indicate the gels could not be easily broken. In summary, the rheological analysis showed pH has an important effect on the elasticity of HEWL/TCEP hydrogels. At pH 5, a strong and turbid lysozyme hydrogel was formed with the highest storage modulus of 4.7 kPa.
5.5.2 Comparing the mechanical properties of HEWL/TCEP and HEWL/DTT gels

This section aims to further investigate whether the choice of reductant (DTT or TCEP) produces a significant difference on the viscoelastic properties of lysozyme gels. Below the LVR and mechanical strength of pH 4 and 5 HEWL/TCEP gels and also pH 4 and 7 HEWL/DTT gels were compared. All of these samples showed the macroscopic formation of self-supporting gels after first heating and first cooling except for the slightly weak HEWL/DTT gel at pH 4.

![Graph showing the comparison of mechanical properties of different pH and reductant combinations.](image)

Figure 5.9 Average storage, \(G'(\text{solid symbols})\) and loss, \(G''(\text{empty symbols})\) moduli of 4 mM lysozyme gels as a function of shear strain at 25°C. Using the symbols, \(\blacktriangle\) represents pH 4 HEWL/TCEP, \(\blacktriangledown\) represents pH 5 HEWL/TCEP, \(\bullet\) represents pH 4 HEWL/DTT and \(\blacksquare\) represents pH 7 HEWL/DTT samples.

In Figure 5.9, all lysozyme gels exhibited a fairly high storage modulus, greater than 1 kPa except for the pH 4 HEWL/DTT gel. At pH 5, the HEWL/TCEP gel showed the highest storage modulus of around 4.7 kPa. This was followed by the pH 7 HEWL/DTT sample.
whose $G'$ (2 kPa) was slightly higher than the pH 4 HEWL/TCEP sample (1.7 kPa). The pH 4 HEWL/DTT gel exhibited the lowest $G'$ of 0.8 kPa.

The results in Figure 5.9 also revealed a large difference between the elasticity of HEWL/TCEP and HEWL/DTT gels at pH 4. TCEP is known as a stronger reducing agent than DTT that promotes a higher degree of thiol-disulphide exchange reactions between the reductant and protein molecules. As a result, this explains the formation of a stronger lysozyme hydrogel at pH 4 under the influence of TCEP rather than DTT which could be related to more fibrils formation.

At pH 4, the $G'$ was approximately 8 times higher than $G''$ for both HEWL/TCEP and HEWL/DTT samples. The difference between their $G'$ and $G''$ was larger than those of pH 5 and 7 samples which showed a magnitude difference of 5 and 6.7 respectively. It can now be correlated that samples with small difference in their moduli often appeared more elastic in nature. This could be due to the high density and connectivity of lysozyme fibrils across the gel network which was seen for pH 7 fibrils in Chapter 4.

In Figure 5.9, the crossing-over points between the $G'$ and $G''$ occurred above 100% strain for all gels with a storage modulus of at least 1 kPa. Only the pH 4 HEWL/DTT sample exhibited a shorter linear viscoelastic region with a crossing-over point at 80% strain. These results indicate the pH 5 and 7 gels were more elastic and hence, able to stretch further than the pH 4 HEWL/TCEP and HEWL/DTT gels.

To conclude, the rheological analysis above suggests the type of reductant used to destabilize the native lysozyme has certainly influenced the storage behaviour of lysozyme gels. Stronger HEWL hydrogels were formed with TCEP rather than DTT addition with the storage modulus reaching up to 5 kPa. The addition of TCEP or DTT increased the elasticity and stability of these gels in comparison to their properties under non-reducing condition.
5.5.3 Relationship between the mechanical and structural properties of lysozyme gels

The next area of interest is to find the link between the mechanical strength of a gel and the protein secondary structural elements found in HEWL/TCEP samples. From the FTIR results in Section 5.4, it is known that lysozyme gelation is promoted by the β-sheet fibrils formation. Therefore, the effect of pH on $G'$ and also on the percentage level of β-sheet was investigated here.

Figure 5.10 Storage modulus, $G'$ (red) and level of β-sheet content (black) as a function of pH for 4 mM lysozyme hydrogels prepared in 15 mM TCEP.

Figure 5.10 shows a sharp increase in $G'$ as the level of β-sheet was significantly increased from pH 3.5 to 4. It is expected strong lysozyme hydrogel was formed at pH 4 due to its high β-sheet content (~45%). The $G'$ was slightly decreasing between pH 4 and 4.5 due to a small decrease in the level of β-sheet. This has so far implies that the elastic strength of a gel is dependent on the number of fibrils formation.
In Figure 5.10, there was a sudden increase in $G'$ between pH 4.5 and 5 even though the level of β-sheet content remained almost the same (~ 45%). Quantitative results from Table 5.2 indicate the presence of a high level of aggregated strands for pH 5 HEWL/TCEP sample. This could lead to the formation of thicker fibril strands and also more stable junction points at pH 5 which will be checked with TEM in Section 5.6.

Above pH 5, the lysozyme gel became less elastic due to the apparent decrease of β-sheet content. It is suspected more α-helix to unordered rather than α-helix to β-sheet structural transition has taken place and this is confirmed with the results in Table 5.2. Nearing to pH 6, the lysozyme structure became highly unordered (~ 25.8%) with less β-sheet fibrils formation and this could attribute to its noticeably high storage modulus of 2.7 kPa. To summarise, the elasticity of lysozyme gels does not merely depend on the quantity of fibrils being formed which is evident at pH 5 and 6 for HEWL/TCEP samples.

5.5.4 Measuring the potential of HEWL/TCEP and HEWL/DTT gels for drug delivery applications

In order to develop an injectable hydrogel material that could be dispensed through a narrow bore syringe, it is important that the gel is able to withstand large strain force and quickly regain its mechanical strength. In this section, the recovery behaviour of 4 mM lysozyme hydrogels formed under different reducing conditions was being investigated.

The storage modulus of sample was measured during the pre-shear, shearing and post-shear stages at 25°C. Samples were initially pre-sheared at 0.5% strain for five minutes. During the shearing stage, samples were exposed to a large strain force i.e. at 150% strain for two minutes. They were then sheared at 0.5% strain for another 15 minutes. Here the $G'$ of pH 4 and 5 HEWL/TCEP gels and also pH 4 and 7 HEWL/DTT gels were monitored over time and shown in Figure 5.11.
Figure 5.11 Storage modulus, $G'$ as time sweeps for lysozyme gels including pH 4 HEWL/TCEP (red), pH 5 HEWL/TCEP (black), pH 4 HEWL/DTT (blue) and pH 7 HEWL/DTT (green) samples.

Figure 5.11 shows the lysozyme gels recovered about ~86% of their original mechanical strength during the beginning of post-shear stage. However, only pH 5 and 7 gels were able to maintain their $G'$ and withstand further deformation during the post-shearing stage. This suggests that the physical crosslinks found in their network were not easily disrupted. It is expected the fibrils would frequently entangled and formed many ribbon-like junctions to cause their rigidity.

In Figure 5.11, it is observed that after the removal of large strain force the $G'$ of pH 4 HEWL/DTT gel was slowly increasing until the 19$^{th}$ minute. This might indicate a breakdown of some of the physical crosslinks in its network. After the 19$^{th}$ minute, the $G'$ started to increase exponentially suggesting the mechanically rigid hydrogel was now capable to flow.
A rapid increase in $G'$ was also observed for pH 4 HEWL/TCEP sample after the 17th minute. However, its ability to restore and maintain $G'$ between 10th and 17th minute might indicate the formation of transient entanglements rather than permanent junction points of fibrils within the sample. In summary, both pH 5 HEWL/TCEP and pH 7 HEWL/DTT gels showed excellent recovery behaviour after an application of mechanical force suggesting the presence of stable junctions in their network.

5.6 Morphology of HEWL/DTT and HEWL/TCEP gels using electron and light microscopy

The main objective of this section is to examine and compare the differences in the microstructure of lysozyme gels in the presence of DTT and TCEP, on the micrometer to nanometer length scales. The effects of varying the sample pH and its degree of dilution on hydrogels microstructure will also be discussed. It is known the high water content of hydrogels made them a fairly delicate material and hence, it is quite difficult to analyse them using electron microscopy. Several modifications were made in the sampling preparation techniques including diluting one sample at different factors.

5.6.1 Microstructure of the HEWL/TCEP hydrogel formed at pH 4 and pH 5

At first, TEM experiments were conducted to reveal differences in the fibrils formation and thus, the microstructure of HEWL/TCEP gels at different pH. Only 4 mM samples were used for better quality of imaging. TEM micrographs in Figures 5.12(a) and 5.12(b) compared the density of lysozyme fibrils formed at pH 4 and 5 in the presence of TCEP.
Figures 5.12 TEM micrographs of a 250-fold diluted HEWL/TCEP hydrogel formed at (a) pH 4 (left image) and (b) pH 5 (right image) which are magnified 18,500 and 23,000 times respectively. The scale bar represents 100 nm.

In Figure 5.12(b), the pH 5 sample exhibits a slightly granular morphology which may be associated with its turbid nature. There was a higher degree of fibrillar entanglement and connectivity at pH 5, forming a denser mesh network than the pH 4 sample when both were diluted at 250-fold. This explains the higher storage modulus of 4.7 kPa and hence, more elastic nature of HEWL/TCEP gel at pH 5 in comparison to pH 4 gel ($G' = 1.6$ kPa). Goodwin and Hughes mentioned the nature of connectivity of the polymer chains affected the rheological properties of the gels [78].

It is assumed there could be differences in the self-assembling pathway between pH 4 and 5 samples resulting in less fibrils formation at pH 4. The fibrils in Figure 5.12 appeared to form several overlapping layers and the thickness of these fibrils was measured to be in the range of 29-55 nm. Therefore, a higher dilution factor (greater than 250-fold) was needed in order to image and estimate the dimension of a single lysozyme fibril in TCEP which will be discussed in the next section.

### 5.6.2 Microstructure of the HEWL/TCEP vs. HEWL/DTT gels

In an attempt to image single fibrils, the pH 4 HEWL/TCEP gel was first vortex and then diluted to 300-fold to allow the fibrils dissociation, while the pH 7 HEWL/DTT gel was diluted to 250-fold. Further comparisons were then made on the detailed structure and arrangement of fibrils chains forming the pH 7 HEWL/DTT and pH 4 HEWL/TCEP gels which were shown in Figures 5.13 and 5.14 respectively.
Figure 5.13 TEM micrograph of a 250-fold diluted HEWL/DTT hydrogel formed at pH 7. It has a magnification factor of 13,000 and the scale bar represents 200 nm.

A very granular morphology was observed in Figure 5.13 with the presence of few small protein aggregates sticking onto fibrils. At pH 7, the lysozyme fibrils formed a repetitive polygon-shaped network with constant gaps between chains in the presence of DTT. These fibrils seemed to be overlapping for several layers forming a mesh-like 3D porous network. The average dimensions of these fibrils were estimated at 13.5 nm in diameter and 457 nm in length. It was quite difficult to extract close-up images of individual pH 7 fibrils due to insufficient dilution of sample (more than 300-fold dilution might be needed).
Figure 5.14 TEM micrograph of a 300-fold diluted HEWL/TCEP hydrogel formed at pH 4. It has a magnification factor of 18,500 and the scale bar represents 100 nm.

In Figure 5.14, the average length and diameter of pH 4 fibrils were calculated as 323 nm and 11.5 nm respectively. It is also observed there were many knot-like junctions present whereby two or more lysozyme fibrils met and entwined to form these permanent junctions. However, there was a distortion in the pore size of the gel’s network which was presumably due to the fibrils distribution. Such irregularity in the arrangement of β-sheet fibrils was also found in the HEWL/water gel network at pH 2.

Lysozyme fibrils formed in the presence of DTT and TCEP were widely distributed, more rigid and relatively shorter than the ones formed in water which measured at ~7 μm in length. It is possible these reduced fibril chains were unintentionally broken into shorter segments during the vigorous sample preparation via dilution and fibrils separation. To summarise, lysozyme gels continued to form a porous structure with numerous strong permanent network junctions regardless of the type of solvent used to dissolve protein. This suggests lysozyme gels have the potential characteristic to mimic the key features of extra-cellular matrix (ECM) and to be used in biomedical applications.
5.6.3 Alignment of fibrils in crystallised lysozyme gels

The presence and alignment of fibrils in the HEWL/TCEP gels was further checked using a light microscope. Here the pH 4 HEWL/TCEP gel was observed under polarised and non-polarised light.

Figure 5.15 Crystallised stalk of pH 4 HEWL/TCEP gel observed under polarised (left) and non-polarised (right) light using the optical microscope.

Figure 5.15 shows the formation of long helical fibrils which seemed to be overlapping several times in the crystal. The wavy nature of the fibrils is in good agreement with TEM result in Figure 5.14 that showed fibrils were quite flexible such that they twisted to form permanent-knot like junctions. In addition, the stack arrangement of fibrils in Figure 5.15 could be related to the fact that they were protofibrils. These protofibrils contained at least two or more fibrils that stick together to form fibrils with a larger diameter which were found in both HEWL/TCEP and HEWL/DTT gels.
5.7 Conclusions

Another HEWL hydrogels system i.e. HEWL/TCEP gels was successfully prepared between pH 3.5 and 6 in the presence of 15 mM TCEP as the reductant. Samples were heated at 85°C for two hours and then cooled for at least 45 minutes at room temperature before their gelation took place. It was found the pI of HEWL in TCEP occurs at pH 6. This explains the formation of self-supporting, turbid hydrogels between pH 4.5 and 6 while strong and clear lysozyme gels were formed at pH 3.5 and 4. The gels were then characterised using a combination of analytical methods.

The addition of DTT and TCEP increased the elasticity of lysozyme gels such that they were able to stretch near or above 10% strain. The HEWL/TCEP gels exhibited higher storage modulus than HEWL/DTT gels, ranging from 1 to 5 kPa. The HEWL/TCEP gels were also able to recover 86% of their mechanical strength after being exposed to a very large strain. However, only pH 5 HEWL/TCEP and pH 7 HEWL/DTT gels were able to maintain their elasticity suggesting the physical crosslinks in their network were not easily disrupted and could form permanent junction points of fibrils.

FTIR results revealed pH 5 HEWL/TCEP sample contained the highest percentage of β-sheet fibrils (about 45%) which contributed to its high storage modulus of ~4.7 kPa. A high level of aggregated strands (between 11 and 21%) was found within HEWL/TCEP samples implying lysozyme fibrils in TCEP were most likely to aggregate and stick together forming thicker fibrils. This agrees with TEM results that showed lysozyme fibrils formed several overlapping layers with a higher degree of entanglement and connectivity. At pH 4, a single HEWL/TCEP fibril was measured as 323 nm and 11.5 nm in length and diameter respectively. At pH 7, lysozyme fibrils formed a dense polygon-shaped network in the presence of DTT. These results further demonstrate the prospective of lysozyme hydrogels to be used as cell scaffolds in tissue engineering and also as an injectable material for drug delivery use.
Chapter 6 Designing temperature-responsive hydrogels from physical mixtures of HEWL and HEWL/PNIPAAm conjugate using thiol-ene click chemistry

6.1 Introduction

In the previous chapter, it was demonstrated that a small quantity of TCEP (15 mM) was used to encourage Hen Egg White Lysozyme (HEWL) gelation by heating samples at 85°C for 2 hours. Stronger lysozyme hydrogels were formed at a lower range of pH (between pH 3.5 and pH 6) in TCEP. The final part of this work aims to combine the thermo-responsive behaviour of poly-N-isopropylacrylamide (PNIPAAm) with HEWL gelling properties by synthesising bioconjugates of HEWL/PNIPAAm. The effect of incorporating fractions of bioconjugates into reduced HEWL solution will be investigated by measuring the thermal behaviour, morphology and mechanical properties of the composite system. It is expected that the protein segment will self-assemble into fibres while the polymer will introduce the thermo-responsive behaviour to the gel [42].

PNIPAAm is one of the most commonly studied polymers since it has a LCST of ~32°C which is near to the body temperature [70]. Its LCST behaviour plays an important role for its application as drug delivery agents [107]. For instance, N-isopropylacrylamide (NIPAAm) was co-polymerised with other hydrophilic co-monomers to form coated tablets with a LCST higher than 37°C. This allows the encapsulated drug to be soluble at 37°C and release into the system above this temperature due to the collapse of co-polymers [107].

Several studies have reported the successful preparation of proteins/PNIPAAm conjugates. Heredia et al. demonstrated the in-situ polymerisation of NIPAAm from site-specific modification of Cys-131 of T4 lysozyme whereby polymerisation has no impact on the bioactivity of lysozyme [14]. The Sumerlin group employed the RAFT polymerisation of NIPAAm to yield NHS-terminated PNIPAAm for conjugation with amine-reactive HEWL [108] and also maleimide-terminated PNIPAAm grafted-to bovine serum albumin (BSA) and ovalbumin (OVA) [109]. The group also reported the modification of BSA with a maleimide-functionalized CTA by grafting-from approach via RAFT polymerization [110]. In this work, a strategy has been developed to prepare this
bioconjugate which involves postpolymerisation modification of the synthesised PNIPAAm [14]. The pre-formed polymer is to become thiol-reactive using aminolysis reaction and TCEP addition [68]. TCEP is also used to cleave the disulfide bonds of lysozyme. A permanent linkage is then formed between the PNIPAAm-SH and the reduced lysozyme by adding a homobifunctional sulfhydryl-reactive crosslinker to the polymer [109].

Section 6.2 discusses the results of PNIPAAm synthesis using controlled radical polymerisation technique and also the characterisation of the pre-formed polymer. Section 6.3 discusses the physical preparation of this conjugate and the presence of a well-defined conjugate is explored using a combination of microDSC, rheology and AFM techniques. The rheological properties of lysozyme-PNIPAAm hydrogels is to be compared to those lysozyme gels formed with TCEP addition.

6.2 Synthesis and characterisation of PNIPAAm polymer

Referring to Figure 3.2, the reversible addition-fragmentation chain transfer (RAFT) radical polymerisation of N-isopropylacrylamide (NIPAAm) was accomplished with 2-(dodecylthiocarbonothioylthio)-2-methylpropanoic acid (DMP) as the RAFT chain transfer agent (CTA) in combination with azobisisobutyronitrile (AIBN) in dimethylformamide (DMF) at 65°C [68, 70]. The reaction was allowed to mix for 8 hours in order to meet the targeted molecular weight of polymer and preserve the thiocarbonylthio end-group at the same time. PNIPAAm batches were synthesised at degree of polymerisation (DP) of 40 and 60 with a targeted molecular weight of 4526 g mol⁻¹ and 6790 g mol⁻¹ respectively and the molar ratio of their feed components was shown in Table 6.1.

Table 6.1 Molar ratio of NIPAAm, CTA, AIBN and DMF used to make PNIPAAm at DP of 40 and 60.

<table>
<thead>
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<th>ID</th>
<th>Molar Ratio Feed</th>
<th>Expected Mr</th>
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</thead>
<tbody>
<tr>
<td></td>
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<td>CTA</td>
</tr>
<tr>
<td>PNIPAAm40</td>
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<td>1</td>
</tr>
<tr>
<td>PNIPAAm60</td>
<td>60</td>
<td>1</td>
</tr>
</tbody>
</table>
The individual masses of monomer, CTA, initiator and solvent used to synthesise PNIPAAm40 and PNIPAAm60 were illustrated in Table 6.2 and Table 6.3.

Table 6.2 Mass of monomer, chain transfer agent (CTA), initiator and solvent used in the radical polymerisation process to form RAFT-PNIPAAm at DP=40.

<table>
<thead>
<tr>
<th></th>
<th>MW</th>
<th>molar ratio</th>
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<th>m / g</th>
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<tbody>
<tr>
<td>Monomer</td>
<td>NIPAAm</td>
<td>113.16</td>
<td>40</td>
<td>35.35</td>
</tr>
<tr>
<td>CTA</td>
<td>DMP</td>
<td>364.63</td>
<td>1</td>
<td>0.88</td>
</tr>
<tr>
<td>Initiator</td>
<td>AIBN</td>
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<td>0.2</td>
<td>0.18</td>
</tr>
<tr>
<td>Solvent</td>
<td>DMF</td>
<td>73.09</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.3 Mass of monomer, chain transfer agent (CTA), initiator and solvent used in the radical polymerisation process to form RAFT-PNIPAAm at DP=60.

<table>
<thead>
<tr>
<th></th>
<th>MW</th>
<th>molar ratio</th>
<th>n / mM</th>
<th>m / g</th>
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</thead>
<tbody>
<tr>
<td>Monomer</td>
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<td>60</td>
<td>35.35</td>
</tr>
<tr>
<td>CTA</td>
<td>DMP</td>
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<td>1</td>
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</tr>
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<td>0.2</td>
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</tr>
<tr>
<td>Solvent</td>
<td>DMF</td>
<td>73.09</td>
<td>4</td>
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</table>

It was initially aimed to explore the effect of increasing the length of polymer chain on the mechanical properties of composite gels. The size of the RAFT-polymer chain is expected to be in the range of 32 - 48% of the protein size assuming 100% conversion took place. It was anticipated that using shorter PNIPAAm chains would increase the number of covalent cross-links form with protein chain by physical mixing at room temperature.

### 6.2.1 PNIPAAm with CTA end (RAFT-PNIPAAm)

The powdered RAFT-PNIPAAm samples were collected as in Section 3.3.2 and now referred to as RAFT-PNIPAAm40 and RAFT-PNIPAAm60. These polymers were characterised using several experimental techniques including mass spectroscopy, $^1$H NMR spectroscopy and microDSC.
6.2.1.1 Distribution of polymer molecules

The Time-of-flight mass spectrometry (TOF-MS) aims to predict the molecular weight of polymers based on the spread or distribution of their m/z values. The results of mass spectroscopy for RAFT-polymers were shown in Figure 6.1 and Figure 6.2.

Figure 6.1 TOF-MS spectrum for RAFT-PNIPAAm40

Figure 6.2 TOF-MS spectrum for RAFT-PNIPAAm60
Figures 6.1 and 6.2 revealed the samples have a relatively broad molecular weight distribution at higher mass-to-charge ratio within the range of 800 to 1400. This implies there was a control over the molecular weight of polymers during RAFT polymerisation and hence, confirms the presence and role of the RAFT agent that is to exert control over the radical polymerisation process [68]. However, TOF-MS is not suitable for the accurate prediction of polymers molecular weight in this case due to the multi-components feed mixture.

6.2.1.2 Degree of polymerisation for RAFT-PNIPAAm

$^1$H NMR spectroscopy was used in Figure 6.3 to confirm that NIPAAm was polymerised and also to determine the resulting degree of polymerisation for the RAFT-PNIPAAm samples. Figure 6.3 shows the $^1$H NMR spectrum of the PNIPAAm within the region of interest: multiplets in the range of 3.6-4.5ppm reflecting the protons from the polymer backbone.

Figure 6.3 $^1$H NMR spectrum, recorded in D$_2$O, of the RAFT-PNIPAAm40 highlighting the presence of end-functional group and the calculation of the absolute degree of polymerisation.

Using the Spinwork software, $^1$H NMR spectroscopic end group analysis was performed to calculate the integral ratio of protons. Two integrals were recorded within this region of
interest; the integral of CH (3.55-3.65ppm) which is labelled as d and also the integral of CH-S (~4.5ppm). Both were compared as in Figure 6.3 and their relative size corresponds to the degree of polymerisation of synthesised PNIPAAm polymer. The degree of polymerisation was estimated to be 24 for RAFT-PNIPAAm40 and 47 for RAFT-PNIPAAm60. Samples are now referred to as RAFT-PNIPAAm24 and RAFT-PNIPAAm47 with estimated molecular weight of 2716 and 5319 respectively. These results indicate the yield was between 60 and 78% and that the reaction conversion was not 100% completed. This is in good agreement with Li et al. who reported the yield as 83% for RAFT-polymerisation of NIPAAm with a trithiocarbonate CTA and AIBN as the initiator in 1, 4-dioxane at 60°C.

There are two possible reasons for this; the first one could be related to the insufficient amount of RAFT agent present due to loss of its mass when transferred into the schlenk tube. The thiocarbonylthio group in RAFT agent is responsible for propagating the living chains in the polymerisation process [67]. Secondly, the growth of polymer chains might be stopped due to irreversible termination reactions from the radicals that initiate the RAFT polymerisation [111]. The radicals on two different growing chains can combine to form a single chain or that a free radical might strip off hydrogen atom from an active chain resulting in two dead chains. Yu et al. reported the presence of terminated products after 2 hours of coupling reaction of NIPAAm [70]. It is highly likely the termination reactions have occurred during the 8 hours of RAFT polymerisation process and to corroborate this, the sample was scanned using microDSC to observe its thermal transition which might show the presence of terminated products.

6.2.1.3 Thermal behaviour of NIPAAm and RAFT-PNIPAAm in water

MicroDSC is employed to monitor the LCST of polymer and also to detect the presence of impurities including terminated products within the temperature range of 20°C to 60°C. A small quantity (~ 0.02g) of the powdered purified NIPAAm monomer and polymerised RAFT-PNIPAAm were dissolved in 0.5mL of distilled water. These samples were then subjected to 3 cycles of heating and cooling and the heating runs of RAFT-polymer were shown in Figure 6.4.
Figure 6.4 MicroDSC curves of RAFT-PNIPAAm47 sample at pH 4 showing the first (blue), second (red) and third (green) heating runs during three heating/cooling cycles at a scanning rate of 1 °C min⁻¹.

In Figure 6.4, two endothermic transitions were observed at 29.6 ± 0.5°C and 36.9 ± 0.5°C during the heating runs of RAFT-PNIPAAm47 which were attributed to the LCST of the polymer. The thermal transitions of RAFT-polymer were fully reversible upon first and second cooling indicating no hysterises (deformation) of polymer. The result of the first endothermic peak is in line with the finding of Bulmus et al who mentioned PNIPAAm has a LCST of 29.9°C at pH 4 [112]. It has also been reported that the polymer molecular weight can affect the LCST of PNIPAAm both inversely and directly [113-115]. Winnik et al. found that the linear PNIPAAm of 6000 g mol⁻¹ has a LCST of 37°C [116] which is comparable to the second peak temperature at 36.9 ± 0.5°C in Figure 6.4 where RAFT-PNIPAAm47 sample has an estimated MW of 5319.

Therefore, it is suspected that the two endothermic transitions in Figure 6.4 could be related to the formation of RAFT-polymers with different molecular weights. This is confirmed with the results in Figure 6.5 that showed the first heating of NIPAAm monomer which was used to synthesise the polymer.
Figure 6.5 MicroDSC curves of NIPAAm monomer during the first heating run at a scanning rate of 1 °C min⁻¹.

Three endothermic transitions were observed in Figure 6.5 where the peak temperatures were higher than the RAFT-PNIPAAm47 sample. This suggests the presence of various average molecular weights of monomer molecules that led to RAFT-polymers with different molecular weights.

In Figure 6.4, the RAFT-polymers in the first and second endotherms are now being referred as P1 and P2 respectively. P1 has an associated enthalpy of 391 ± 30 mJ g⁻¹ which is larger than the enthalpy of P2, 87 ± 30 mJ g⁻¹. It is estimated that the ratio of P1 to P2 is 4.5:1 based on the size of their enthalpies indicating a higher proportion of the RAFT-polymer has a LCST of 29.6 ± 0.5°C. In addition, it is quite difficult to detect the presence of impurities or terminated products from the microDSC curves at this stage. To conclude, the RAFT-polymerisation of NIPAAm with the choices of DMP as the RAFT agent and AIBN as the initiator produced samples of 2716 g mol⁻¹ and 5319 g mol⁻¹ despite their higher targeted DP of 40 and 60 respectively.
6.2.2 Thiol-terminated PNIPAAm (PNIPAAm-SH)

Both RAFT-PNIPAAm24 and RAFT-PNIPAAm47 were subjected to aminolysis reaction (Figure 3.3) in order to cleave the S-C=S-S group in the polymer and promote its thiol-end (S-H) functionalisation. The aminolysis of RAFT-PNIPAAm was accomplished with TCEP as the reducing agent in combination with butylamine as the source of amine in 8 mL DMF solution at 30°C. The mass of RAFT-polymer and TCEP and also volume of butylamine used are shown in Table 6.4.

Table 6.4 Mass of RAFT-PNIPAAm and TCEP and also volume of butylamine used during aminolysis to form thiol-terminated polymers.

<table>
<thead>
<tr>
<th></th>
<th>MW</th>
<th>DP = 24</th>
<th></th>
<th>DP = 47</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>molar ratio</td>
<td>m / g</td>
<td>V / mL</td>
<td>molar ratio</td>
<td>m / g</td>
</tr>
<tr>
<td>RAFT-PNIPAAm</td>
<td>5319</td>
<td>1</td>
<td>0.50</td>
<td>1</td>
<td>0.50</td>
</tr>
<tr>
<td>TCEP</td>
<td>73.14</td>
<td>1.2</td>
<td>0.06</td>
<td>1.6</td>
<td>0.04</td>
</tr>
<tr>
<td>Butylamine</td>
<td>286.65</td>
<td>6</td>
<td>0.08</td>
<td>7.8</td>
<td>0.07</td>
</tr>
</tbody>
</table>

The reaction was allowed to mix for 4 hours until the yellow RAFT-PNIPAAm solution became colourless indicating the S-C=S-S group was successfully broken. The dried powdered thiol-end polymers were collected and referred as PNIPAAm24-SH and PNIPAAm47-SH. They were subjected to further characterisation using the TOF-MS analysis in Figure 6.6 and microDSC analysis in Figure 6.7.

Figure 6.6 TOF-MS spectra for (a) RAFT-PNIPAAm47 and (b) PNIPAAm47-SH
Figures 6.6(a) and 6.6(b) show the distribution of molecules for RAFT-PNIPAAm47 and PNIPAAm47-SH respectively which are quite similar in pattern. The PNIPAAm47-SH molecules in Figure 6.6(b) were found at higher mass-to-charge ratio but at lower percentages than the RAFT-PNIPAAm47 sample in Figure 6.6(a). This implies the possibility of unwanted products removal during aminolysis and the formation of thiol-end polymer with just one molecular weight which would be checked with microDSC. The PNIPAAm47-SH sample was then subjected to three heating runs which were shown in Figure 6.7.

![MicroDSC curves of PNIPAAm47-SH sample (at pH 3.78) showing the first (blue), second (red) and third (green) heating runs during three heating/cooling cycles at a scanning rate of 1 °C min⁻¹.](image)

In Figure 6.7, a single endothermic transition was observed at 37.6 ± 0.5°C during the first heating of PNIPAAm47-SH with an associated enthalpy of 295 ± 30 mJ g⁻¹. This confirms that the thiol-terminated polymer chains exhibit an average molecular weight. The second and third endothermic transitions occurred at 35.0 ± 0.5°C and 34.6 ± 0.5°C with associated enthalpies of 296 ± 30 mJ g⁻¹ and 302 ± 30 mJ g⁻¹ respectively. The endothermic enthalpy of PNIPAAm47-SH is smaller than RAFT-PNIPAAm47 which could be related to the small quantity of PNIPAAm47-SH formed in Figure 6.6(b).
In general, the thermal transitions were fully reversible for PNIPAAm47-SH and the size of their enthalpies were almost similar. It is suspected there is a difference on the interactions between polymer segments and water molecules during the first heating in comparison to second and third heating which affect its LCST. Taylor and Cerankowski also mentioned the LCST of polymer in solution is dependent on its hydrophilic and hydrophobic balance [117].

However, it is not fully understood at this stage the reason for the higher LCST during first heating. It is assumed that PNIPAAm47-SH has a LCST within the range of 34.6 - 35.0°C with an expected molecular weight of 5427. These results are comparable to Schild and Tirrell who reported PNIPAAm sample of 5400 g mol⁻¹ has a LCST of 34.3°C [113]. In summary, PNIPAAm exhibits different LCST depending on the functional end group it has. RAFT-PNIPAAm47 shows two LCST at 29.6 ± 0.5°C and 37.9 ± 0.5°C while PNIPAAm47-SH exhibits a LCST in between 34.6 and 35.0°C.

6.3 Formation of bioconjugate and also the composite gel of HEWL/PNIPAAm by disulfide cross-linking

This section discusses the formation of protein-polymer bioconjugate by reacting the thiol-terminated polymer chains with excess bismaleimidohexane (BMH) and readily coupling the product to thiol-containing HEWL. It also demonstrates the incorporation of a small quantity of the bioconjugate into the protein matrix by grafting-to approach yielding the final composite HEWL/PNIPAAm gel.

6.3.1 Bioconjugates formation via stable thioether linkages

Referring to Figures 3.5 and 3.6, PNIPAAm-SH was first mixed with excess BMH crosslinker at a ratio of 1:2 in minute quantities. The resulting maleimide-terminated polymer was reacted with reduced HEWL in TCEP and subjected to physical mixing for one day. The ratio of HEWL to PNIPAAm used is 0.1:1. It is aimed to introduce a permanent cross-link between the reduced protein and the thiol-terminated PNIPAAm by forming the stable thioether linkages and this is illustrated in Figure 6.8.
PNIPAAm with attached BMH was used in excess of HEWL in order to favour the formation of lysozyme-polymer bioconjugates. It also led to better removal of the unwanted products (unreacted PNIPAAm-BMH) from the wanted bioconjugate through water dialysis. The dialysed bioconjugate was dried, collected and analysed using TOF-MS in Figure 6.9. This is to confirm its presence and observe the distribution of molecules. At this stage, it was noticed that there was a massive loss of dialysed conjugate at DP of 24.

In Figure 6.9, it is observed that there was a poor signal to noise ratio for the mass spectrum which could be due to the significantly low percentages of dialysed conjugate molecules present. The signals found at higher mass-to-charge ratio indicate the presence of polymer molecules. The list of these m/z ions was used in Equations 3.8 and 3.9 in order to estimate the molecular weight of sample. In Figure 6.9, the molecular weight of dialysed bioconjugate at DP of 47 was calculated as 20,874 g mol\(^{-1}\)which is comparable to its

Figure 6.9 TOF-MS spectrum for dialysed bioconjugate at DP of 47.
expected weight of 20,182 g mol\(^{-1}\). At DP of 24, the calculated molecular weight of bioconjugate was 15,843 g mol\(^{-1}\) while the expected weight was 17,579 g mol\(^{-1}\). The standard error of these arithmetic measurements is within ±10% from the targeted values.

6.3.2 Formation and characterisation of the composite HEWL/PNIPAAm gel

6.3.2.1 Visual observation

The main aim of this section is to confer the thermoresponsive nature of PNIPAAm to the model protein HEWL by introducing a small quantity of the bioconjugate into the major protein matrix. The idea is to use any of the free sulfhydryl groups of lysozyme in the bioconjugate (assuming one has been used for thioether bond and the other seven –SH groups are free) and form a disulphide (S–S) linkage with thiol-containing HEWL in TCEP solution (Figure 6.10).

![Figure 6.10 Schematic representation of the bioconjugate forming disulphide bonds (circled) with thiol-containing HEWL that constitutes the major protein matrix. The dotted lines are examples of unreacted thiol-groups in protein.](image)

Since lysozyme in bioconjugate can have up to 7 thiol groups reacting with thiol-groups of HEWL, it is likely that multiple-points covalent attachment of bioconjugate chains will take place rather than just a single-point. However, this work focuses more on combining the thermoresponsive property of PNIPAAm with HEWL instead of locating the exact points of attachment.

A small volume (0.2 mL) of the dialysed bioconjugate sample with DP of 47 and concentration of 2 mg mL\(^{-1}\) was added to 1 mL of reduced HEWL in TCEP. The reduced protein was prepared at 4 mM as in Section 3.3.1. The resulting mixture or also known as the composite HEWL-PNIPAAm47 solution was vortexed for 2-3 minutes and prepared at pH 4.5 since strong HEWL/TCEP gel was formed at this pH in Section 5.
Figure 6.11 Typical photographs of (a) HEWL/TCEP gel and (b) the composite gel containing mixture of HEWL matrix and small amount of bioconjugates and these samples were prepared at pH 4.5.

Figures 6.11(a) and 6.11(b) show both HEWL/TCEP and composite samples formed self-supporting gels after heating at 85°C and then cooling at ambient temperature. This implies the protein within the composite was able to self-assemble even if the bioconjugate chains may be grafted to the protein surface at multiple points. It is also observed that no macroscopic aggregates were formed within the composite sample during heating and this might be related to the low polymer density used. To confirm the incorporation of bioconjugate molecules to the protein matrix, the composite sample was characterised using several analytical techniques including microDSC, AFM and rheology.

6.3.2.2 Thermal behaviour of composite HEWL/PNIPAAm solution

This section aims to establish that covalent cross-links have been formed between the bioconjugate and reduced HEWL within the composite sample. Using microDSC, the thermal transitions of composite solution was monitored to detect the presence of PNIPAAm and HEWL and also explore the reversibility of transitions which relate to its gelling behaviour. 0.5 mL of HEWL-PNIPAAm47 composite solution was prepared at pH 4.5. It was subjected to three heating and cooling cycles and the heating runs are shown in Figure 6.12.
In Figure 6.12, two endothermic peaks were observed at 35.7 ± 0.5°C and 61.3 ± 0.5°C during the first heating run. The melting temperature and areas of these peaks are associated with the LCST of PNIPAAm and the denaturation of lysozyme respectively. De et al. reported a similar LCST of 35°C for BSA-PNIPAAm conjugates formed by grafting-from approach via RAFT polymerisation [110]. In Figure 6.12, the enthalpy of the first endotherm, 58.1 ± 10 mJ g⁻¹ was smaller than the denaturation enthalpy of HEWL during first heating, 351.9 ± 20 mJ g⁻¹. The size of these enthalpies might reflect the relative amount of interactions between the polymer and protein during first heating.

It is also observed that the thermal transitions of first heating were not reproducible during the second and third heating. This implies a large portion of lysozyme unfolded during the first heating and gelation of the sample took place after first heating/cooling cycle. On further heating, the temperatures and enthalpies of protein denaturation transitions were observed to be reproducible suggesting further melting and re-forming of gel by simple heating and cooling. Yan et al. reported the lysozyme/DTT gel would only be fully melted

Figure 6.12 MicroDSC curves of HEWL-PNIPAAm47 composite sample showing the first (blue), second (red) and third (green) heating runs during three heating/cooling cycles.
at 90°C [2]. These results show that protein gelation for the composite sample occurred after the first heating and the gel could be further melted if heated.

Figure 6.12 shows the peak temperature of first endotherm decreases from 35.7 ± 0.5°C during the first heating to 29.5 ± 0.5°C on the second and third heating. It is suspected that upon first heating the protein part of the protein-polymer bioconjugate will self-assemble with the protein that forms the major matrix causing the polymer to be distributed along the fibres [42]. Hence, the peak temperature at 29.5 ± 0.5°C during second and third heating might represent the actual LCST of PNIPAAm with attached BMH crosslinker. The temperature of this transition agrees well with Maslovskis et al. who reported the LCST of PNIPAAm as ~31°C for PNIPAAm-FEFEFKFK hydrogels [118]. It is interesting that at this point the polymer in the composite sample has a LCST similar to RAFT-PNIPAAm which also exhibited a peak temperature at 29.6 ± 0.5°C.

To summarise, both synthesised polymer and lysozyme protein were detected in the composite sample based on their LCST and denaturation temperature respectively. However, it has not been confirmed that the polymer is clicked with the protein at high temperature especially during fibrils formation. To solve this, AFM imaging on protein-polymer particle might be necessary.

6.3.3 PNIPAAm attachment onto lysozyme fibrils

Atomic Force Microscopy (AFM) is a powerful surface characterisation technique that gives direct measurements of height and volume with 3D display [87]. It is often used to target individual particle imaging and characterisation. This method requires minimal sample preparation at ambient temperature and it is also cost-effective [87]. 4 mM HEWL/PNIPAAm composite and HEWL/TCEP gels were prepared at pH 4.5 and they were diluted 500-fold in water and agitated vigorously to separate the fibrils. The results of AFM imaging on these samples were shown in Figures 6.13 and 6.14.
Figure 6.13 AFM images of lysozyme fibrils found in (a) HEWL/TCEP and (b) composite HEWL/PNIPAAm gels. The scan size is 5 µm with a scanning rate of 0.501 Hz.

Figures 6.13(a) and 6.13(b) show the formation of clusters of entangled fibrils within the network and such distribution could be due to the high dilution factor used. These results indicate physical gels were formed even if the polymer chains are successfully cross-linked to the surface of protein fibres. The fibrils in Figure 6.13(a) have an estimated diameter of ~8.6 nm and this is comparable to the width of pH 4 HEWL/TCEP fibrils in Figure 5.15 which is approximately around ~11.5 nm when diluted 300-fold. For the composite sample, the fibrils average width is estimated to be ~14.5 nm. Such increase in the diameter size could be due to the sticking of two or more fibrils to form thicker strands or the attachment of polymer chains onto the fibres. Further analysis was then performed on the composite gel at a different scan size.
Figure 6.14 AFM 3D-imaged of lysozyme fibrils in (a) HEWL/TCEP gel and (b) HEWL/PNIPAAm composite sample with suspected polymer deposits. The scan size is 1.02 µm with a scanning rate of 0.501 Hz.

Figure 6.14(b) shows the fibrillar network of the composite gel with some “globular features” present on the outer surface of the yellow fibres. It is assumed that these “globular features” correspond to the distribution of polymer segments along the protein fibres. The suspected polymers exhibit random distribution throughout the network. In Figure 6.14(b), the width of at least 15 fibrils with and without “globular features” was directly measured. The average diameters of fibrils with and without “globular features” are estimated to be ~21 nm and ~16 nm respectively. Hence, this suggests the length of polymer chains with cross-linker to be ~5 nm with an expected MW of 5600 g mol\(^{-1}\).

It is noted there is the difficulty to measure the exact length of composite fibrils in Figure 6.14(b) but they were found in the range of 100 - 400 nm. This is comparable to the average length of pH 4 lysozyme fibrils i.e. ~323 nm as reported in Section 5.6.2.

Lysozyme fibrils were found repeatedly twisting and linking with each other and hence, forming many permanent junction points. This was in good agreement with TEM result in Figure 5.5 that shows the frequent entanglements of fibrils with disparities in the pore size of the network. In Figure 6.14(b), the network pore size varied from 20 to 40 nm but larger distances were also found in the range of 75 - 100 nm. These results have also been
reported by Maslovskis for the peptide system FEFEFKFK and the composite gels of FEFEFKFK and PNIPAAm [42].

![Diagram](image)

Figure 6.15 Mechanism showing the self-assembly of lysozyme (black arrows) and their conjugates with attached polymer chains (blue) that form mesh network of fibrils (black thread-like structure) with polymers lining on its surface within the composite gel. Diagram adapted from [42].

In summary, AFM images revealed the random formation of “globular features” on the fibres surface possibly indicating the ‘hit and miss’ attachment of polymer segments onto protein fibres that led to an increase in their diameter by \(~5\) nm. To confirm such attachment has taken place, the mechanical properties of the samples were measured and compared in the next section.

### 6.3.4 Rheological properties of HEWL/PNIPAAm composite hydrogel

This section aims to investigate and evaluate the mechanical behaviour of HEWL/TCEP and HEWL/PNIPAAm composite gels in response to changing temperature. The samples were subjected to two heating and cooling cycles within the temperature range of 25-85°C and their \(G'\) were closely monitored in Figure 6.16.
Figure 6.16 Storage modulus, $G'$ as temperature sweeps for HEWL/TCEP gel (blue) and HEWL/PNIPAAm composite (red) during (a) 1st heating, (b) 1st cooling, (c) 2nd heating and (d) 2nd cooling run at a rate of 1°C min$^{-1}$.

Figure 6.16(a) shows the composite gel has a lower $G'$ than the pure protein gel at 25°C which measured at 2.3 kPa and 6.5 kPa respectively. It is suspected there is a difference in the network structure of these gels. Few studies have reported that the presence of polymer in the conjugates influenced the conformational behaviour of the peptide/native protein and thus, the differences in their network [109, 119]. During the first heating, both gels were able to maintain their elasticity as the temperature increases from 25°C to 60°C due to the presence of many strong physical junctions in their network as shown in previous AFM and TEM images. Such characteristic proves to be valuable if the hydrogels are to be used for therapeutic delivery at the body temperature of 37°C. Above 60°C, there is a significant decrease in $G'$ of both systems during first heating and they are still decreasing.
at 85°C. For examples, the $G'$ decreased from 6.5 kPa to 1.8 kPa for pure protein matrix while the $G'$ of composite matrix decreased from 2.3 kPa to 0.65 kPa. Both gels have lost about 72% of their original elasticity upon first heating. Yan et al. also reported a large decrease in $G'$ (from 800 to 20 Pa) from 55°C onward during the heating of lysozyme/DTT/water system [2]. These results could have been associated with the melting of links or aggregates found within the gel network and hence, the macroscopic melting of the gels occurred.

Figure 6.16(b) shows an exponential increase in $G'$ from 85°C to 48°C on the first cooling of both gels. It is suspected some of the physical entanglements or cross-links have been reformed upon cooling resulting in stronger gel network (higher $G'$ than first heating) especially from 60°C to 44°C. Below 44°C, the pure protein matrix showed a significant loss in its elasticity which might be related to changes in intermolecular fibre-fibre interactions. This is in contrast to the composite sample which exhibits constant $G'$ (4.3 kPa) between 37°C and 44°C presumably due to the intramolecular cross-linking between collapsed polymer segments and protein fibres.

However, the composite gel shows a small decrease in $G'$ between 25°C and 37°C where the LCST of PNIPAAm lies. Shakya et al. reported the PNIPAAm constantly changes its conformation between dense globule and loose coil around its LCST and hence, this might affect the stiffness of protein fibres which explains the slower decrease in $G'$ in comparison to the pure lysozyme gel [120]. At 25°C, the composite gel was able to restore its original elasticity (before 1st heating) with $G'$ of 2.3 kPa while the pure lysozyme gel exhibits a major loss in its elasticity when $G'$ decreased from 6.5 kPa to 2.2 kPa.

In Figure 6.16(c), there was a very small decrease in $G'$ of both samples as the temperature increases from 25°C to 60°C during the second heating. Such rheological behaviour suggested some disruption on the physical crosslinks that define the gels network. It is also observed there was an exponential increase in $G'$ of pure lysozyme gel between 60°C and 68°C which has not been fully understood yet. However, the incorporation of polymer segments into the composite matrix seems to limit the drastic increase of $G'$. The melting of these gels is believed to occur at and above 68°C that resulted in continuous decrease of their $G'$. 

150
A similar pattern was observed between the first and second cooling of both gels in Figures 6.16(c) and 6.16(d) respectively. However, during second cooling the highest $G'$ was recorded at 52°C instead of 44°C and thus, there is presumably a slight change in the re-formation of physical crosslinks and the gel network after two successive heating and cooling processes. One notable observation in Figure 6.16(d) is the composite gel has regained its elasticity (2.3 kPa) even after the end of second cooling. Hence, these results demonstrate the thermoreversibility of the composite gel which has higher storage and thermal stability in comparison to pure lysozyme gel. It also suggests the reusability of lysozyme-PNIPAAm composite gel in its application. In summary, the covalent attachment of small quantity of responsive PNIPAAm to HEWL protein results in excellent storage stability and also more thermally stable composite sample in comparison to pure lysozyme matrix.

6.4 Conclusions

This chapter reported a simple strategy or approach to synthesise a temperature responsive lysozyme-PNIPAAm composite gel by using post-modification of preformed polymer and thiol chemistry in the covalent cross-linking of bioconjugates to HEWL. PNIPAAm was prepared at degree of polymerisation of 47 using free-radical RAFT polymerisation. MicroDSC results revealed the formation of RAFT-PNIPAAm47 with two different MW and hence, two LCST were observed at 29.6°C and 36.9°C. End group aminolysis of RAFT-PNIPAAm was performed to facilitate the formation of its thiol end-group. Thiol-terminated PNIPAAm47 shows a LCST of ~35°C and it was subsequently reacted with excess bismaleimidohexane to yield maleimide-terminated polymers that were readily attached with thiol-containing HEWL.

A small fraction of the bioconjugate mixture was incorporated into lysozyme in TCEP solution that would form the major matrix after heating the sample at 85°C. It is hypothesised the protein segments from the bioconjugate would self-assemble and form fibres together with the major protein matrix and thus, the polymer segments are attached and distributed along the fibres. The presence of both polymer and protein within the composite solution was detected at 35.7°C and 61.3°C respectively during their first heating using microDSC.
AFM imaging demonstrates the formation of “globular features” on the surface of protein fibres which are suspected to be segments of synthesised PNIPAAm chains. Further rheological studies revealed the composite gel has excellent storage stability. The gel was able to maintain its elasticity at 2.3 kPa when subjected to two successive heating and cooling cycles within the temperature range of 25-85°C. This is in contrast to the pure lysozyme matrix that exhibits a large decrease in $G'$ from 6.5 kPa to 2.2 kPa by the end of second cooling. These preliminary studies indicate the successful attachment of responsive PNIPAAm chains to HEWL which gives the protein higher stability in response to changing temperature.
Chapter 7  Conclusions and Future Work

7.1 Conclusions

Hen Egg White Lysozyme (HEWL) was used as a model protein to study protein gelation under reducing and non-reducing conditions. Samples were heated at 85°C for different times at different pH and subsequently cooled at room temperature for 45 minutes in order to encourage gelation. Self-supporting gels were formed after 3 days of heating the protein in water at pH 2 and also after an hour of heating in the presence of DTT at pH 7.

Using a combination of analytical techniques, the physical properties and morphology of these gels were investigated. MicroDSC results revealed lysozyme denaturation in water was quite slow indicating there is a lag time to form fibrils. The pH 2 HEWL/water sample exhibits a high storage modulus of 6 kPa with shorter linear viscoelastic region (LVR), suggesting the gel is strong but brittle in nature. The pH 2 fibrils were several microns in length, reasonably flexible and distributed irregularly, which might contribute to their brittleness.

The addition of DTT has encouraged the formation of weak gels at pH 3 and pH 4 after 24 hours of heating at 85°C. MicroDSC results confirmed lysozyme becomes less stable with DTT addition and high pH resulting in smaller denaturation enthalpies in comparison to lysozyme in water. FTIR results revealed more α-helix to β-sheet structural transitions occurred between pH 3 and pH 7. The highest β-sheet to α-helix ratio was observed at pH 7, indicating extensive fibril formation, which promotes strong gelation. The pH 7 fibrils were found to be shorter and stiffer, forming a dense polygon-shaped porous network with a storage modulus in the range of 0.75-1 kPa.

The addition of a small quantity (15 mM) of TCEP encouraged HEWL gelation in between pH 3.5 and 5.5. These HEWL/TCEP gels exhibit rather high storage modulus ranging from 1 to 5 kPa. They were able to recover 86% of their mechanical strength after being exposed to a very large strain. The highest percentage of β-sheet (about 45%) was found at pH 5 which explains its high storage modulus of 5 kPa. It is suspected that lysozyme in TCEP formed thicker fibrils with more permanent junction points than lysozyme in DTT.
A temperature responsive lysozyme-PNIPAAm composite gel was formed by incorporating small fraction of the PNIPAAm-BMH-HEWL bioconjugate into lysozyme in TCEP solution that would form the major matrix after heating the sample at 85°C. To form the bioconjugate PNIPAAm was prepared at degree of polymerisation of 47, using free-radical RAFT polymerisation. The RAFT-PNIPAAm was aminolysed to form thiol-terminated PNIPAAm47 that shows a LCST of ~35°C. It was subsequently reacted with excess bismaleimidohexane to yield maleimide-terminated polymers that were readily attached with thiol-containing HEWL.

MicroDSC results revealed the presence of both polymer and protein within the composite solution at 35.7°C and 61.3°C respectively during their first heating. AFM imaging shows the formation of “globular features” on the protein fibres surface which are suspected to be segments of synthesised PNIPAAm chains. The composite gel exhibits high storage stability by maintaining its elasticity at 2.3 kPa when subjected to two successive heating and cooling cycles within the temperature range of 25-85°C. This implies the attachment of responsive PNIPAAm chains onto HEWL fibres promotes higher protein stability which will be useful for future drug delivery and tissue engineering applications.

### 7.2 Future Work

There are several areas of the HEWL systems that can be further developed and improved. These include finding the number of disulfide bonds that are partially reduced at different conditions. Such quantitative measurements can be done by using the Ellman’s assay to quantify the number of thiols and relate it to the level of oxygen during sample reduction (a redox reaction) with the help of oxygen probe. The effect of higher protein concentration (above 4 mM) and different ionic strengths on the formation of reduced HEWL gels are yet to be explored.

Further work is still required in the area of lysozyme-PNIPAAm conjugation. More analytical techniques could be used to further characterise the synthesised polymers, including High Performance Liquid Chromatography (HPLC), Matrix Assisted Laser Desorption/Ionisation (MALDI) and Gel Permeation Chromatography (GPC). The degree of polymerisation of polymer and the ratio of incorporation of conjugate to protein matrix could be varied in order to observe their influence on the gelation of composite samples.
Chapter 8  References

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